Study of the involvement of autophagy in the acquisition of tumor resistance to Natural Killer-mediated lysis

Joanna Baginska

Insanity: doing the same thing over and over again and expecting different results.

Albert Einstein

REMERCIEMENTS

First of all, I am grateful to all of the members of the jury for accepting to evaluate my thesis work

I am thankful to Prof. Salem Chouaib for honoring me to preside over my thesis jury

I am also thankful to Prof. Patrice Codogno and Prof. Daniel Olive for accepting to review my thesis manuscript.

I would like to say that it was a pleasure for me to work with all the wonderful people in our lab here in Luxembourg. I am really grateful to all the members of CRP-Santé for supporting me scientifically and personally during my thesis. I wish all of you all the best for future.

I would like to express special thanks to my thesis director Dr Bassam Janji for being a great advisor. His ideas and tremendous support had a major influence on all my life. After four years I need to say, that he is a hard boss. Hard boss that teached me how to behave in scientific life, how to be a researcher and how to deal with regular day problems. Bassam become my YODA, which I respect and admire. Thank you Bassam, thank you 100 times again… for everything.

To answer power with power, the Jedi way this is not.

In this war, a danger there is, of losing who we are.

--YODA, Star Wars: The Clone Wars, "Lair of Grievous"

I would like to thank Professor Christian Auclair, the person that give me an ultimate chance to start my scientific, professional and personal life when I came to France in 2007 without speaking a word in French! Thank you for your trust Christian. Thank you for all the opportunities you gave me, which I tried to use in a best way.

My sincere thanks to Guy Berchem, The Big Boss of the lab, for being always there for me when I needed you, for all your support and for having wonderful wife Manon that was for me like a mother while working in the lab !

I would like to thanks Kris, that teached me lot of techniques and support me in every way.

Thanks to Petit&Grand – Jerome and Etienne – for being! What would be my life without you? Who I would « nuire » to?? Hehe…. Jerome, thank you to be the person that I really liked to make bad jokes to □

Angelina, you as well, I want to thank you – for your uninhibited help, your friendship and mental support in most difficult situations!

Sandrine M. – thanx dude – for handling me each day in the same office

Takouhie – thanks for being my innocent victim that I appreciate very much – but then still – I will keep my eye on you and your thesis ;)

Thanks to Sandrine Pierson for spending lot of time with me in the beginning of my thesis.

Nassera, Victoria and Valerie – Thank you for your kindness and all support

I would like to say Villmools Merci to Aurelie Wagner for your support and friendship ;)

Big thanks to great, beautiful people that I am graced to have as friends: Kai Schäfer, Mor Tal and Raz Bachar.

I would like to say VERY BIG and VERY SPECIAL thank you to ELODIE VIRY: For being always calm, handling me each day, for all work that we performed together, for being a great person and great friend, and for helping me in each situation, professional and personal.

One personal THANK YOU for Somebody Del Particular, because you restored my trust in mankind. And I would even say, kocham cię in the most pure way…

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ABBREVIATIONS

ADAM	A Disintegrin And Metalloproteinase
ADCC	antibody-dependent cell-mediated cytotoxicity
ALDOA	aldolase A, fructose-bisphosphate
AMBRA1	activating molecule in Beclin 1-regulated autophagy
AML	acute myeloid leukemia
ANGPT	angiopoietin
ASCT	allogeneic stem-cell transplantation
Atg	autophagy-related gene
BC	breast cancer
BECN1	Beclin 1
BNIP3	Bcl-2/adenovirus E1B 19-kDa interacting protein 3
BNIP3L	Bcl-2/adenovirus E1B 19-kDa interacting protein 3 like
CCR	chemokine (C-C motif) receptor
ccRCC	clear cell renal cell carcinoma
CD	cluster of differentiation
cFLIP	cellular FLICE-like inhibitory protein
CMA	chaperone-mediated Autophagy
CQ	chloroquine
CTL	cytotoxic T lymphocyte
CXCR	C-X-C chemokine receptor
DAPK	Death-associated protein kinase
DC	dendritic cell
DNA	deoxyribonucleic acid
DNAM-1,	DNAX accessory molecule-1;
E-cadherin	epithelial cadherin
EDN1	endothelin 1
EEA-1	early endosome antigen 1
EMAP	echinoderm microtubule-associated protein
EPO	erythropoietin
ER	endoplasmic reticulum
ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
FCγRIII	Fc fragment of IgG, low affinity IIIa, receptor
FIH1	factor inhibiting hypoxia-inducible factor 1 alpha
FLICE	caspase 8, apoptosis-related cysteine peptidase
GABARAP	gamma-aminobutyric acid receptor associated protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	gastric carcinoma
GIST	gastrointestinal stromal tumors
GLUT1	glucose transporter 1

GVHD	graft-versus-host disease
GVT	graft-versus-tumor
HER2	tyrosine kinase-type cell surface receptor HER2
HIF1	hypoxia inducible factor 1
HK1	hexokinase 1
HLA	human leukocyte antigen
HNSCC	head and neck squamous carcinoma
HRE	hypoxia response element
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HSP	heat-shock protein
HuR	embryonic lethal, abnormal vision, Drosophila-like 1 Hu antigen R
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
iNKR	inhibitory natural killer cell receptor
IS,	immunological synapse
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK1	mitogen-activated protein kinase 8
KIR	killer cell immunoglobulin-like receptor
KLRG1	killer cell lectin-like receptor subfamily G, member 1
Kv1.3	potassium voltage-gated channel, shaker-related subfamily, member 3
LAMP	lysosomal-associated membrane protein 3
LC3	microtubule-associated protein 1 light chain 3
LDHA	lactate dehydrogenase A
LFA1	integrin beta 2
LILRB1	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM do member 1
LIR1	protein leukocyte immunoglobulin-like receptor
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MIC	MHC class I chain-related protein;
MICA	MHC class I polypeptide-related sequence A
MIIC	MHC class II loading compartment
miRNA	microRNA
MM	multiple myeloma
MMP-7	matrix metallopeptidase 7
MTOC	microtubule-organizing center
MUC	mucin protein
NAF-1	nutrient-deprivation autophagy factor-1
NANOG	homeobox transcription factor Nanog
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity triggering receptor

NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells 1
NK	natural killer
NKG2C	natural killer group 2C
NKG2D,	natural killer group 2D
NKT	natural killer T cell
NSCLC	non-small cell lung carcinoma
p14ARF	cyclin-dependent kinase inhibitor 2A
PAS	pre-autophagosomal structure
PAS	pre-autophagosomal structure
PBMC	peripheral blood mononuclear cells
PDGFB	platelet-derived growth factor beta polypeptide
PDK1	pyruvate dehydrogenase kinase, isozyme 1
PE	phosphatidylethanolamine
PFKL	phosphofructokinase, liver
PFN	perforin 1
PGF	placental growth factor
PGK1	phosphoglycerate kinase 1
PHD2	egl-9 family hypoxia-inducible factor 1
PI	propidium iodide
PI-9	serpin peptidase inhibitor, clade B (ovalbumin), member 9
PKM1	pyruvate kinase, muscle
pSMAC	peripheral supramolecular activation cluster
PTEN	phosphatase and tensin homolog
RAG	recombination activating gene
RB1CC1/FIP200	RB1-inducible coiled-coil 1
SCT	stem cell transplant
SDF1	stromal cell-derived factor 1
SHIP1	phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
SLUG	snail family zinc finger 2
SNAIL	snail family zinc finger 1
Src	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
ssRNA	single-stranded RNA
STAT-3	signal transducer and activator of transcription-3
TAM	tumor-associated macrophages
TAP	transporter associated to antigen processing
TCA	tricarboxylic acid
TCR	T-cell receptor
TGF	transforming growth factor
Th17	T helper 17 cells
TIL	tumor infiltrating lymphocyte
TLR	Toll-like receptor
TME	tumor microenvironment
TRAIL	tumor necrosis factor (ligand) superfamily, member 10
Treg	regulatory T cells

TVCs	tubulovesicular clusters
TWIST	basic helix-loop-helix transcription factor twist
UBL	ubiquitin-like
ULBP, UL16-binding protein;	tumor infiltrating lymphocytes
ULK	UNC-51-like kinase
Ulk1	Unc-51-like kinase family
VAM	c-Jun N-terminal protein kinase 1
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
VHL	TNF-related apoptosis-inducing ligand
ZEB1	zinc finger E-box binding homeobox 1

ABSTRACT

Natural killer (NK) cells are effectors of the innate immune system, able to kill cancer cells through the release of the cytotoxic protease granzyme B. NK-based therapies have recently emerged as promising anticancer strategies. However, it is well established that hypoxic tumor microenvironment interferes with the function of antitumor immune cells and constitutes a major obstacle for defining cancer immunotherapies. Recent studies demonstrated that autophagy is an important regulator of innate immune response within the tumor microenvironment, but the mechanism by which autophagy plays such a role remains elusive. We demonstrate that hypoxia impairs breast cancer cell susceptibility to NK-mediated lysis in vitro by a mechanism involving the activation of autophagy, as this impairment was reverted by inhibiting autophagy. The resistance of hypoxic tumor cells to NK-mediated killing was neither related to a defect in target cell recognition by NK cells nor to a defect in their cytolytic function toward hypoxic cells. We provided a strong evidence that autophagy activation in hypoxic cells degrades NK-derived granzyme B in lysosomes of hypoxic cells making these cells less sensitive to NK-mediated killing. Genetic and pharmacological inhibition of autophagy restored granzyme B levels and revert the resistance of hypoxic cells in vitro. Our results highlight autophagy as a critical factor in modulating NK-mediated anti-tumor immune response. We have validated this concept in vivo by showing that targeting autophagy significantly improves NK-mediated tumor shrinking in breast and melanoma models.

This study provides a cutting-edge advance in our understanding of how hypoxia-induced autophagy impairs NK-mediated lysis and paves the way for formulating more effective NK-based antitumor therapy by combining autophagy inhibitors.

1 INTRODUCTION

1.1 HYPOXIC TUMOR MICROENVIRONMET AND CANCER CELL

In multistep development of human tumors the driving force of progression are cancer cells. For several decades, cancer research was focused on the understanding of how the activation of oncogenes and loss of function of tumor suppressor genes induce carcinogenesis in normal cells [1]. Despite our knowledge about oncogenes and activated oncogenic signaling pathways in tumor cells, targeted anti-cancer therapies remain not completely effective [2]. Genomic instability and inflammation foster multiple adaptations of cancer cells. It is now widely accepted that cancer cells are not the only part of disease, but also exploit and take advantage of surrounding normal cells, embodied in the concept of tumor microenvironment (TME) [3].

Tumors are composed of a complex network of tumor cells, lymphocytes, stromal components including fibroblasts, immune cells and a complex vasculature which bring the immune cells to the tumor site and provide necessary nutrients for tumor growth [1]. The tumor microenvironment is a major factor influencing the initiation, growth and metastatic spread of tumors, as well as the effectiveness of cancer therapy [4]. The microenvironment of solid tumors contains regions of poor oxygenation, and tumor cells must adapt to exist in this microenvironment [5]. Most pro-survival mechanisms in hypoxic TME are orchestrated by the transcription factor, Hypoxia Inducible Factor-1 alpha (HIF-1α) [6]. HIF-1α pathway enables tumor cells to survive by changing glucose metabolism toward a glycolytic phenotype, by inducing angiogenesis and by regulating pH balance and proliferation rate [7].

1.1.1 Hypoxia

In tumors, cell growth and proliferation exceed the development of local vasculature responsible for supplying oxygen and nutrients. As a result, tumors form disorganized angiogenic vessels that cause the oxygen within the tumor to range heterogeneously from anoxic and hypoxic to normoxic levels [8]. The tumor microvasculature induced, in part, as a

response to hypoxia, is highly abnormal and fails to rectify the oxygen deficit. Hypoxia within tumors results in a metabolic switch towards glycolysis and is a negative prognostic and predictive factor. [9]. Hypoxia plays a role in the activated expression of genes involved in the inhibition of apoptosis, angiogenesis, invasiveness and metastasis, epithelial-to-mesenchymal transition, and genomic instability. Moreover, tumor hypoxia influences immune response by decreasing tumor cell sensitivity to cytotoxic effectors or by inactivating immune effectors [10]. Given its central role in tumor progression and resistance to therapy, tumor hypoxia might well be considered as the important target in cancer treatment. The delivery and consumption of O2 pressure in human tumors are tightly regulated through the activity of hypoxia-inducible factors (HIFs). HIF activity in intratumoral hypoxia region mediates angiogenesis, epithelial-mesenchymal transition, stem cell maintenance, invasion, metastasis, and resistance to therapy. Obviously, HIFs links hypoxia with chronic inflammation, metabolic adaptation, and tumor progression [11].

1.1.2 HIF-1α regulation by O2 level

HIF transcription factors consist of an oxygen- sensitive alpha-subunit (HIF-1α, HIF-2α and HIF-3α subunits) and a constitutively expressed beta-subunit (HIF-β). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. The expression level of HIF-1 is regulated by O2 concentration through hydroxylation of proline and asparagine [12]. The hydroxylation of two proline residues in HIF-1α by prolyl hydroxylase domain protein 2 (PHD2) is necessary for the binding of the von Hippel-Lindau protein (VHL) and leads to ubiquitination of HIF-1α and its subsequent degradation in proteasome [13]. In hypoxic condition, PHD2 is inhibited, thereby stabilizing the α-subunits of HIF. Hydroxylation of an asparagine residue in HIF-1α by factor inhibiting HIF-1α (FIH-1) blocks the recruitment of the coactivator p300. These hydroxylation reactions use O_2 and α-ketoglutarate as substrates and enzyme activity is inhibited under hypoxic conditions, leading to increased HIF-α stability and transcriptional activity. In those

circumstances, HIF-1α accumulates and translocates to the nucleus to finally binds HIF-1β and thereby form the active transcription factor HIF-1 [14]. The HIF-α/β dimer binds to Hypoxia Response Element (HRE) sequence (5′-G/ACGTG-3′). The activated HIF complex associates to HREs in the regulatory regions of target genes and induces gene expression critical to systemic hypoxia responses, such as angiogenesis and erythropoiesis, and cellular hypoxia responses involving metabolism, proliferation, motility, and autophagy [15]. HIFs play a critical role in cancer progression and its accumulation has been associated with poor patient survival in early stage oligodendroglioma, oropharyngeal squamous cell carcinoma, cervical, breast, ovarian, and endometrial cancer. [16]

1.1.3 HIF regulates cell proliferation and survival

HIF transcription factors regulate the expression of genes involved in the control of anaerobic energy metabolism, angiogenesis, proliferation, apoptosis, and differentiation. In neoplasia formation, the secretion of growth/survival cytokines results in increased rate of cell proliferation and a decreased rate of cell death [17]. Hypoxia-inducible factor 1 (HIF-1) is responsible for the transcriptional activation of the Vascular Endothelial Growth Factor (VEGF) gene in hypoxic cells by binding to a hypoxia response element located 1 kb 5′ to the transcription initiation site [18]. VEGF is a potent mitogen specific for endothelial cells and a key mediator of hypoxia-induced angiogenesis [19] Once transcribed, VEGF mRNA is bound by human antigen R (HuR) protein and other proteins that inhibit its degradation under hypoxic conditions. Several growth/survival factors participating in autocrine signaling are encoded by HIF-regulated genes, such as transforming growth factor-α (TGFα) in clear-cell renal carcinoma [20] ; insulin-like growth factor-2 (IGF2) in colorectal carcinoma, VEGF in gastric, pancreatic and colorectal cancer; [21]; endothelin 1 (EDN1) in breast, prostate, and ovarian cancer [22] and erythropoietin (EPO) in breast, prostate, and renal cancer and melanoma [23].

1.1.4 HIF regulates angiogenesis

The regulation of angiogenesis by hypoxia is an important component of homeostatic mechanisms that link vascular oxygen supply to metabolic demand. A large number of genes involved in different steps of angiogenesis are responsive to hypoxia [24]. HIF-1 directly activates transcription of the multiple genes, including VEGF, stromal-derived factor 1 (SDF1), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and angiopoietin (ANGPT) 1 and 2 [25]. Above mentioned genes are encoding angiogenic growth factors essential for initiating and maintaining new vessel formation. In addition to activation by hypoxia, the HIF system is also induced or amplified by a wide range of growth-promoting stimuli and oncogenic pathways such as the insulin, insulin-like growth factor-1, epidermal growth factor and mutant Ras and Src kinase pathways [24]. Tumor suppressor mutations, including PTEN, p53, p14ARF, and pVHL, also activate the HIF system [26].

1.1.5 HIF in epithelial-mesenchymal transition

The epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose their polarity and shift to a mesenchymal phenotype. EMT is a critical event in cancer progression and is involved the initial steps in metastasis [27]. EMT converts cells from an epithelial morphology to become migratory and prone to invade other tissues. EMT is accompanied by specific changes in gene expression, such as down-regulation of E-cadherin [28]. The E-cadherin promoter is repressed by several transcriptional repressors, including the zinc finger transcription factor Snail-1 [29]. Snail factors are potent inducers of EMT and its up-regulation correlates with metastasis and poor prognosis, whereas silencing of Snail-1 is critical for reducing tumor growth and invasiveness [30]. Under hypoxic conditions, the tumor microenvironment generates and sustains major EMT-triggering pathways for facilitating tumor growth and metastasis, such as transforming growth factor (TGF) β, Notch and nuclear factor kappaB (NFκB) signaling pathways [31]. HIF modulates the EMT by regulating the

expression and the activity of major transcription factors including TWIST, SNAIL, SLUG, SIP1 and ZEB1. These EMT regulators subsequently bind to the promoters of E-cadherin, vimentin, and N-cadherin to mediate EMT. [32-34]

1.1.6 HIF and metabolic reprogramming

In cancer cells, reprogramming of cellular metabolism drives substrate utilization toward a dependence on glucose [35]. HIF plays a central role in the regulation of cellular metabolism, by inducing a switch from oxidative phosphorylation to glycolysis. [36]. This switch is mediated by HIF-1-dependent expression of genes encoding glucose transporters (GLUT1, GLUT3) and glycolytic enzymes (ALDOA, ENO1, GAPDH, HK1, HK2, PFKL, PGK1, PKM2, LDHA) involved in glucose conversion to lactate [37]. Moreover, HIF expression under hypoxia increases the expression of pyruvate dehydrogenase kinase 1 (PDK1) which inhibits the conversion of pyruvate to acetyl CoA, thus reducing the consumption of oxygen in mitochondria [38]. HIF-1 also decreases metabolism rates by regulating BNIP3 and BNIP3L expression involved in the activation of autophagy [39].

1.2 Hypoxia dependent regulation of immune response

1.2.1 Myeloid cells

The evolution of immune escape mechanisms by tumor cells is also dependent on hypoxia. Recent evidence indicates that hypoxia modulates the differentiation and functions of infiltrating immune cells in the tumor microenvironment [40]. Macrophages are frequently the most abundant myeloid cells to invade the hypoxic intratumoral environment [41]. The production of stromal cell–derived factor 1α (SDF1α) by hypoxic tumor cells induces homing of bone marrow–derived CD45+ myeloid cells to tumor areas. Invasion of myeloid cells in tumor microenvironment is known to be a highly immunosuppressive factor [42]. Myeloid derived suppressor cells (MDSCs) suppress innate and adaptive immunity, thereby limiting

anti-tumor immune responses in cancer patients. MDSCs are known to directly promote immune tolerance by inducing T cell anergy, restraining the effector phase of the CD8+ T cell, and promoting antigen-specific Regulatory T (Treg) cell proliferation [43]. MDSCs downregulate production of Interleukin (IL)-12 by macrophages and increase their own production of IL-10 in response to signals from macrophages [44]. IL-10 polarizes activated macrophages (of M1 phenotype) to an immunosuppressive phenotype type 2 (M2) [45]. Although in the early immune response to neoplasia, macrophages contribute to the death of tumor cells, the presence of macrophages is correlated with a poor prognosis for patients in advanced stages. Macrophages, which constitute a major component of the myeloid infiltrating tumors, comprise up to 80% of the cell mass in breast carcinoma. In the tumor microenvironment, they differentiate into $CD206^{+}$ Tumor Associated Macrophages (TAM) and accumulate in hypoxic areas of endometrial, breast, prostate and ovarian cancers [41]. This accumulation is due to the release in hypoxic microenvironment of macrophage chemoattractants such as endothelial monocyte-activating polypeptide (EMAP) II, endothelin 2 and VEGF [46]. Moreover, hypoxia inhibits macrophage-dependent expression of the chemokine receptors such as CCR5 and CCR2, thus immobilizing TAMs in hypoxic microenvironment [47]. High TAM numbers in tumors in the majority of cases are correlated with reduced survival. Exposure of TAM to tumor-derived cytokines such as IL-4 and IL-10 is able to convert them into polarized type II or M2 macrophages with immune-suppressive activities and pro-angiogenic effects, resulting in tumor progression [48]. Subsequently, M2 macrophages establish an environment that skew CD4+ and CD8+ T cell immunity toward a tumor-promoting type 2 response [49]. Moreover hypoxia upregulates the expression of the matrix metalloprotease MMP-7 protein on the TAM surface leading to cleavage of Fas ligand from neighbouring cells, and thereby making tumor cells less responsive to NK and T cell-

mediated lysis. Thus, myeloid cells directly suppress both adaptive and innate antitumor immunity thereby facilitating tumor growth [50].

1.2.2 NK cells

Hypoxia has also been reported to affect NK cells by increase the shedding of MHC class I polypeptide-related sequence A (MICA), a ligand for activating receptor Natural Killer Group 2, member D (NKG2D), at the surface of prostate cancer cells via impaired NO signaling, leading to tumor escape from NK cells and CTLs [15]. More recently, it has been shown that HIF-1 is able to down-regulate MICA expression in osteosarcoma cells resulting in tumor resistance to NK-mediated lysis [51]. In addition, hypoxia contributes to tumor cell resistance to lysis mediated by NK cells through stimulation of A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) expression and, consequently, decreased levels of MICA on the tumor cell membrane. This mechanism has been shown to be blocked by inhibition of HIF-1α accumulation [52].

Recently, a link between tumor hypoxia and immune tolerance to NK through the recruitment of regulatory T cells (Tregs) has been established. Hypoxia induces secretion of immunosuppressive cytokine TGF-β1 which subsequently induces the proliferation and the accumulation of Tregs in tumor microenvironment [53]. Moreover, cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-β. Indeed, TGF-β downregulates the activating receptor NKG2D on the surface of NK, thus resulting a poor antitumor response [54].

HIF-1 has also been involved in the regulation of tumor cell response to cytotoxic effectors independently of the oxygen level but in the context of inactivating VHL mutations. These mutations of VHL induce a constitutive stabilization of HIFs, a process known as pseudo-hypoxia [55]. In this context, HIF-1 has been associated with increased sensitivity to NK cells in clear-cell renal cell carcinoma (ccRCC) mutated in VHL compared to the same cells

expressing wild type VHL gene. The proposed mechanism involves a stronger expression of Human Leukocyte Antigen (HLA)-class I molecules at the surface of VHL mutated-RCC cells [56].

1.3 Hypoxia dependent regulation of T- cell response

The tumor microenvironment includes a complex network of immune T-cell subpopulations. The types of T cells infiltrating the TME correlate with different clinical outcomes, which reflect their diverse roles in tumor progression [57]. Tumor-infiltrating lymphocytes (TIL), comprising CD3+CD4+ and CD3+CD8+ T cells, are recruited to hypoxic tumor microenvironment. Compelling evidence point to a clinical relevance for high numbers of T cells at the tumor site with CD8 memory T cells as a key denominator for overall survival in patients with breast, colorectal and lung cancers [58]. In addition, the assessment of TIL density and distribution was shown to independently predict sentinel lymph node status and survival in patients with melanoma [59]. The tumor microenvironment is hostile to T cell function due to expression of enzymes that deplete the amino acids tryptophan and arginine, high concentration of tumor secreted lactate, and presence of innate cells or regulatory T cells both with suppressive activity. CD8 T cells has been shown to be important in spontaneous tumor control. CD4 T cells are more plastic and play dual roles; thus CD4 T cells can convert from anti-tumor to pro-tumor [58]. Although accumulation of effector T cells in the tumor is a good prognostic factor, TIL are largely ineffective in killing of cancer cells. It has become clear that hypoxic tumor microenvironment plays a determining role in neutralizing the immune system of the host and negatively impacting the outcome of T cell lysis [60].

1.3.1 Regulatory T cells

Regulatory T cells (Treg) are part of CD4+ T cells present in the tumor. Treg are characterized as CD4+CD25high Foxp3+ cells and are expanded in patients with cancer. Treg cells suppress

proliferation of other T cells in the microenvironment through different mechanism dependent on cell-cell contact or through the secretion of IL-10 and TGF-β [61]. Treg accumulation is a characteristic feature of the human tumor microenvironment [62]. Hypoxia plays an important role in regulation of Treg dependent immune suppression. HIF-dependent pathways activate multiple anti-inflammatory mechanisms affecting T cell mediated immune response [63]. Moreover, it has recently been proposed that the activation of the glycolytic pathway by HIF-1α leads to enhanced Th17 and decreased Treg cells differentiation from naïve T cells. HIF-1α has also been shown to induce Th17 differentiation and to inhibit Treg by targeting FOXP3 for proteasomal degradation [64].

1.3.2 Cytotoxic T Lymphocytes

Hypoxic environment influences CTL-mediated lysis of tumors by different mechanisms involving HIF-1 [65]. Upon entry of T cells into hypoxic sites, HIF-1α accumulation enables the cells to survive within regions of low oxygen, but also suppresses T cell function avoiding an excessive immune response. Suppression of T cell response under hypoxia depends on the modulation of calcium homeostasis in T cells via potassium voltage-gated channel, shaker-related subfamily, member 3 (Kv1.3) channel inhibition and membrane depolarization [66, 67]. In addition to the direct suppression of T cell activity, HIF-1α was also demonstrated to indirectly modulate T cell mediated killing by influencing the differentiation of CD4+ T cells [68]. The subsets of functional CD4+ (Th1, Th2, Th17, and T regulatory cells) have different levels of glycolytic activity and it has been reported that HIF-1α controls differentiation into these effector subsets by modulating their metabolic signatures [69]. It has been also shown that hypoxia decreases tumor susceptibility to CTL-mediated lysis via HIF-1α stabilization and phosphorylation of STAT3 [65] . Moreover, hypoxia increases homeobox transcription factor Nanog (NANOG) [70], microRNA-210 [71], and CD137 [40] thus contributing to hypoxia-induced tumor target resistance to CTL-mediated lysis.

1.4 Immune response in the tumor microenvironment

Innate and adaptive immune effectors are able to infiltrate solid tumors and thereby control cancer cell growth or promote tumorigenesis. It is now admitted that immune system, along with mesenchymal-derived cells and stromal cells, plays a critical role in oncogenesis, and tumor response to therapy. Several immune cell types can infiltrate tumors such as Natural Killer (NK) cells, Cytotoxic T-Lymphocytes (CTL), macrophages, dendritic cells (DC), eosinophils, mast cells, and myeloid-derived suppressor cells. The complex interactions between various cell types and plasticity of immune system within tumor microenvironment are important new emerging hallmark of cancer.

1.4.1 Cancer immunoediting – from surveillance to escape

Immune system plays a fundamental role in protection against cancer by elimination of pathogens and viruses, thus preventing inflammation and virus-induced tumors, respectively. Furthermore, by recognition and elimination of transformed cells in immunosurveillance process, immune system can block or control long term cancer development [72]. This concept was validated by experiments showing that immunodeficient mice are more susceptible to the development of cancers. For example, mice lacking responsiveness to interferon gamma (IFN-γ) or Recombination-Activating Gene (RAG)-2, resulting in the absence of T-, B- and NKT cells , showed increased neoplasia [73]. Deletion of TCRβ and γ resulting in absence of γδ and αβ T cells, or lack of the joining segment Jα281 leading to increased number of NKT cells, showed the similar effect [74]. Decrease in production of perforin or tumor-necrosis factor(TNF)-realted apoptosis-inducing ligand (TRAIL) resulted in decreased ability to induce apoptosis in target cell and thus higher risk to develop cancer [75]. Additionally, depletion of NK cells, NKT cells, or neutralization of NK-activating ligand NKG2D increased tumor growth [76]. There are several lines of evidence indicating strong immune response in patients in early stages of cancer development. It has been reported that specific bone marrow CD8+ T-cells

from breast cancer patients recognizing mucin-1 (MUC-1) and Human Epidermal Growth Factor Receptor 2 (HER2) ERBB2 peptides derived from the breast-cancer-associated proteins can mediate regression of autologous human tumors if transplanted into immunodeficient mice [77] [78]. Similarly, bone marrow tumor-reactive T cells from patients with pancreatic cancer are able to reject tumor xenotransplants in recipient animals [79]. Nevertheless, cancer cells can still develop by induction of immune tolerance and immune system may promote the development of primary tumors with reduced immunogenicity, able to escape immune surveillance. These results led to development of cancer immunoediting concept, a complex process of three phases: elimination, equilibrium and escape. The immunosurveillance, described above, represents the classical concept of elimination of newly formed cancer cells [73].

Equilibrium is the period of latency mediated by immune cells. In the equilibrium phase, the host immune system and any tumor cell variant that have survived the elimination phase enter into a dynamic balance, wherein lymphocytes and IFN-γ exert potent and relentless selection pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed containing many genetically unstable and mutating tumor cells. Although many of the original tumor cell escape variants are destroyed, new variants arise carrying different mutations that provide them with increased resistance to immune attack. The end result of the equilibrium process is a new population of tumor clones with reduced immunogenicity by the sculpting forces of the immune system [80]. Equilibrium is probably the longest of the three phases and may occur over a period of many years in humans. Indeed, it has been estimated that for many solid human tumors twenty years are needed between initial carcinogen contact and clinical detection of the tumor. During this period, the heterogeneity and genetic instability of cancer cells that survive the elimination phase are possibly the principal forces that enable tumor cells to resist the immune response [81].

In the escape phase, tumor cell variants selected in the equilibrium phase now can grow in an immunologically intact environment, when genetic and epigenetic changes in the tumor cell confer resistance to immune detection and/or elimination, allowing the tumors to expand and become clinically detectable. Because both the adaptive and innate compartments of the immune system function in the cancer immunosurveillance network, tumors avoid either one or both arms of immunity in order to achieve progressive growth. Individual tumor cells may employ multiple immunoevasive strategies to elude the powerful integrated innate and adaptive antitumor immune responses to their immunogenic progenitors. Thus, it is possible that several distinct immunological events must occur before the final immunogenic phenotype of a malignant cell is ultimately established [82]. The phases of immunoediting are represented in **Figure 1.**

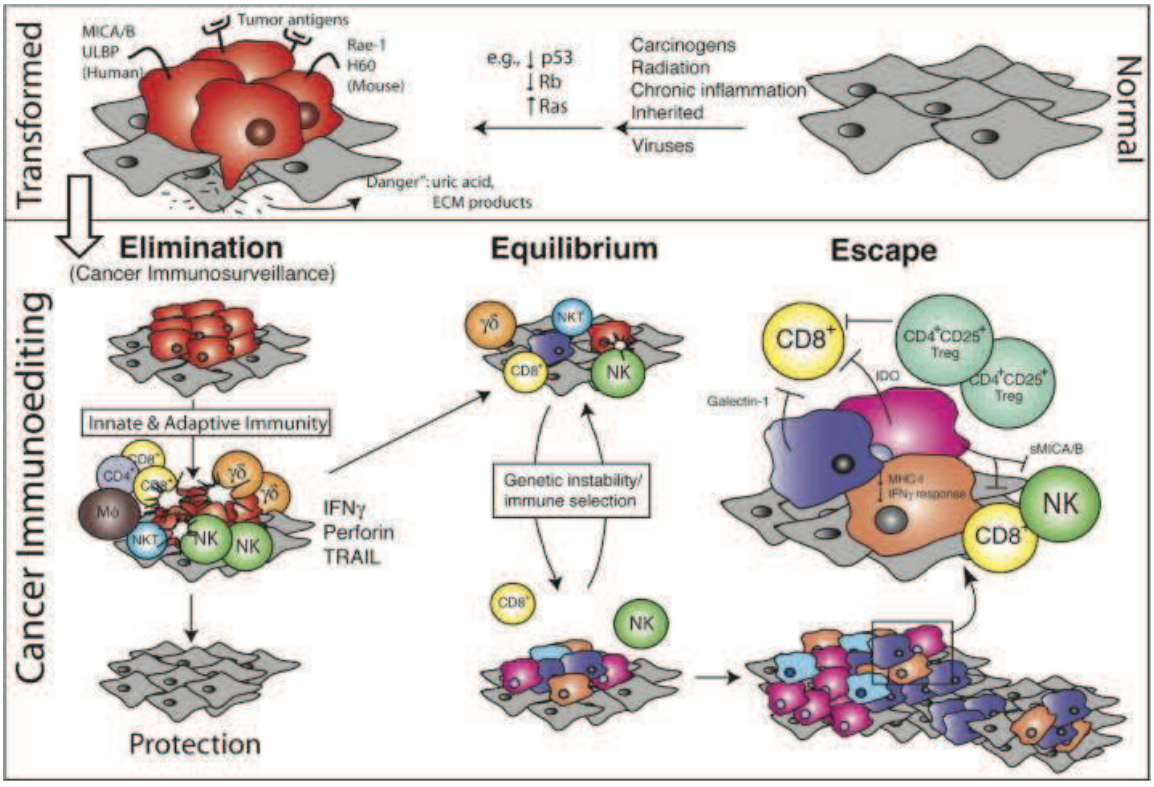

Figure 1: The three phases of the cancer immunoediting process.
Normal cells subject to common oncogenic stimuli ultimately undergo transformation and become tumor cells. Even at early stages of tumorigenesis, these cells may express distinct tumor-specific markers and generate proinflammatory "danger" signals that initiate the cancer immunoediting process. In the first phase of elimination, cells and molecules of innate and adaptive immunity, which comprise the cancer immunosurveillance network, may eradicate the developing tumor and protect the host from tumor formation. However, if this process is not successful, the tumor cells may enter the equilibrium phase where they may be either maintained chronically or

immunologically sculpted by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system by a variety of mechanisms and become clinically detectable in the escape phase. (From Avin P. Dunn, Immunity, Volume 21, Issue 2, August 2004, Pages 137–148).

1.4.2 Cancer immunoediting by Natural Killer cells

NK cells were identified in mid-1970s, and first described as lymphoid cytotoxic cells able to lyse tumor cells without prior sensitization of the host [83] . NK cells comprise 15% of all circulating lymphocytes and are also found in peripheral tissues, including liver, peritoneal cavity and placenta [84]. Although resting NK cells circulate in the blood, , they are capable to infiltrate most tissues that contain pathogen-infected or malignant cells following activation by cytokines. NK cells can be rapidly activated in the periphery by NK-cell stimulatory factors, such as IL-12, IFN-α, IFN-beta, IL-15 or IL-2. Hematopoietic stem cell (HSC)-derived human natural killer cells, represent a subset of CD3-CD56+ lymphocytes which can be subdivided in two types on the basis of expression level of CD56 and CD16 - the low affinity receptor for FcγRIII[85]. The neural cell adhesion molecule (N-CAM/CD56) is a member of the immunoglobulin supergene family that has been shown to mediate homophilic interactions between NK and target cell. CD16 receptor (FCγRIII) binds to the Fc portion of IgG antibodies11. At the surface of NK cells, CD16A, is a transmembrane protein that co-localizes with CD3ζ and Fc-εRI-γ. Upon ligation, it induces a potent series of signals resulting in cytokine production and cytotoxic effector activity via antibody-dependent cellular cytotoxicity (ADCC) [86].

$CD56^{bright}$ $CD16^{-}$ NK cells predominate in lymph nodes, and express little cytolytic activity. This subset of NKs possess little or no killer immunoglobulin-like receptors (KIRs), and is able to rapidly proliferate, produce high levels of proinflamatory Th1 cytokines and mediate cross-talk with other innate and adaptive immune cells [87]. In turn, $CD56^{bright}$ peripheral blood NK cells are thought to differentiate into $CD56^{dim}$ cells. $CD56^{dim}CD16^{+}$ NK cells with high KIR expression, predominantly found in peripheral blood and inflamed tissues are highly cytotoxic

but display lower cytokine production than $CD56^{bright}$ $CD16^{-}$ NK cells [88]. In vivo, human $CD56^{bright}$ NK cells may undergo progressive differentiation towards an $CD94^{low}CD62L^{neg}CD56^{dim}$ phenotype. $CD56^{dim}$ NK cells continue to differentiate. During this process, they lose expression of NKG2A and acquire inhibitory killer cell inhibitory immunoglobulin-like receptors and CD57 [89]. Furthermore, $CD57+CD56^{dim}$ NK cells express lower levels of the chemokine receptors, CXCR3, CXCR4, and CCR5, as well as the activation receptors, NKp46 and NKp30, compared with $CD57-CD56^{dim}$ NK cells. Finally, the expression of CD57 as well as the acquisition of multiple KIRs was associated with changes in the expression of LILRB1, NKG2C, CD62L, and KLRG1 [90].

Bone marrow is the main site of NK-cell generation, providing growth factors and cytokines required for NK-cell development, and containing stromal cells necessary to their maturation. Regulation of NK cells activity depends on repertoire of germline encoded activating and inhibitory receptors. The activating receptors recognize stress-induced, pathogen-derived or tumor specific ligands, whereas, the inhibitory receptors bind self-molecules presented on normal cells [91].

Type 1 natural killer T cells (NKT cells) are defined as CD1d-restricted T cells that express an invariant T cell antigen receptor (TCR; variable (V) and joining (J) Vα24Jα18 in humans combined with a limited but not invariant TCR β-chain repertoire, usually Vβ11 in humans. This unusual TCR αβ-chain combination endows NKT cells with specificity for glycolipid antigens presented by CD1d. NKT cells produce a broad range of cytokines within minutes to hours of antigenic stimulation, which allows them to modulate immunity in a broad spectrum of diseases, including cancer, autoimmunity, infection, allergy, allograft rejection and graft-versus-host disease [92].

1.4.3 Negative regulation of NK activity

NK cells discriminate between normal host and invading cells by recognition of Major Histocompatibility Complex (MHC) class I molecules. MHC proteins are involved in antigen presentation and immune responses and are classified as class I, class II and class III [91]. NK cell responses are negatively regulated by MHC class I molecules presented on all nucleated cells. MHC class I molecules are cell surface heterodimers consisting of a chain associated with β2-microglobulin. MHC class I specific inhibitory receptors expressed on the NK cells allow self-tolerance, and are needed for the acquisition of NK cell functional competences, a process named NK education. The highly polymorphic MHC class I proteins are ligands for the inhibitory NK-cells receptors (iNKR), however, non-engagement of iNKR by MHC class I molecules leads to NK cell activation and killing of target cell. Most of the iNKR belong to the Killer cell Immunoglobulin-like Receptors (KIR) family, recognizing allelic determinants of MHC-class 1 molecules **(Table 1)**.

NK-cell inhibitory receptors

NK-cell receptor	Ligand
For MHC class I molecules	
Human KIR2DL	HLA-C
Human CD158/KIR3DL	HLA-Bw4, HLA-A
Human CD158i/ILT2	HLA class I
Mouse Ly49	H-2K, H-2D
For non-classical MHC class I molecules	
CD49/NKG2A (mouse: Qa-1b)	HLA-E

Table 1: NK-cell inhibitory receptors.
(Adapted From Mark J. Smyth, Nature Reviews Cancer 2, 850-861, November 2002)

NK cells express also a lectin-like receptor heterodimer composed of CD94 coupled with member of Natural killer group 2, member D (NKG2) family, able to induce activatory and inhibitory signals [93]. NKG2A, NKG2C and NKG2E proteins are all highly related in sequence and are present as heterodimers with CD94 protein. They recognize HLA-E, a non-classical MHC class I molecule in humans [94].

Human MHC - Human Leukocyte Antigen – HLA, is encoded by six functional HLA class I genes (HLA-A, -B, -C, -E, -F and –G). HLA polymorphisms confer susceptibility and resistance to numerous diseases, particularly the chronic inflammatory and autoimmune diseases and are important in clinical transplantation. HLA class I molecules consist of a MHC-encoded heavy chain, light chain β_2 microglobulin (β_2M), and a peptide [95]. The proteasome is responsible for the generation of the majority of peptides presented by MHC class I molecules. These peptides are transported into the endoplasmic reticulum from the cytosol by transporters associated with antigen processing protein (TAP). Peptides binding to MHC class I molecules trigger their release from TAP and subsequently their transport to the cell surface. Peptide free MHC class I molecules are unstable and fall off the cell surface [96].

KIR molecules are monomeric type I glycoproteins which contain 2 (KIR2D) or 3 (KIR3D) Ig-like domains in the extracellular region. Killer cell inhibitory receptors (KIRs) inhibit NK cytotoxicity when recognizing MHC class I molecules on target cells. They possess two tandem intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) that, when phosphorylated, each bind to the two Src-homology 2 domain-bearing protein tyrosine phosphatases SHP-1 and SHP-2 in vitro [97]. Recruitment and activation of phosphatases SHP-1 and SHP-2 through ITIMs block the activating signals delivered upon engagement of activating receptor [98]. Other inhibitory receptors are characterized by broad specificity for different HLA-class I molecules such as LIR1/ILT2 or recognize HLA-E molecules such as CD94/NKG2A. It is important to note that each inhibitory receptor is expressed by unique subsets but not all NK cells, and all mature NK cells express at least one receptor specific for self HLA-class I molecules, generating divers' repertoires of human NK cells [99].

The failure to express one or more HLA-class I alleles is one of the strongest signals leading to NK killing of target cells. Interaction between HLA-class I molecules and iNKR is the best know check-point controlling NK cell activation and target killing. It has been reported that

85% of metastatic carcinomas display altered surface expression of HLA-class I molecules, making them potential target of NK-mediated lysis [100]. In addition, transplantation across incompatible HLA class I may trigger alloreactive NK cells responses in recipients lacking one or more ligands for donor inhibitory KIRs. For example, the primary role of NK cells as allogenic therapy of leukemia patients by exerting graft versus tumor (GVT) effect was demonstrated [101]. The success of hematopoietic stem cell transplantation (HCT) is due, at least in part, to NK cells exerting a graft versus leukemia (GVL) effect capable of eradicating remaining acute myeloid leukemia (AML) cells [102]. It has been shown, that AML patients undergoing haploidentical KIR ligand mismatched stem-cell transplantation (SCT) had a greatly improved disease free survival and reduced relapse rates [103]. SCTs that involved NK-cell alloreactivity were associated with higher rates of bone-marrow engraftment and reduced rates of graft-versus-host disease (GVHD) as a consequence of the inefficient priming of alloreactive donor T cells owing to NK-cell killing of recipient antigen presenting cells [104]. Additional work demonstrated an important role of NK cells in HLA-molecules-dependent elimination of experimentally established pulmonary metastases of melanoma [105].

1.4.4 Positive regulation of NK cell activity

To kill potential target, NK cells need to generate activating signal involving activating receptors which can be classified in two groups: 1) receptors interacting with immunoreceptor tyrosine-based activation motif (ITAM)-bearing peptides such as CD16, NKG2C/CD94 and natural cytotoxic receptors (NCRs); 2) receptors interacting with non-ITAM-bearing proteins, such as NKG2D, 2B4 or CD2 [93]. Only the NKG2D and NCRs which include NKp30, NKp44 and NKp46 will be described in this section as a major NK-activating receptors in human NK cells mediating tumor cell lysis [106].

1.4.4.1 The NCRs family: NKp30, NKp44 and NKp46

NK activation requires a positive signal delivered by the engagement of activating receptors, more particularly of the natural cytotoxicity receptors (NCRs), directly involved in the natural cytotoxicity of Natural Killer **(Table 2)**. The NCR family including NKp46, NKp44 and NKp30 belongs to the Ig-superfamily and contains amino acid in transmembrane domain, associating with ITAM bearing adaptor molecules [107]. NK cells from most healthy donors express a high quantity of NCRs on their surface, corresponding to the NCR^{bright} phenotype while only 20 % present the NCR^{dull} phenotype. In sharp contrast, most patients (80%) with leukemia express NCR^{dull} phenotype [108]. It is well documented that a functional dependency exists between the three members of the NCRs family. Indeed, the engagement of a single NCR leads to the activation of signaling cascade involving the other members. It has been shown that blocking NCR in lung adenocarcinoma lead to inhibition of lysis, thus highlighting the important role of NCR signaling in NK cell-mediated cancer immunosurveillance [109]. A strict relationship between NCR density on the surface of NK cells and their ability to kill tumor targets was reported [93].

The activation receptors involved in NK-cell function

Receptor	Species	Ligand	Signal transduction
NKG2D	Human Mouse	MIC, ULBP Rae-1, H60	Associated with DAP10 and activates PI3K pathway
NCR			
NKp30	Human	Unknown	Associated with ITAM-bearing CD3-ζ or FcRγ and activate ZAP70/SYK pathway
NKp46	Human/mouse	Unknown	
NKp44	Human	Unknown	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
Others			
KIR2DS	Human	HLA-C	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
CD94/NKG2C	Human/mouse	HLA-E/Qa-1	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
Ly49D	Mouse	H-2D^{d}	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
Ly49H	Mouse	MCMV m157	Associated with ITAM-bearing DAP12 and activates ZAP70/SYK pathway
NKR-P1C (CD161c)	Mouse	Unknown	Associated with ITAM-bearing CD3-ζ or FcRγ and activates ZAP70/SYK pathway
CD16	Human/mouse	IgG	Associated with ITAM-bearing CD3-ζ or FcRγ and activates ZAP70/SYK pathway
Co-stimulatory molecules			
CD28	Human/mouse	CD80, CD86	Activates PI3K pathway
CD27	Human/mouse	CD70	Activates TRAF pathway and ZAP70/SYK pathway
2B4 (CD244)	Human/mouse	CD48	Associated with SAP

ITAM, immunoreceptor tyrosine-based activating motif; PI3K, phosphatidylinositol-3 kinase; SAP, SLAM-associated protein; SYK, spleen tyrosine kinase; TRAF, tumour-necrosis-factor-receptor-associated factor; ZAP70, zeta-associated phosphoprotein 70.

Table 2: NK-cell activating receptors.
(From Mark J. Smyth, Nature Reviews Cancer 2, 850-861, November 2002)

1.1.1.1. NKG2D

NKG2D is NK receptor associated with responses to cellular distress, such as transformation, infection or cell stress. NKG2D is a type II transmembrane-anchored C-type lectin-like molecule expressed as a disulfide-linked homodimer. NKG2D is present as a homodimeric receptor and recognizes cell-surface molecules distantly related to MHC class I molecules In humans, NKG2D binds to MHC class I-related chain A and B (MICA or MICB) a non-classical class I molecule that is encoded in the human MHC, and human cytomegalovirus glycoprotein UL16 binding protein (ULBP) family members [110]. The first evidence for a protein that binds to NKG2D came from a study showing that a soluble form of MICA binds to various lymphocyte subsets and that monoclonal antibody blocking this interaction, bound also NKG2D. Further analysis showed that MICB — a close relative of MICA — also binds

NKG2D [94]. Expression of NKG2D on the cell surface requires its association with hematopoietic cell signal transducer protein (HCST, also known as DAP10) a transmembrane-anchored adaptor protein expressed as a disulfide-linked homodimer. NKG2D represents an important activating receptor involved in NK-mediated lysis tumor cell lysis [111]. Recently, it has been shown that the expression of human NKG2D ligands is increased by chronic activation of the DNA damage response. In general, the lysis of tumor cells that naturally express NKG2D ligands is partially inhibited by NKG2D-specific antibodies, indicating that NKG2D is an important receptor in the recognition of target cells by NK cells. Nevertheless, tumors evade NK cell response by release of NKG2D ligands or by TGF-β-dependent inhibition of NKG2D activity [112].

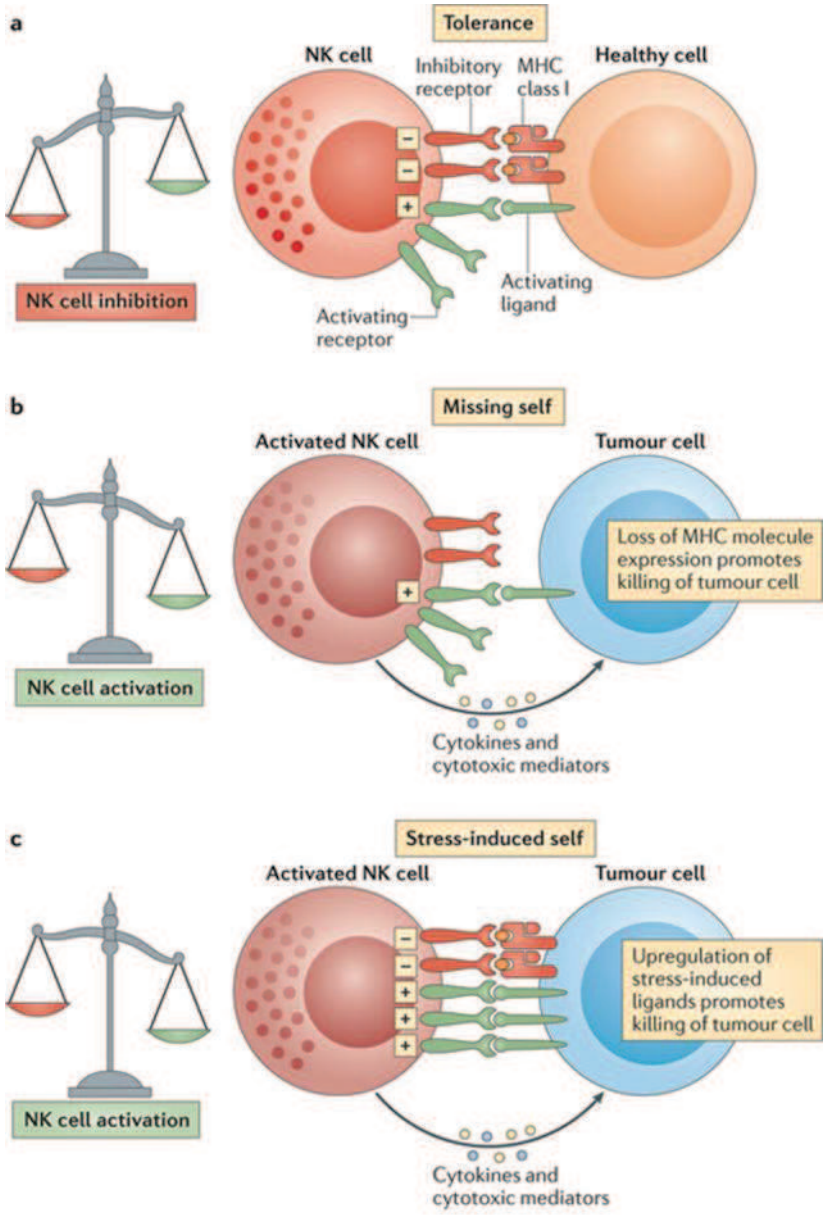

Figure 2: Recognition of tumor cells by NK cells
a) Natural killer (NK) cells are tolerant to healthy host cells, as the strength of the activating signals they receive on encountering these cells is dampened by the engagement of inhibitory receptors (tolerance). **b)** Tumor cells may lose expression of MHC class I molecules. NK cells become activated in response to these cells, as they are no longer held in check by the inhibitory signal delivered by MHC class I molecule engagement. This is known as 'missing-self' triggering of NK cell activation. **c)** In addition, NK cells are selectively activated by 'stressed' cells, which upregulate activating ligands for NK cells and thereby overcome the inhibitory signaling delivered by MHC class I molecules. This is known as 'stress-induced self' triggering of NK cell activation. In both conditions, NK cell activation leads to tumor elimination directly (through NK cell-mediated cytotoxicity) or indirectly (through the production of pro-inflammatory cytokines, such as interferon-γ). (From Eric Vivier, Nature Reviews Immunology 12, 239-252, April 2012).

1.4.5 Natural Killer-Immunological Synapse (NKIS)

Activation of NK cells induces a highly regulated response resulting in release of cytotoxic granules and induction of target cell death. The activation take place at the specialized interface at the cell-to-cell contact point, called immunological synapse (IS), a crucial zone of recognition between ligands and receptors **(Figure 3)** [113].

1.4.5.1 Activating NK cell immunological synapse (aNKIS)

The formation of activating synapse is a multistep process requiring contact and adhesion, ligation of receptor and segregation of signal, rearrangement of actin cytoskeleton and the formation of tight conjugation with the target, polarization of microtubule-organizing center (MTOC), to allow granule polarization and degranulation. After secretion of cytotoxic granules, immunological synapse is disassembled [113].

Initiation stage

In the first step of IS formation, NK cell in response to chemotactic signals, encounters its potential target cell [114]. Molecules involved in the recognition of target depends on interactions with members of selectins family or on recognition of CD15 by CD2 on NK cells [115]. In the next step, firm adhesion is established, owing to high affinity receptor-ligand interactions. In this process are implicated the adhesion molecules of integrin family, such as lymphocyte function-associated antigen1 (LFA1; CD11a-CD18) and macrophage receptor 1 (MAC1; CD11b-CD18). Upon initial contact, LFA1 and MAC1 rapidly segregate to a peripheral supramolecular activation cluster (pSMAC). The mature activating NKIS contains a central and peripheral supramolecular activation cluster (SMAC), and includes polarized surface receptors, filamentous actin (F-actin) and perforin. Evaluation of the NKIS in human NK cells revealed CD2, CD11a, CD11b and F-actin in the peripheral SMAC (pSMAC) with perforin in the central SMAC **(Figure 3a)** [116].

Effector stage

The effector stage begins by LFA1-dependent accumulation of actin at the pSMAC. Actin polymerization and accumulation at the aNKIS is necessary for NK cell cytotoxicity. Fibrous (F)-actin reorganization occurs downstream of activating-receptor-induced guanine nucleotide exchange factor VAV-1 activity and is dependent on Wiskott-Aldrich syndrome protein (WASP) involved in promoting F-actin branching [117]. Simultaneously, activating receptors accumulate in a central supramolecular activation cluster (cSMAC) and form a signalosome, a putative signalling complex, which assembles near the plasma membrane in response to external signals received at cell surface receptors and then migrates towards downstream effectors. Formation of signalosome is involved in the signal amplification and inititation leading to cytokine secretion [113]. Cytolytic response depends on the activation of phosphatidylinositol 3-kinase/ extracellular signal-regulated kinases (PI3K/ERK) and phosphoinositide phospholipase C γ /c-Jun NH(2)-terminal kinase (PLCγ-JNK) pathways, and leads to MTOC polarization to the NKIS and finally the delivery of cytotoxic granules to the target cell, known as process of degranulation [116]. Cytotoxic granules containing perforin and granzymes, are indispensable for NK cell cytotoxicity because their release results in the induction of target-cell apoptosis [118]. Lysosome-associated membrane protein (LAMP) 1 is widely used as a marker for the identification of NK-cell degranulation, as it appears on the cell surface following the fusion of lysosomes with the plasma membrane, but its role in NK-cell biology is still unknown **(Figure 3b)** [119].

Termination stage

After degranulation NK cell detaches from its target and the NKIS is disassembled. This step correlates with a downregulation of the activating receptors such as NKG2D, NKp46 and 2B4. Once NK detached from its target, NK cytolytic potential is restored **(Figure 3c)** [113].

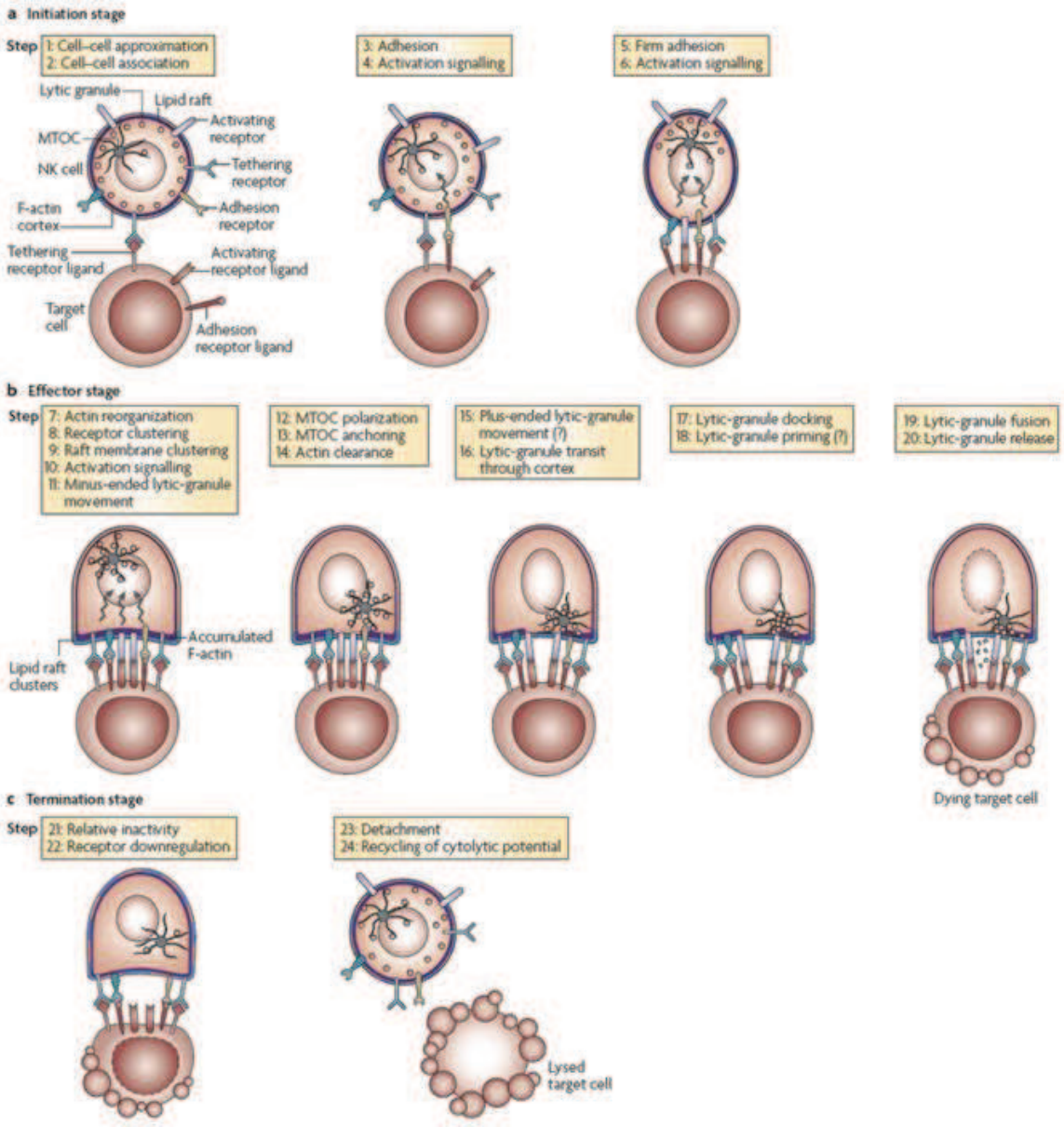

Figure 3: Model for sequential stages in the formation and function of the NK-cell lytic synapse.
The formation of a functional natural killer (NK)-cell lytic synapse can be divided into three main stages — initiation (a), effector (b) and termination (c) — that are each subdivided into multiple steps. Important steps that are proposed to occur in the initiation stage include adhesion and initial signaling for cell activation. In the effector stage, key steps include actin reorganization, receptor clustering, polarization of the microtubule-organizing center (MTOC) and lytic granules, and lytic-granule fusion with the plasma membrane. The crucial steps of the termination stage are proposed to include a period of inactivity and detachment. The specific time required to progress through the various stages varies and is likely to be a feature of the given target cell and the activation state of the NK cell. A linear progression between certain steps (such as the requirement of actin reorganization for MTOC polarization) has been proven experimentally, whereas the linearity between other steps remains hypothetical at present. The inhibitory synapse (see Box 3) has been shown to halt the progression of lytic-synapse formation by interfering with the late steps of the initiation stage (activation signaling) and early steps in the effector stage (actin reorganization and receptor clustering). F-actin, filamentous actin.(From Jordan S. Orange, Nature Reviews Immunology 8, 713-725,September 2008)

1.4.5.2 NK cells functions

NK cell-mediated killing depending on TNF pathway

NK cells express at least three TNF- family ligands able to induce apoptosis: FAS ligand (FASL), Tumor Necrosis Factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL). These ligands bind to and activate their receptors, FAS and TRAIL receptor (TRAILR), respectively, which are expressed by tumor cells. Sensitivity to each of these ligands is controlled by cancer-cell expression of the receptors for these ligands and intracellular protective mechanisms against apoptosis [120]. NK cells that express TRAIL have been shown to kill tumor cells that are sensitive to recombinant TRAIL in vitro. Some NK cells express FAS ligand (FASL) or tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL). This interaction induces tumor-cell apoptosis. The protein c-FLIP (FLICE inhibitory protein) blocks apoptosis induction through this pathway [121]. NK cells secrete various effector molecules, such as IFN-gamma. IFN-gamma exerts antitumor functions in many different ways [122]. Nitric oxide (NO) is one of the most powerful effector molecules in the immune system, and can regulate the cytotoxic function of NK cells against tumor cells. It remains unclear in this situation whether NK cells or another cell type produces NO [123].

NK cell-mediated killing depending on cytotoxic granule endocytosis pathway

NK cells can use the perforin/ granzyme-containing granule exocytosis pathway, the death-receptor-ligand pathway or the nitric oxide pathway to kill tumor cells. NK cells can also produce cytokines such as interferon (IFN)-gamma that restrict tumor angiogenesis and stimulate adaptive immunity [124]. Within a few minutes of effector–tumor-cell interaction, cytotoxic granules are secreted into the intercellular cleft between effector and tumor cells in a calcium-dependent manner. These granules contain perforin, which disturbs the tumor-cell membrane and allows the entry of serine proteases known as granzymes [123]. The two most-

abundant granzymes, A and B, have been implicated in mediating apoptosis of target cells through caspase-dependent and -independent pathways. Signalling through activating receptors induce this activity, whereas inhibitory receptors prevent it [125].

The different NK cell effector pathways to eliminate tumor cells are represented in **Figure 4.**

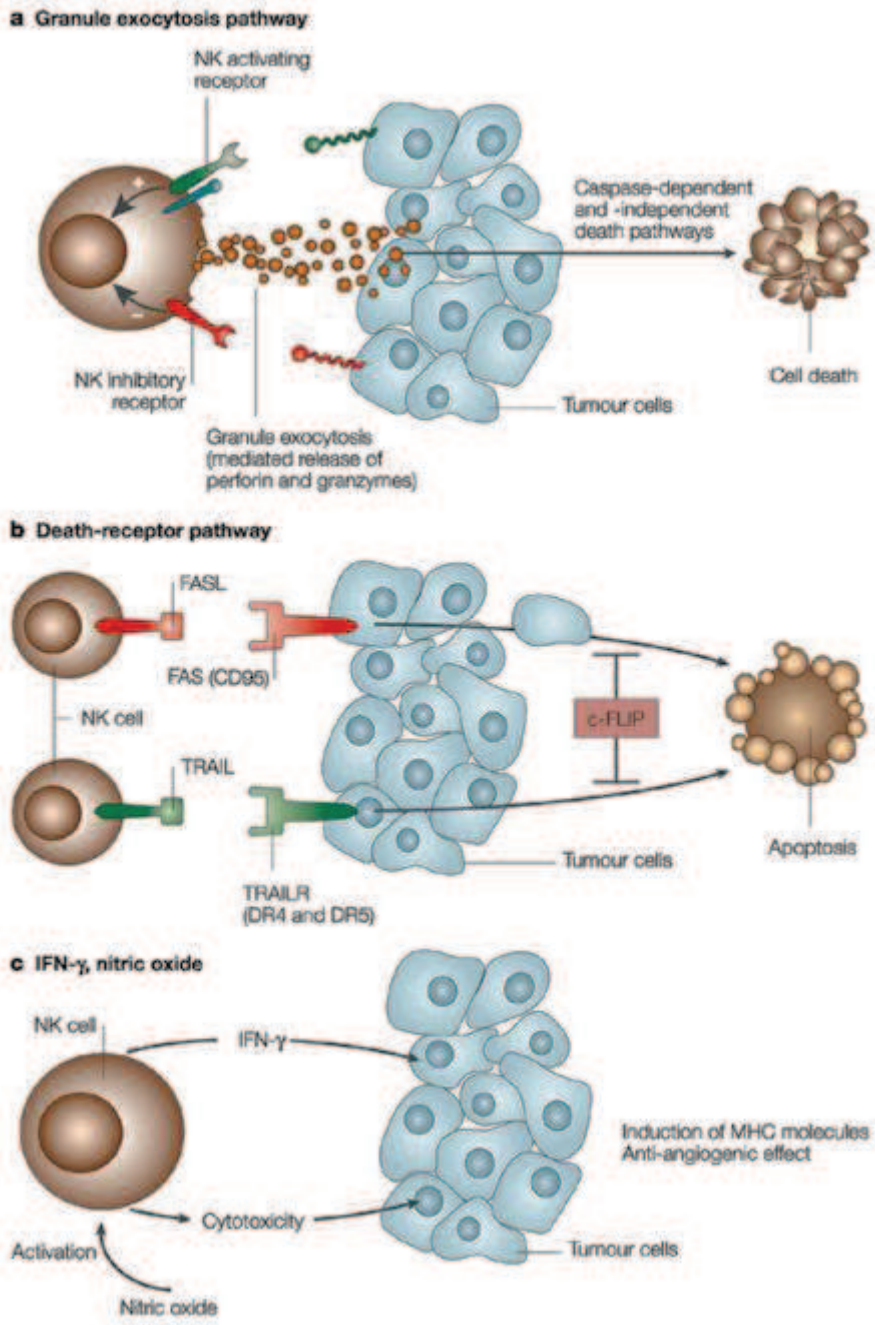

Figure 4: NK cell effector pathways to eliminate tumor cells.
a. Granule exocytosis pathway. **b.** Death receptor pathway. **c.** IFN-γ pathway. (From Mark J. Smyth, Nature Reviews Cancer 2, 850-861, November 2002)

Granzyme B

Granzyme B (Granzyme B) is a caspase-like serine protease inducing programmed cell death after its delivery into target cells [126] . Granzyme B pre-pro-enzyme is activated by two proteolytic cleavages releasing an 18-amino-acid leader sequence coded by exon 1 and a dipeptide motif (Gly-Glu) at the NH_2 terminus. These cleavages are required for its full maturation and for its folding into catalytically active conformation [127]. The delivery of the granzymes to target cells can occurs by: 1)uptake by fluid-phase endocytosis [128], 2) mannose 6-phosphate receptor (M6PR)-mediated endocytosis [125] or 3) mechanism involving heat shock protein 70 (HSP70) bounding Granzyme B at the target cell surface [129]. It has been widely described that in the absence of receptor-mediated uptake, fluid phase uptake can still efficiently deliver Granzyme B into the target cell. Perforin perturbs the target plasma membrane by creating pores allowing, transient calcium influx. This process induces reparation of damaged membrane and endocytosis of cytotoxic granules [118]. Recent studies indicated that perforin activates clathrin- and dynamin-dependent endocytosis, which removes perforin and granzymes from the plasma membrane to enlarged early endosomes - gigantosomes [130]. Early Endosome Antigen (EEA-1) positives gigantosomes are formed by Rab5-dependent fusion of early endosomes with cytotoxic granule. Granzymes and other cargo from this granule are released into the cytosol through perforin (PFN) acting in the gigantosome membrane. Subsequently, PFN forms pores in the gigantosome membrane, allowing release of Granzyme B into the cytosol [131].

Granzyme B-induced apoptosis

Once released inside target cells, Granzyme B initiates apoptotic cell death by cleavage of substrates at the carboxyl side of aspartic acidic residues. Granzyme B-induced target cell death occurs by different mechanisms including mitochondrial disruption, direct activation of

caspases in the cytosol, inducing DNA fragmentation or cleavage of proteins in nuclear membrane and cytoskeleton. In mitochondria, Gzm B cleaves and activates proapoptotic member of Bcl-2 family, Bid [132]. Truncated Bid associates with Bax to induce the release of cytochrome c, the activation of caspases 9 and 3 and the increase in the permeability of the mitochondrial membranes and subsequently induces apoptosis [133]. Importantly, in absence of caspases activation, Granzyme B is still able to kill cells. It has been reported that effector cells lacking Granzyme B displayed impairment in the killing activity, thus highlighting the important role of this protease in executing of cell death in tumor cells. Cancer cells could evade NK mediated immunosurveillance by preventing the initiation of apoptosis by inhibiting Granzyme B. The intracellular serine proteases PI-9 (serpin B9) has been identified as the only natural and irreversible inhibitor of Granzyme B. PI-9 is highly expressed in the cytosol of NKs to protect them from exposure to their own Granzyme B. PI-9 overexpression has been shown in different types of cancer, such as cervical, lung, prostate and colon cancer. Moreover, estrogen-mediated induction of PI-9 protects breast cancer cells against apoptosis mediated by natural killer cells [134].

1.5 Interactions of NK cells with other immune cells

NK cells interact with a number of other cells of the immune system, most notably, DCs, macrophages and T cells. It has been described that NK cells can eliminate DCs loaded with antigens to facilitate the contraction phase where activated T cells undergo apoptosis, and to selectively kill immature DCs through the TNF-related apoptosis-inducing ligand (TRAIL)-mediated pathway [135]. Moreover, IL-12 and IL-15 derived from DC are important cytokines involved in the activation and the secretion of IFN-γ by NK cells. NK cells require multiple contacts with DCs to upregulate CD69, NKp30, NKp46 and NKG2D and to achieve full activation in vivo [136].

Macrophages are another type of immune cells implicated in immune response cross talk with NK. Macrophages display a coordinated sequence of events between the upregulation of MHC-I chain related molecule A (MICA) expression on their surface and the secretion of NK-stimulating proinflammatory cytokines (IL-12, IL-18, TNF, type I IFN) [137].

In addition to the cross talk between myeloid and NK cells, emerging data highlight a bidirectional cross-talk between NK and T cells. NK cells expressing CD86 upon ligation of their activating receptor CD16 induce IFN-γ production and proliferation of autologous T cells [138]. NK cells also kill activated T cells, as following activation, T cells upregulate NKG2D ligands and become susceptible to NK cell-mediated lysis. This mechanism allows NK cells to regulate the quantity of T regulatory cells [139].

1.6 NK cells in cancer treatment

NK cells can protect against experimental tumor growth by direct killing and by producing cytokines involved in the limitation of tumor angiogenesis [140]. Beyond innate activity, NK cells are important for regulation of antitumor adaptive immunity. Recent data confirm that NK cells are required for the elicitation of potent tumor-specific CTL responses [141]. Moreover, significant correlation between high intratumoral levels of NK cells and improved disease outcome was shown in esophageal carcinoma [142], head and neck squamous cell carcinoma [143], gastric cancer [144] and gastrointestinal stromal tumor patients [145]. NK cell activity was described to be decreased in non-small cell lung carcinoma (NSCLC) [146]. Carrega et al. showed that NK cells infiltrating NSCLC are mainly $CD56^{bright}$ CD16- capable of producing relevant cytokines rather than exerting direct cancer cell killing [147].

NK abnormalities in cancer patients

Recent studies of tumor-associated NK cells demonstrated a striking NK-phenotype, supporting the notion that tumor-induced alterations of activating NK cell receptor expression may hamper

immune surveillance and promote tumor progression. Decreased cytotoxic activity of NK cells was observed in different types of human cancer [148].

The mechanisms of cancer immunosurveillance mediated by NK cells depends on the expression of activating and inhibitory receptors. Indeed, the upregulation of NKG2D ligands on stressed cells clearly influences NK-cell mediated tumor immunosurveillance [149]. The cytotoxic responses mediated by NKG2D protect the host from tumor initiation, as supported by reduced expression of NKG2D ligands during tumor progression. In NK-mediated immune responses against cancer, NKG2D is an important receptor, as tumor-associated expression of NKG2D ligands promotes destruction of malignant cells [150]. However, in advanced human cancers NKG2D and its ligands are targeted or exploited for immune evasion and suppression [151]. For example, NKG2D ligands MICA/B and various ULBPs are negatively regulated by cytokines, including TGF-β and IL-1 [54]. Other mechanism involves glycosylation and intracellular transport of NKG2D ligands mediated by carcinoembryonic Ag-related adhesion molecule 1 [152]. Of interest is a report of O-glycan modification of MICA by core2 β-1,6-N-acetylglucosaminyltransferase in bladder tumor cells confering poor prognosis and highly metastatic behavior of bladder cancers [153]. It has been proposed that tumors developed mechanism to escape NKG2D-dependent immune response by shedding of soluble MHC class I-related chain (MIC) proteins [154]. The MICA and MICB ligands for the activating receptor NKG2D shedding from tumor cells and their presence as soluble molecules in sera have been reported to compromise immune response and progression of disease. MICA/MICB shedding reduces cell surface density of NKG2D ligands leading to a reduced susceptibility to NKG2D-mediated cytotoxicity and systemic downregulation of NKG2D on NK cells in cancer patients [153]. Elevated levels of soluble MICA have been detected in the sera of patients with various types of cancer, including gastrointestinal malignancies, breast and lung tumors, melanoma, prostate cancer, pancreatic carcinomas, hepatocellular cancer, and leukemia [155] [156-158].

Moreover, the levels of soluble MICA and MICB in patient sera can be used as diagnostic markers for cancer progression, where their elevated levels often correlate with a poor prognosis for the patient [159].

Other factor influencing NK-mediated tumor killing is the expression of NCRs, such as NKp30. The major role of NKp30 is the regulation of NK-dependent DC killing and promotion of tumor recognition and lysis [160]. The B7 family consists of structurally related, cell-surface protein ligands, which bind to receptors on lymphocytes that regulate immune responses. Recently, the novel B7 family member B7-H6, which is expressed on the cell surface of various tumor cells, was identified as an activating ligand for NKp30 [161]. It has been shown that the down-regulation of NKp30 expression correlates with decreased NK cytotoxicity and decrease the survival of patients with acute myeloid leukemia (AML) [162].

Other important NK-related factors influencing cancer patients' outcome is expression of KIR [163]. KIR recognition of specific human histocompatibility leukocyte antigen (HLA) class I allotypes contribute several complex receptor-ligand interactions that determine NK cell response to its target. Inhibitory receptors prevent NK cell activation directed against cells expressing self-MHC class I molecules. Consequently, cells that do not express self-MHC class I molecules become susceptible to NK cell-mediated attack. It has been shown that expression of activating KIR3DS1 has a protective effect against the development of hepatocellular carcinoma, whereas it results in increased risk of cervical cancer [164]. Moreover, fully humanized anti-KIR therapeutic antibodies blocking KIR/HLA-C interaction boost the reactivity of NK cells against tumor cells expressing ligands for activating receptors [165]. Thus, blocking KIR/HLA-C interaction using IPH2101, a human IgG4 monoclonal antibody against KIR2DL-1, -2, and -3 was shown to augment NK cell killing of autologous tumor cells and enhance NK cell cytotoxicity against multiple myeloma (MM) and AML [166] [167].

HLA-E/peptide complexes are ligands of the CD94/NKG2A inhibitory or the CD94/NKG2C stimulatory complex, expressed on the majority of NK cells [168]. The presentation of "self" signal peptides by HLA-E enables NKs to recognize the overall MHC class I expression on the surface of target cells. CD94/NKG2A expression can be induced in response to cytokines such as IL-15 and transforming growth factor β (TGF-β) [169]. These cytokines are frequently present in the tumor microenvironment, suggesting that CD94/NKG2A inhibitory receptors play a role in immune escape by tumor cells. While the secretion of IFN-γ by NK cells in general is a negative prognostic factor in solid malignancies, in imatinib mesylate-treated gastrointestinal stromal tumors (GIST) NK-mediated IFN- γ secretion leads to antitumor effects [170].

Other mechanism was described in acute myeloid leukemia (AML) cells expressing CD137 ligand (CD137L). Interaction of CD137L with CD137 on activated NK cells induced the release of the immunomodulatory cytokines IL-10 and TNF by AML cells and directly decrease granule mobilization, cytotoxicity, and IFN-γ production by human NK cells, indicating that the immune evasion of AML cells occurs by impairing NK-cell tumor surveillance [171].

Prognostic value of NK infiltration in cancer

Several recent studies correlated tumor infiltration by NK cells to improve disease outcome. High tumor infiltration by NK cells has been associated to a significant improvement of clinical outcome in patients with head and neck squamous carcinoma (HNSCC). Indeed, Van Herpen et al. showed that the $CD56^{+}$ NK cells in IL-12-treated lymph nodes produced considerable amounts of IFN-γ. Thus, increase in intratumoral NK cells infiltration and the high IFN-γ production by NK cells in the lymph nodes, implied a prominent role of the NK cells in antitumor immunity after IL-12 treatment in HNSCC patients [143]. In oral squamous carcinoma, the density of CD57+ NK cells was statistically lower in poor prognostic compared

to good prognostic tumors [172]. In gastric carcinoma (GC) tumor, it has been shown that patients with a high level of NK infiltration had limited lymphatic invasion and metastases compared to patients with a low level of NK infiltration. NK cell infiltration was found to correlate also with depth of invasion, clinical stage, and venous invasion. Consequently, the 5-year survival rate of GC patients with a high rate of NK infiltration was significantly better than that of GC patients with a low level of NK infiltration [173]. In GIST), the 1-year immunomonitoring follow up of NK cell effector functions in 2 phase III trials revealed an enhancement of the NKG2D-dependent cytotoxicity and IL-2/IL-12–dependent IFN-γ production. In particular, this study showed an increase in IFN-γ production, resulting from the Imatinib Mesylate -induced DC/NK cell cross talk, act in concert to block the proliferation of GIST cells and dictate the clinical outcome [174]. Moreover, alternative splicing of the *NKp30* gene was found to affect the signaling of the NK-specific NKp30 protein, and thereby considered as another factor determining the prognosis of patients with metastatic GIST treated with imatinib mesylate [145]. Moreover, recent studies have shown that several molecules abundantly found in tumor microenvironment, such as IDO1 and TGFβ, can negatively influence NK cell phenotype and function [175].

1.7 Strategies for NK-therapy

1.7.1 Activation of endogenous NK cells.

It has been proposed that the enhancement of the anti-tumor NK cell responses can be achieved by the use of several cytokines including IL-2, IL-12, IL-15, IL-18 and IL-21 [176]. Few of them were already used in treatment of human cancers, as they are able to directly or indirectly activate NK cells. Beside the use of cytokines, anticancer chemotherapy can also induce the activation of endogenous NK cells, for example thalidomide and lenalidonide in the treatment of myeloma, increasing effectivity of treatment. NK cells play also important role in well-established clinical efficacy of Mycoacterium bovis BCG treatment of bladder cancer [177].

1.7.2 Allogenic Stem Cell Transplantation

Allogeneic stem-cell transplantation (ASCT) in cancer treatment consists on the transfer of haematopoietic stem cells from unrelated or familial donors to patients with malignancies. Transfusion of donor-derived haematopoietic stem cells restores the function of bone marrow and generates new immune cells including NKT cells. Donor-derived NK cells may recognize and kill residual tumor cells in 'graft-versus-tumor' (GVT) reaction [178]. Interestingly NK cells may mediate GVT effects and has been shown effective in AML [179] and CML [180] .

1.7.3 Adoptive NK-cell immunotherapy

Lack of significant clinical effects using autologous NK cells transfer leads to the use of allogeneic NK cells in adoptive cell therapy for the treatment of cancer. Based on our understanding of the molecular mechanisms that regulate NK-cell function, it has been anticipated that the use of allogeneic NK cells can provide more effective strategy [181]. As most NK cells express inhibitory KIRs, Miller et al. hypothesized that the susceptibility of recipient cells to donor NK cell–mediated lysis is genetically predetermined by the absence of known KIR ligands in AML. In this report a total of 43 patients with advanced cancer (10 with metastatic melanoma, 13 with metastatic renal cell carcinoma, 1 with refractory Hodgkin's disease, and 19 with poor-prognosis AML) were given haploidentical NK-cell infusions together with IL-2 in a non-transplantation setting to determine the safety and the in vivo NK-cell expansion. Using such protocol, 5 out of 19 patients with AML achieved complete remission. Only 4 of the 19 patients were KIR-ligand-mismatched and interestingly, 3 out of these 4 patients achieved complete remission. This study highlighted the fact that complex KIR interactions may influence NK-cell recovery and function and explain failure of clinical improvement in AML. These findings suggest that haploidentical NK cells can persist and expand in vivo and may have a role in the treatment of selected malignancies used alone or in association with haematopoietic stem cell transplantation [179].

NK cells can eliminate cancer cells and it has been shown in clinical studies that NK cells can be used in therapeutic regimens against cancer. Importantly, data from haploidentical haematopoietic SCT and NK-cell-based adoptive immunotherapy indicate potent antitumoral effect mediated by NK cells [181].

1.8 Immune microenvironment in breast cancer

More than 1.3 million women worldwide are diagnosed with breast cancer (BC) each year, making it the second most common form of cancer behind lung cancer [182]. Breast cancer can be successfully treated when detected early, but patient survival markedly decreases with metastatic dissemination to tissues such as bone marrow and lung. Intrinsic breast cancer factors, tumor microenvironment, angiogenesis and tumor-specific immune responses are recently proposed as key factors influencing patients outcome. It is now well admitted that lymphocytes and regulatory T (Treg) cells infiltrating tumor have a great significance in the final clinical outcomes of cancer patients [57] .

Deregulation in antitumor immunity is a common feature in advanced breast cancer patients. Indeed, Perez et al. found that advanced HER2+ breast cancer patients exhibited an overall significantly increased quantity of circulating Treg which can be restored by Trastuzumab treatment. Recently, it has been shown that higher total CD8+ lymphocyte number was associated with longer breast cancer specific survival. Based on these studies, it is tempting to speculate that TIL (Tumor infiltrating Lymphocytes) may represent not only a prognostic but rather a predictive factor of clinical response in breast cancer [77].

The common alterations in anti-tumor immunity in breast cancer patients are characterized by an altered maturation of DCs and an increased infiltration by plasmacytoid DC (pDC) and regulatory T cells. Tumor infiltration by pDC may merely mark a subset of aggressive primary breast cancers with an intrinsic propensity to metastasize. In addition, tumor-infiltration by pDC enhances tumor growth through the production of angiogenic factors, proteases and/or motility

factors that can modify the tumor microenvironment of breast cancer. Moreover, tumor-infiltration by pDC may also induce T cell tolerance or deviate the tumor-specific T-cell response toward a Th2 phenotype and subsequently alter the presentation of tumor-associated antigens to T cells by myeloid DC. Indeed, pDC may inhibit the differentiation of monocytes into DC, reduce the antigen-presentation capacity of myeloid DC, or even convert myeloid DC into mediators of immune tolerance [183].

Other important immune effectors infiltrating BC are regulatory T cell (Treg). Treg represent around 10% of CD4 T cells and express the forkhead box P3 transcription factor (FOXP3). Recent studies have shown that Treg play an essential role in sustaining self-tolerance. Treg-mediated immune suppression is dependent on the expression of the transcription factor FOXP3 which controls some genes encoding proteins like CD25, GITR, CTLA-4, and others, capable of mediating Treg suppressive functions. Recent studies have shown that Treg play an essential role in sustaining self-tolerance and controlling immune suppression in a FOXP3 dependent manner. Indeed, FOXP3 has been shown to control the expression of CD25, GITR, CTLA-4 encoding genes involved in the suppression of Treg functions [184].

Increase in Treg infiltration is correlated with poor prognosis in breast cancer. Treg mediated immunosuppression can also be achieved by the secretion of immunosuppressive cytokines such as IL-10, TGF-β, and others. TGF-β plays a central role in the generation and the function of CD4+CD25+ Treg and in the suppression of IFN-γ production by Th1 and CD8+ T cells. In breast cancer, the increased number of FOXP3 CD4+CD25+ Treg and decreased number of CD8 T cells are correlated with a poor prognosis. Bates et al. showed that elevated number of Treg confers a significant decrease in overall and recurrence-free survival of breast cancer patients and correlates with more aggressive breast cancer features [185]. One of the characteristics is lower absolute number of peripheral blood lymphocytes but increased numbers of functionally suppressive CD4+CD25+ Treg and dysfunctional dendritic tumor

microenvironment. As the depletion of FOXP3 Treg can enhance antitumor immunity, different strategies based on the attenuation of the suppressive function of Treg are being used. Denileukin diftitox (Ontak), a recombinant fusion protein consisting of IL-2 and diphtheria toxin, has been shown to deplete Treg and thus reducing the immune suppression and boosting the antitumor immunity in breast cancer [186] .

Tumor-associated macrophages (TAM) and myeloid-derived suppressive cells (MDSC) within the tumor microenvironment seem to play a critical role in the progression of tumor development. In early stages, tumors are infiltrated by type 1 macrophages (M1) releasing proinflammatory cytokines and chemokines. However, in advanced stages, TAMs polarize to a type 2 macrophage (M2) releasing cytokines such as transforming growth factors β1 (TGF β1) and IL-10, thus favoring the development of Treg and promoting tumor development through the inhibition of anticancer immune responses [187]. In breast cancer, an increased level of M2-TAM was significantly associated with more aggressive phenotype, increased micro vessel density, and decreased overall survival [188].

MDSC represent a heterogeneous population of immature myeloid cells in different stages of myeloid cell differentiation. Within the tumor microenvironment, these cells exert a variety of immune suppressive functions by impairing innate and adaptive immune responses [189]. The immune suppressive function of MDSC depends on the secretion of cytokines (TGF-β, IL-10, and IL-6) and cellular factors. High percentages of circulating MDSC in metastatic breast cancer significantly correlate with a decrease in overall survival and worse outcome, highlighting MDSC level as a good biomarker and a therapeutic target in breast cancer [190].

1.8.1 NK cell infiltration in breast cancer

Natural Killer cells are an important component of the antitumor response and control tumor progression and metastasis in breast cancer (BC) [181]. Gene expression profiling studies have shown a correlation between massive NK infiltration and good outcome in BC patients. It has

been shown that peripheral natural killer (pNK) cells display functional and phenotypic alterations in breast cancer patients [191]. Chemotherapy treatment resulted in a dramatic decrease of the total number of lymphocytes including NK cells. It has been reported that changes in NK functions were more pronounced in advanced stages of cancer [192]. Based on level of early activation marker CD69, it has been proposed that NK cells in breast cancer patients can be still activated. This important finding suggests that immune cells of cancer patients could be responsive and an appropriate immunotherapeutical approach might be useful to restore compromised immune functions [193]. Other important feature of pNK cells in breast cancer patient is the increased proportion of $CD56^{bright}CD16+/-$ and $CD56^{dim}CD16-$ subsets, which are poorly represented in healthy donors. This increase was correlated with high plasma levels of TGF-β, described as a major inhibitors of immune response in tumor microenvironment. The alteration in NK phenotype in invasive BC patients has been characterized by an upregulation in the expression of NK inhibitory receptors NKG2A and CD85j and a downregulation in the expression of NK activating ligand such as NKp30, NKG2D, 2B4 and CD16 in metastatic compared to noninvasive tumors. It has been demonstrated that the ability of NK cells to kill target cell, to degranulate and to secrete IFN-γ were also altered in invasive BC. In this regards, the involvement of the tumor microenvironment in the alteration of NK phenotype and function has been proposed. NK cells activity and function can be impaired by soluble factors secreted by tumor cells. Indeed, tumor supernatants are able to dramatically inhibit all NK cell functions by inducing the secretion of IFNγ. Moreover, it has been shown that blocking of TGFβ1 in tumor supernatants partially restored NK cell function. More interestingly, it has been shown that these alterations are reversible in long-time remission patients with invasive tumors. These observations highlight the tumor microenvironment as an important immune suppressive mediator of the NK cell activity [194].

1.8.2 NK-Cell Therapy of Breast Cancer

1.8.2.1 Antibody-Dependent Cellular Cytotoxicity

Approximately 15%-25% of primary breast cancers overexpress the HER2 protein due to amplification in the HER2 gene. The overexpression of HER2 is associated with an aggressive clinical phenotype, increased disease recurrence, and an unfavorable prognosis. The anti-HER-2/neu monoclonal antibody trastuzumab has been shown to engage both activatory and inhibitory NK receptors. When trastuzumab (Herceptin®, Genentech), an IgG1-type antibody, binds to HER2 overexpressing target cells, immune effector cells such as NK cells, actively recognize these tumor cells via the Fcγ-part of trastuzumab. Subsequently, NK cells induce tumor cell death through antibody-dependent cell-mediated cytotoxicity (ADCC). It is administered for the treatment of patients with HER2-positive breast and gastric cancers [195]. Other monoclonal antibody inducing potent ADCC is Cetuximab [196]. Cetuximab is a chimeric mAb that binds EGFR, and some reports suggest its efficacy in BC. It has been shown that NK cells were able to kill more efficiency metastatic breast cancer cell line IIB-BR-G MT displaying low EGFR expression level in presence of Cetuximab (ccr 2011). Moreover, recent study has shown that the NK-mediated ADCC of a breast cancer cell line mediated by either trastuzumab or cetuximab was enhanced by lenalidomide [197]. ADCC-mediated activation of an innate immune response results in cytokine secretion and antigens liberation, which subsequently lead to the induction of a potent host adaptive immune response against tumor antigens. Recently, the positive effect of monoclonal antibodies in inducing NK-dependent ADCC was confirmed by clinical studies several clinical studies such as phase II and III CLEOPATRA and NEOSPHERE clinical studies [195].

1.8.2.2 Adoptive Cell Transfer

NK cells represent a promising weapon for effective adoptive immunotherapy. Adoptive transfer of NKs has shown remarkable responses in different hematological malignancies. Early

clinical trials showed modest clinical success using autologous NK with high-dose IL-2 in different types of cancer. It is well admitted that the failure of autologous NK therapy is due, at least in part, to the down-regulation of NK cell killing. This was related to the recognition of self-class I MHC on tumor cells, making allogeneic cell transfer more attractive option for cancer immunotherapy [181]. Sustained in vivo NK cell expansion may be limited by host rejection, competition with host lymphocytes or suppression by recipient Treg or MDSC. New clinical trial is now recruiting patients to test the effect of allogeneic NK transfer in metastatic breast cancer in combination with IL-2 following a preparative regimen of cyclophosphamide, fludarabine and cyclosporine (NCT01105650). More effective strategies to augment in vivo NK cell persistence, expansion and effectiveness of killing are needed to improve patient outcome in NK-based cancer immunotherapy [198] .

1.9 AUTOPHAGY

To adapt to hypoxic stress, cancer cells activate autophagy and undergo a metabolic shift to increase their dependency on anaerobic metabolism [199]. Upregulation of autophagy in cancer cells increases cell survival through liberation of nutrients, decreases the buildup of reactive oxygen species, and induces a clearance of misfolded proteins [200]. Autophagy is a cellular degradation pathway highly conserved in all eukaryotes, controlling degradation of proteins and organelles. Autophagy plays an essential role in survival, development and homeostasis [201]. Post-translational modulation of the Atg (autophagy-related genes) proteins adds additional complexity to autophagic pathway entry points for crosstalk with other cellular processes and helps to define cell-type-specific regulations of autophagy [202]. Beyond the simplistic view of a process dedicated to the turnover of proteins and cellular components, recent data have shown that autophagy and autophagy-related genes are implicated in regulation of immune responses in cancer [203].

1.9.1 Types of autophagy

There are at least three different types of autophagy described so far: macroautophagy (hereafter referred to as autophagy), microautophagy and chaperone-mediated autophagy. We will describe briefly micro and chaperone mediated autophagy and provide more detail for the macroautophagy [201].

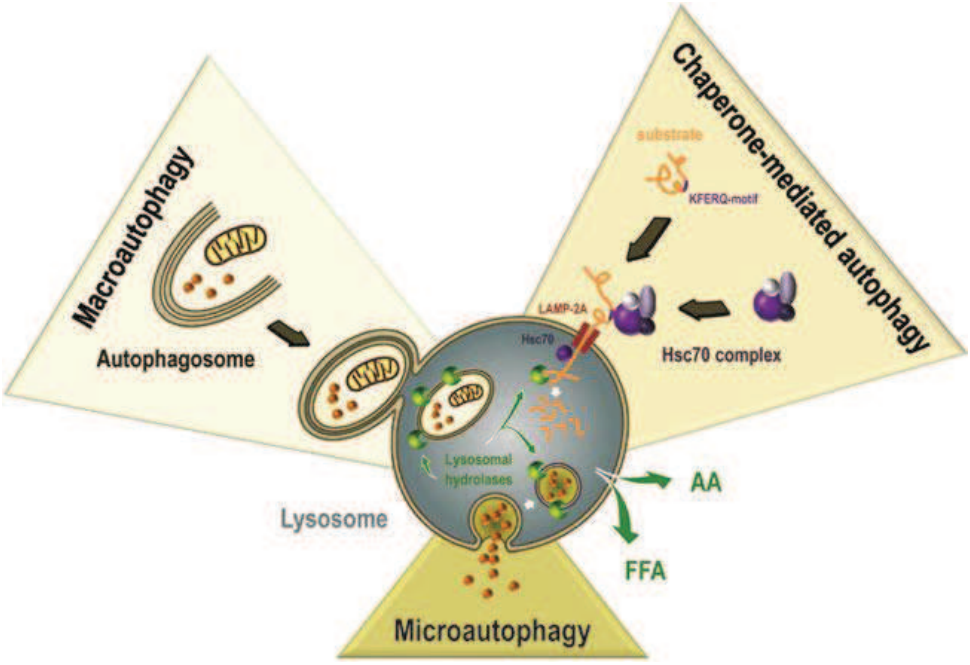

Figure 5: Schematic representation of the different types of autophagy.
Chaperone-mediated autophagy sequesters proteins harboring a KFERQ-like motif that, mediated by the Hsc70 complex, are directly targeted to the lysosomes for degradation. During microautophagy the lysosomal membrane invaginates to engulf portions of the cytoplasm, which are consequently broken down once entirely enclosed. During macroautophagy specialized vacuoles are formed for cargo transportation. These vacuoles, called autophagosomes, are double membrane bound and deliver proteins, lipids and organelles to the lysosome.

1.9.1.1 Microautophagy

Microautophagy is a process by which cytoplasmic contents enter the lysosome through an invagination or deformation of the lysosomal membrane. A recent study provided evidence that microautophagy transports soluble cytosolic proteins directly to late endosomal multivesicular bodies [204].

1.9.1.2 Chaperone-Mediated Autophagy (CMA)

In contrast to microautophagy and macroautophagy which can both nonspecifically engulf bulk cytoplasm, CMA is highly specific type of autophagy in which unfolded proteins are translocated through the action of cytosolic chaperones to the lumen of lysosome to be degraded in this compartment. CMA degrades a wide range of substrate proteins, including some glycolytic enzymes, transcription factors and their inhibitors, calcium and lipid binding proteins, proteasome subunits, and proteins involved in vesicular trafficking [205]. Moreover, CMA eliminates oxidized and misfolded proteins and provides amino acids during prolonged periods of starvation. Cytosolic substrates for CMA contain a KFERQ-related motif recognized by the cytosolic constitutive chaperone hsc70 (heat shock cognate of the Hsp70 family). This recognition allows the translocation of unfolded polypeptides across the lysosomal membrane through the lysosomal membrane protein, LAMP-2A. Binding of substrate to LAMP2A, leads to its multimerization. Following transport of the substrate into the lysosomal lumen, LAMP2A returns to a monomeric state where it can bind new substrate and initiate a new round of translocation [206].

1.9.1.3 Macroautophagy

Initiation

The typical inducers of autophagy are nutrient starvation, hypoxia and other types of cellular stress. Membrane dynamics during autophagy are highly conserved from yeast to plants and animals. Exocytic membrane traffic begins with polypeptide translocation into the ER and their subsequent export from the ER to the Golgi apparatus [201]. Autophagy involves the de novo formation of a double-membrane autophagosome which sequesters cytoplasmic material and fuses with lysosomes for degradation. Autophagosomes are generated from sites where autophagy- related (Atg) proteins come together, in yeast this is known as the phagophore

assembly site (PAS). Although this could potentially occur from multiple membrane sites, the ER is a prime location [207].

In the first step of autophagosome formation, cytoplasmic constituents, including organelles, are sequestered by an isolation membrane called phagophore. Complete sequestration by the elongating phagophore results in formation of the autophagosome, a double-membraned organelle. One of the most substantial molecules in the maturation of autophagosomes/endosomes is Rab7, a member of small GTPases. Rab7 designates the maturation of endosomes and also autophagosomes, directing the trafficking of cargos along microtubules, and finally, participating in the fusion step with lysosomes. Rab7 is an effective multifunctional regulator of autophagy and endocytosis [208]. In yeast, induction of autophagosome formation is regulated by the formation of complex between several Atg proteins (Atg1-Atg13-Atg17-Atg31-Atg29). In mammalian cells the complex consist of an Atg1 homolog Unc-51-like kinase family (either ULK1 or ULK2), the mammalian homolog of Atg13 (ATG13), and RB1-inducible coiled-coil 1 (RB1CC1/FIP200) [209]. Under nutrient-rich conditions, MTORC1 associates with the complex and inactivates ULK1/2 and ATG13 through phosphorylation. During starvation, MTORC1 dissociates from the complex and ATG13 and ULK1/2 become partially dephosphorylated allowing the induction of macroautophagy. The ULK1/2-ATG13-RB1CC1 complex is stable and its formation occurs independently of the nutrient status [210]. The association of the induction complex with the mammalian target of rapamycin complex 1 (MTORC1) depends on nutrient status. Under nutrient-rich conditions, MTORC1 associates with the complex, and phosphorylates ULK1/2 and ATG13, inactivating them. However, treatment of cells with rapamycin or starvation of nutrients induces dissociation of MTORC1 from the induction complex, resulting in induction of macroautophagy [211].

Nucleation

The next complex recruited to the putative site of autophagosome formation is the ATG14-containing class III phosphatidylinositol 3-kinase (PtdIns3K) complex. This complex is involved in the nucleation of the phagophore and consists of PIK3C3/VPS34, PIK3R4/p150 (Vps15 in yeast), and BECN1 (Vps30/Atg6 in yeast) [212]. The complex can be positively regulated by AMBRA1 and negatively regulated by BCL2 binding to BECN1 preventing its association with the complex. It has been shown that starvation activates autophagy by dissociating the Beclin1/Bcl-2 (or Bcl-XL) complex [213]. Therefore, it was proposed that Bcl-2 is not only an anti-apoptotic but also an anti-autophagic protein. Beclin 1 is a mammalian Atg6/Vps30 (vacuolar protein sorting 30) ortholog and a subunit of the class III PI3-kinase complex. Beclin 1 was originally identified as an interacting partner of Bcl-2. The antiapoptotic protein BCL2 binds BECN1 and prevents its interaction with PIK3C3; thus, inhibiting macroautophagy [214]. The interaction between Bcl-2/Beclin 1 has been reported to be mediated by BH3 domain in Beclin 1. In addition to Beclin 1 and Bcl-2 expression levels, cellular localization is a determining factor regarding the effects of Bcl-2/Beclin 1 interaction on autophagy and apoptosis [215]. Initial studies demonstrated that Bcl-2 and Beclin 1 interact at the endoplasmic reticulum (ER) and mitochondria. However, it appears that only Bcl-2/Beclin 1 localization to the ER results in inhibition of autophagy. This cellular location and complex formation is regulated by phosphorylation of key kinases. The dissociation of Bcl-2/Beclin 1 complex can occur by phosphorylation of Bcl-2 by stress-activated c-Jun N-terminal protein kinase 1 (JNK1). Furthermore, only the ER-enriched pool of Bcl-2 is subjected to regulation of Beclin 1 binding by JNK1-mediated phosphorylation, another indication that regulatory mechanisms occur at particular subcellular localization. Once Bcl-2 is phosphorylated and dissociates from Beclin 1, autophagy can occur. Upon continuous JNK pathway activation and Bcl-2 phosphorylation, the cells will eventually undergo apoptosis via

caspase 3 activation [216]. Death-associated protein kinase (DAPK) is also involved in regulating the Bcl-2/Beclin 1 complex. DAPK phosphorylates a threonine within the BH3 domain of Beclin 1, which induces dissociation and allows autophagy to proceed. Interestingly, a recent study demonstrated that NAF-1 (nutrient-deprivation autophagy factor-1) binds Bcl-2 at the ER, independent of a BH3 domain, and is required for Bcl-2 to inhibit Beclin 1-mediated autophagy. Beclin 1 and Bcl-2 can form complexes with additional autophagy regulatory proteins at different cellular locations [217]. AMBRA1 (activating molecule in Beclin 1-regulated autophagy) is a key regulator of autophagy and has been shown to interact with both Beclin 1 and Bcl-2. In response to autophagic stimuli, unc-51 -like kinase 1 (Ulk1) phosphorylates AMBRA1, which results in AMBRA1 dissociation from the Dynein motor complex. After dissociation, AMBRA1 translocates to the ER, binds to Beclin 1 in the autophagy initiation complex, which results in the induction of autophagy. Alternatively, a recent study demonstrated that Bcl-2 localized to the mitochondria can also bind AMBRA1, but ER-localized Bcl-2 does not. The Bcl-2:AMBRA1 interaction at the mitochondria is downregulated during autophagy, as well as during apoptosis. Therefore, Bcl-2 can regulate Beclin 1-induced autophagy by direct binding to Beclin 1, as well as by sequestering the activator of Beclin 1 [218]. UV-irradiation-resistance-associated gene (UVRAG) was shown to be a member of the class III PI3-kinase complex and a positive regulator of autophagy. Moreover, UVRAG complex (UVRAG/BECN1/PIK3C3/PIK3R4) is involved in the endocytic pathway and also participates in macroautophagy. In yeast, several proteins, such as Atg18 and Atg21 are able to bind PtdIns3P generated by the Vps34 complex [212]. Mammalian cells express two Atg18 orthologs, WIPI1 and WIPI2, which, through its binding to PtdIns3P, are associated with phagophores during amino acid starvation [219]. Another PtdIns3P-binding protein in mammalian cells is the zinc finger, FYVE domain containing 1 (ZFYVE1/DFCP1), which associates with PtdIns3P-enriched omegasomes. Omegasomes aid in autophagosome

biogenesis in two ways: in addition to attract down-stream effectors, some of which are direct targets of PI3P (e.g. members of the WIPI family of proteins) omegasomes also physically demarcate the outer edges of the growing autophagosomal membrane until those edges fuse to generate the double membrane vesicle [220]. However, the connection of omegasomes to upstream components such as the ULK1 complex is not clear. While down-regulation of proteins of the ULK1 complex inhibits the formation of omegasomes, inhibition of Vps34 has been reported not to prevent the starvation-induced translocation of ULK1 to puncta. At the same time, components of the ULK1 complex and omegasomes have been reported to be close but physically distinct during autophagy, suggesting that the ULK1 complex may leave the pre-autophagosomal sites before omegasomes are formed. The precise functions of WIPI1/2 and ZFYVE1 in autophagy are still unknown [221].

Elongation

In yeast and mammals two conjugation systems involving ubiquitin-like (UBL) proteins contribute to the expansion of the phagophore [201]. The first system is involved in the formation of Atg12/Atg5/Atg16 complex. In yeast, the UBL protein Atg12 is covalently conjugated to Atg5. This conjugation depends on E1 activating enzyme Atg7 and E2 conjugating enzyme Atg10 [222]. This process differs from ubiquitination by the fact that the conjugation of Atg12 to Atg5 is irreversible and does not require an E3 ligase enzyme. Following Atg12/Atg5 conjugation, Atg16 noncovalently binds Atg5 and dimerizes to form a larger complex. Mammalian orthologs of ATG5, ATG12 and ATG16L1, have been identified, and the complex formation of ATG12/ATG5/ATG16L1 with phagophore membrane has been reported. Following autophagosome completion the ATG12/ATG5/ATG16L1 complex is dissociated from following. This complex is regulated through the Golgi protein RAB33A, which can bind to and inhibit ATG16L1 [223].

The second UBL system involved in phagophore expansion is the Atg8/LC3 system. This conjugation pathway in yeast begins with processing of Atg8 by the cysteine protease Atg4 to expose a glycine residue at the C terminus of Atg8 [224]. The E1-like enzyme Atg7 activates the processed Atg8 and transfers it to the E2-like enzyme Atg3. Finally, the C-terminal glycine of Atg8 is covalently conjugated to the lipid phosphatidylethanolamine (PE). The Atg12/Atg5 conjugate, which may act as an E3 ligase, facilitates this final step. Atg8–PE is membrane-associated, but can be released from membranes as a result of a second Atg4-mediated cleavage. The mechanism of regulation of the second Atg4-dependent processing event, referred to as deconjugation, is not known; however, this appears to be an important step in autophagy because defects in cleavage result in partial autophagic dysfunction [225].

Mammalian homologs of the Atg8/LC3 system function much like their yeast counterparts. Unlike yeast, which have only one Atg4 and one Atg8, mammals have four isoforms of ATG4 and several Atg8-like proteins, the latter of which are divided into the LC3 and GABARAP subfamilies. Whereas both subfamilies can localize with autophagosomes, it has been proposed that they function at different steps in phagophore elongation and completion, with the LC3 subfamily acting before the GABARAP subfamily [226]. Among the Atg8-like proteins in mammals, LC3 has been the best characterized. The ATG4-processed form of LC3 is referred to as LC3-I and the PE-conjugated form is called LC3-II. Lipidation of LC3 in mammalian cells is accelerated under conditions of nutrient starvation or other types of stress. Only LC3 has been identified on the autophagosomal inner membrane. LC3 has been proposed to function as a receptor for the selective substrate, p62/SQSTM1. p62/SQSTM1 binds to LC3 and is preferentially degraded by autophagy and accumulates in autophagy-deficient cells. Furthermore, since p62/SQSTM1 has a ubiquitin-binding domain, it has been proposed that ubiquitinated proteins and inclusion bodies can be recruited to the autophagosome membrane via p62/SQSTM1 [227].

Another protein thought to function in elongation of the phagophore is the transmembrane protein ATG9. In yeast, Atg9 may cycle between the pre-autophagosomal structure (PAS) and peripheral sites. These peripheral sites are referred to as Atg9 reservoirs or tubulovesicular clusters (TVCs) [207]. The TVCs may be direct membrane precursors to the PAS, and thus, to phagophores . The movement of Atg9 is dependent on the Atg1-kinase complex, as well as multimerization of Atg9. The abilities of Atg9 to traffic and multimerize are necessary for autophagosome formation, suggesting that these properties of Atg9 contribute to a role for this protein in recruiting membrane to the expanding phagophore [228]. Atg9 is also seen to shift localization within the cell and is proposed to have a similar role in membrane recruitment. Under nutrient-rich conditions, ATG9 localizes to the trans-Golgi network and late endosomes. When cells are starved for nutrients, however, ATG9 colocalizes with autophagosomal markers. This cycling to autophagosomes is dependent on both ULK1 and PtdIns3K activity and is negatively regulated by MAPK14/p38α. The exact functions of ATG9 in the cell, and how the ULK1 complex regulates ATG9 movement, are poorly understood [229].

Autophagosome completion and fusion

The expanding phagophore mature and close to form a completed autophagosome, which traffics to and fuses with an endosome and/or lysosome, becoming an autolysosome. The inner membrane of the autophagosome and the cytoplasm-derived materials contained in the autophagosome are then degraded by lysosomal/vacuolar hydrolases [201]. One unique enzyme in this process is yeast Atg15/Aut5/Cvt17, which is a putative lipase that is likely involved in the intravacuolar lysis of autophagic bodies [230]. In addition, it has been proposed that autophagosomes fuse with endosomes to become amphisomes before fusion with lysosomes. Fusion with endosomes is believed to provide nascent autophagosomes with machinery that is required for lysosome fusion. Movement of autophagosomes to lysosomes is dependent on microtubules. Fusion of autophagosomes with endosomes involves the protein VTIlB.

UVRAG, which can associate with the PtdIns3K complex, can activate the GTPase RAB7, which promotes fusion with lysosomes. It has also been suggested that components of the SNARE machinery, such as VAM7 and VAM9, have a role in fusion. Recent work has identified another SNARE, syntaxin 17, which localizes to completed autophagosomes and is required for fusion with the endosome/lysosome through an interaction with SNAP29 and the endosomal/lysosomal SNARE VAMP8. Once macromolecules have been degraded in the lysosome/vacuole, monomeric units (e.g., amino acids) are exported to the cytosol for reuse [231].

Autophagosomes may fuse in certain circumstances with late endosomes, resulting in hybrid organelle formation, called amphisome. Amphisomes then fuse with lysosomes to be degraded. Autophagosome-derived MVBs can fuse with the plasma membrane, which releases the internal vesicles of the MVBs to the extracellular milieu [2 3]. The different steps of the autophagic process are reported in **figure 6.**

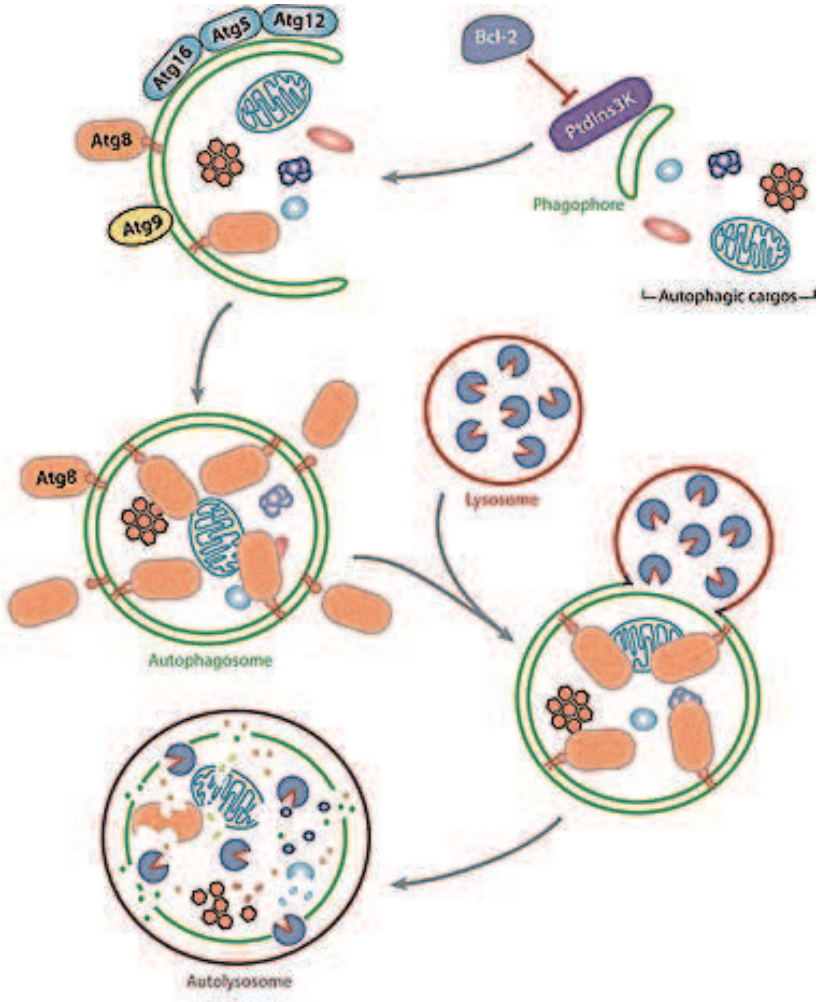

Figure 6: Schematic model of autophagy.
The class III PtdIns3K complex mediates nucleation of the phagophore membrane, enwrapping cytosolic proteins, protein aggregates, and organelles (such as mitochondria). Bcl-2 blocks this step by binding and inhibiting Beclin 1, a component in the PtdIns3K complex. Atg12–Atg5-Atg16 and Atg8–PE conjugates are recruited to the phagophore, together with the transmembrane protein Atg9, facilitating the phagophore expansion step. Upon vesicle completion, most of the Atg proteins are dissociated from the autophagosome, allowing autophagosome-lysosome fusion and cargo degradation by lysosomal proteases.

1.9.2 Principal functions of autophagy

1.9.2.1 Adaptation to metabolic stress: starvation and hypoxia

During prolonged starvation necessary amino acids are produced by autophagy, which is up-regulated as an adaptive response. Autophagy is a major contributor to cycle in which glucose from carbohydrate stores is supplied through gluconeogenesis in the liver [232]. Furthermore, amino acids can be used as an energy source through the tricarboxylic acid (TCA) cycle and recent studies have suggested that energy can be produced through autophagy. Then, amino

acids produced by autophagy can be used to synthesize proteins, which are important for adaptation to starvation environments [210]. In addition to nutrient limitation, autophagy is an adaptive response to hypoxia. HIF-1-mediated autophagy is induced in a mouse cerebral ischemia-hypoxia model and in ischemic myocardium[233]. Furthermore, autophagy occurs in an in vitro metabolic stress model, as well as in the center of tumors where ischemic conditions exist until angiogenesis is established. Such metabolic stress typically induces apoptosis, but apoptosis-defective cells can survive under hypoxic conditions. Since cell survival depends on autophagy, excess amino acid generation is likely important under conditions of metabolic stress. If both apoptosis and autophagy are suppressed, cell survival is severely impaired. Intriguingly, the resulting necrotic cell death promotes tumorigenesis, which is probably mediated by the inflammatory response. Thus, tumorigenesis may be a secondary effect of autophagy suppression in this context [234].

1.9.2.2 Elimination of cytoplasmic contents

Autophagy participates in intracellular clearance or protein/organelle quality control. The most direct evidence is the accumulation of abnormal proteins and organelles in autophagy-deficient hepatocytes, neurons, and cardiomyocytes even in the absence of any disease-associated mutant protein. Soluble ubiquitinated proteins, ubiquitin-positive inclusion bodies, and deformed organelles accumulate in these cells. Since induced autophagy is not observed in the brain during starvation, low levels of basal autophagy are likely sufficient for quality control [235].

The defects in the elimination of cytoplasmic contents by autophagy cause various cellular malfunctions, leading to neurodegeneration and tumorigenesis. The accumulation of autophagic vacuoles has been observed in many human neurodegenerative diseases, including Alzheimer's disease, polyglutamine (CAG) repeat diseases, and Parkinson's disease. Another possible outcome of defects in autophagic degradation is tumorigenesis. Although autophagy may be a survival mechanism for tumor cells, there are many reports indicating that autophagy may act

as a tumor suppressor [236]. Monoallelic deletions of Beclin 1 are frequently observed in human breast, ovarian, and prostate cancers. Like Beclin 1, UVRAG is mutated in human cancers [237]. DNA damage and genomic instability were demonstrated in mammary epithelial cells in response to metabolic stress when both autophagy and apoptosis were suppressed. This genome damage and genetic instability promoted by defective autophagy may drive tumor progression by elevating the mutation rate. It may be that autophagy prevents the accumulation of abnormal proteins and organelles that are harmful to genomic stability [238].

1.9.2.3 Autophagy in antigen presenatation

Autophagy present endogenous antigens on major histocompatibility complex (MHC) class II molecules, recognized by CD4+ T cells. The antigen-binding site of MHC class II molecules is blocked by the invariant chain until they reach the MHC class II loading compartment (MIIC), related to the lysosome. Recent evidence suggests that autophagy (both macroautophagy and CMA) accounts for the delivery of these peptides. Furthermore, it was shown that influenza antigen fused to LC3 was preferentially incorporated into autophagosomes and very efficiently presented on MHC class II molecules [239].

The autophagy pathway is used by plasmacytoid dendritic cells to recognize viral single-stranded RNA (ssRNA) [240]. Toll-like receptors (TLRs) are key molecules in innate immunity, which recognize various molecules derived from microbes. Some TLRs localize to endosomes (TLR3, TLR7, TLR8, and TLR9). Among these, TLR7 recognizes viral ssRNA in endosomes and triggers immune responses, such as induction of inflammatory cytokines. It was recently demonstrated that cytosolic replication intermediates are also recognized by TLR7 following transportation to endosomes/lysosomes via autophagy, leading to interferon-α secretion by plasmacytoid dendritic cells [241].

1.9.2.4 Sequestration/packing

Autophagy can be induced by ER stress. ER stress-induced autophagy is basically protective against cell death in both yeast and mammals. How autophagy protects cells during ER stress is not exactly known, but it was suggested that sequestration of ER into autophagosomes might be sufficient to relieve ER stress. Furthermore, vacuolar proteases are not required for this protective effect, confirming that ER sequestration, rather than degradation, is important in the response to ER stress. It is possible that stressed ER is segregated into ERAs so that it does not disseminate cytotoxic signals. Whether this is also the case in mammalian cells remains unknown [242].

1.9.3 Hypoxia induction of autophagy

To adapt hypoxia, cells can activate autophagy through HIF-1α-dependent or -independent pathways. The induction of autophagy in HIF-1-dependent manner is associated with the induction of atypical Bcl-2/adenovirus E1B 19-kDa interacting protein 3 and Bcl-2/adenovirus E1B 19-kDa interacting protein 3 like (BNIP3/BNIP3L) proteins. BNIP3 and BNIP3L are members of the so-called BH3-only subfamily of Bcl-2 family proteins that heterodimerize and antagonize the activity of the prosurvival proteins (Bcl-2 and Bcl-XL), thereby activating authophagy independently of mTOR [244]. In normoxia, the atypical BH3 domain in Beclin 1 forms low affinity complexes with Bcl-XL and Bcl-2 decreasing the rate of autophagy. In hypoxia, induction of the atypical BH3-only proteins (HIF-dependent gene products, BNIP3 and BNIP3L) disrupts Beclin 1/Bcl-XL/Bcl-2 complex, leading to autophagy. The affinity of the BH3 domains of the BNIPs is too low to form tight complexes with Bcl-XL. Therefore, BNIP3/BNIP3L fail to induce cell death. Interestingly, severe hypoxic conditions or anoxia are capable to induce HIF-independent autophagic response, implicating the AMPK/mTOR and

unfolded protein response (UPR) pathways. The autophagic cell death that is often observed in these extreme stress conditions should be seen as the outcome of failed adaptation [245].

In the context of cancer, hypoxia-induced autophagy in tumor cells is now well described to be a new mechanism of tumor resistance and survival. Recently, the role of autophagy in regulating the anti-tumor immune response has been reported. Therefore, understanding how autophagy modulates tumor immune response represents a major challenge in the field of tumor immunotherapy [243].

1.9.4 Autophagy regulation of the immune cell activity in tumor microenvironment

Despite the hostile hypoxic microenvironment, multiple cell types of the innate and adaptive immune system are capable to recognize and eliminate tumor cells. This is related to their capacity to adjust their metabolic dependency by activating autophagy [246].

Neutrophils

Neutrophils are the first type of immune cells to migrate to the inflammatory site of the tumor where they promote inflammation and activate macrophages and dendritic cells (DCs). Neutrophils display high glycolytic rate making them resistant to hypoxic condition. This resistance has been shown to be dependent on autophagy activation. It has been shown that autophagy-related form of programmed neutrophil necrosis plays an important role in decreasing inflammation and ultimately lead to limit tumor growth [247].

Antigen Presenting Cells (APCs)

APCs such as macrophages and dendritic cells (DCs) adapt their metabolism to hypoxia through stabilization of HIF-1α. Hypoxia decrease phagocytosis in macrophages and DCs, reduces their migratory capacity, and increases production of proangiogenic and proinflammatory cytokines such as VEGF, tumor necrosis factor (TNF)-α, IL-1, and IL-12 [248]. While, hypoxia is involved in dampening APC activity, autophagy in tumor-infiltrating APCs is involved in their survival. This occurs likely by liberation of nutrients required to support the energy demands

of activated cells and is important for the cell's antigen presentation capabilities. Autophagy is important for the process of antigen presentation and activation of T cells through improved MHC expression [249].

T lymphocytes

Autophagy is activated in T cells upon T cell receptor engagement in both $CD4^+$ and $CD8^+$ subtypes. Targeting autophagy by silencing ATG5 or ATG7 during T cell receptor stimulation leads to a significant decrease in cellular proliferation, highlighting the importance of autophagy during T cell activation [250]. It has been shown that when mature T cells traffic to the periphery, autophagy is required for their survival. The role of autophagy in promoting mature T cell survival has been attributed to the ability of autophagy to degrade essential components of the apoptotic cell death machinery and maintain mitochondrial turnover [251]. Moreover autophagy supplies the metabolites necessary to generate ATP production required for cell survival during sustained growth factor withdrawal in bone marrow hematopoietic cells [252].

1.9.5 Effect of autophagy on tumor cell susceptibility to immune cells

Autophagy has been described as a mechanism of cell survival under stress condition and thereby as a mechanism tumor resistance to therapies. Based on this statement, the role of autophagy inhibition in the anti-tumor immune response has recently received widespread attention.

It has been shown that targeting autophagy in hypoxic tumor cells restores CTL-mediated killing [253]. It appears that HIF-1α and autophagy coordinately operate to induce and stabilize a survival pathway involving the activated signal transducer and activator of transcription-3 STAT-3. Furthermore, targeting autophagy *in vivo* enhances the anti-tumor effect of tumor vaccine [253]. These findings provide important clues to cancer immunotherapy and extend the

notion that simultaneously boosting the immune system and targeting autophagy could enhance the therapeutic efficacy of cancer vaccine and may prove beneficial in cancer immunotherapy. It has been recently proposed that chemotherapy-induced autophagy causes the release of ATP from tumor cells, thereby stimulating antitumor immune response. Indeed, autophagy-dependent extracellular ATP release recruits DCs into tumors and activates anti-tumor T cell response. it seems that targeting autophagy blunted the release of ATP by tumor cells in response to chemotherapy [254].

It is now well established that immune effector cells integrate signals that define the nature and magnitude of the subsequent immune response. In this context, it has been shown that at high effector-to-target ratios, autophagy was induced in several human tumors by Natural Killer (NK) cells. Importantly, immune cell-mediated autophagy in target cells promoted resistance from treatment modalities designed to eradicate tumor. Thus, the lymphocyte-induced cell-mediated autophagy promotes cancer cell survival and may represent an important target for development of novel anti-tumor therapies [255]. Moreover, it has recently been shown that the combination of IL-2 with autophagy inhibitor chloroquine increased long-term survival, decreased toxicity associated with vascular leakage, and enhanced immune cell proliferation and infiltration in the liver and spleen[256].

Different immune subsets cooperatively and coordinately act to influence the immune response through the secretion of cytokines and other soluble factors. Thus, the antitumor immune responses is highly influenced by tumor microenvironment and rely on the balance between immune effectors versus immune suppressors and. Autophagy is now recognized as a new important factor influencing immune response in tumor microenvironment, and is probably interesting target in future anticancer treatment.

2 RESULTS

2.1 Hypoxia-induced autophagy decreases breast cancer cells susceptibility to NK lysis

We first investigated whether hypoxia impairs NK-mediated lysis of MCF-7 cells derived from pleural effusion breast adenocarcinoma. To address this issue we used NK cells isolated from the peripheral blood mononuclear cells (PBMC) of eight healthy donors (D1 to D8). MCF-7 cells were cultured under hypoxic environment (0.1% pO_2) for 16h and then presented for 4 hours to NK cells to assess the percentage of tumor cell lysis. We observed variable efficacy of lysis between donors which is most likely due to a differential expression of the NK cell activating and inhibiting receptors between individuals. We demonstrated **(Figure 7)** that, despite the inter-individual variability of NK cell cytolytic potential, the percentage of hypoxic MCF-7 cell lysis by NK cells was decreased compared to that of normoxic MCF-7 cells in all cases.

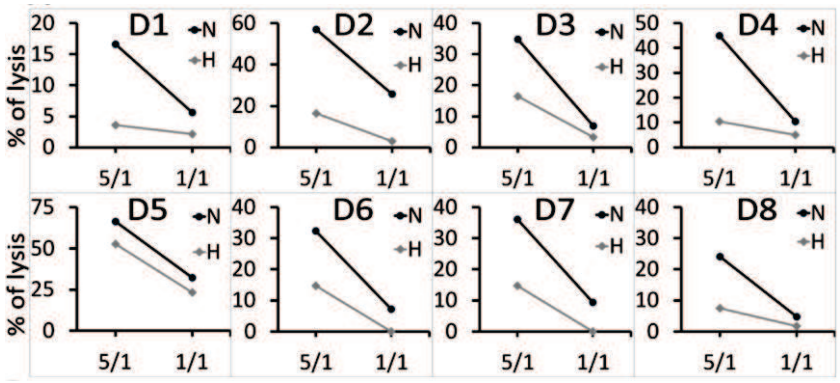

Figure 7: NK-mediated lysis of normoxic and hypoxic MCF-7 cells.
Cytotoxicity assays were performed in duplicate using NK cells isolated from 8 healthy donors (D1 to D8) at 5/1 or 1/1 effector (E) / target (T) ratios on normoxic (N) or hypoxic (H) MCF-7 cells. Cell death was assessed by flow cytometry using TO-PRO®-3.

As autophagy is activated under hypoxic stress, we assessed the autophagic status of hypoxic cells. Our results showed that the impairment of NK-mediated lysis in hypoxic cells was correlated with the induction of the autophagic flux. The induction of autophagy was characterized by the degradation of p62/SQSTM1 **(Figure 8, left panel)**, and the accumulation of microtubule-associated protein light chain-3 II (LC3-II) in chloroquine (CQ)-treated cells **(Figure 8, right panel)**.

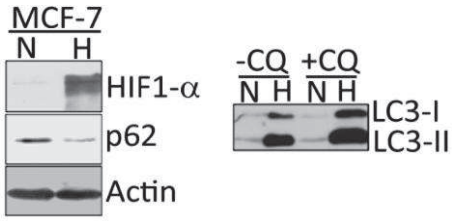

Figure 8: Induction of autophagy in hypoxic MCF-7 cells
Left panel: Cells were cultured under normoxic (N) or hypoxic (H) conditions for 16 h. The expression levels of HIF-1α, p62 and LC3 were assessed by immunoblot.

The formation of autophagosomes in hypoxic cells was visualized by confocal microscopy using MCF7 cell line stably expressing GFP-LC3 **(Figure 9 left panel)**. LC3 is a reliable marker of autophagosomes in mammalian cells, and its localization changes from a diffuse cytosolic pattern to a punctuate pattern representing its recruitment to the autophagosomal membrane during the induction of autophagy. An accumulation of autophagic vacuoles was observed in hypoxic cells incubated for 16h in 0.1% O2 atmosphere **(Figure 9 middle panel).** The quantification of the number of autophagosomes is represented in the **Figure 9 right panel.**

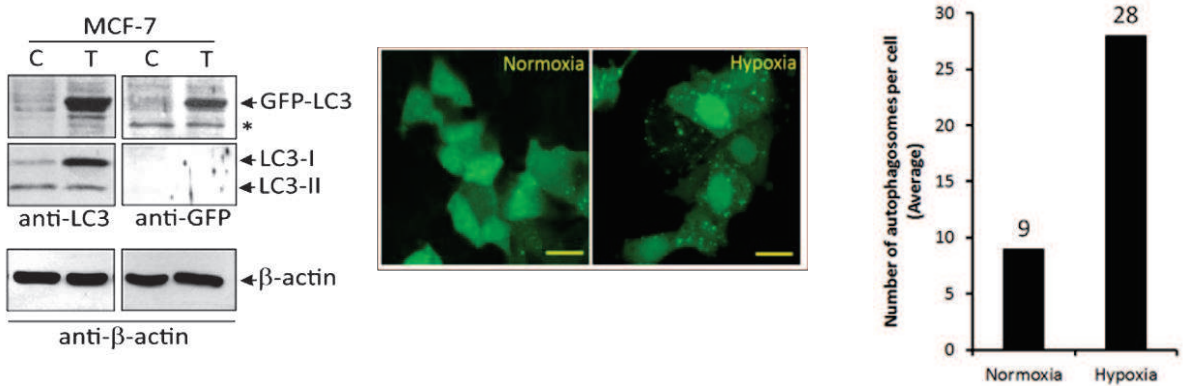

Figure 9: Formation of autophagosomes in hypoxic MCF-7 cells.
Left panel: MCF-7 cells were stably transfected with GFP-LC3 cDNA and maintained in RPMI/10% FBS supplemented with geneticin (0.5µg/ml). The expression of exogenous GFP-LC3 was assessed by immunoblot using anti-LC3 or anti-GFP antibody as indicated. Actin antibody was used for loading control. C: Control untransfected cells; T: Cells transfected with GFP-LC3 encoding vector. *Unspecific band. Middle panel: GFP-LC3 expressing normoxic or hypoxic MCF-7 cells were analyzed by fluorescence microscopy for the autophagosome formation (dots). Bar: 10 µm. Right panel: Quantification of autophagosomes number in normoxic and hypoxic MCF-7 cells

To visualize the NK-mediated killing of normoxic and hypoxic MCF-7 cells, we performed time-lapse video microscopy using GFP-LC3-expressing normoxic or hypoxic MCF-7 cells co-

cultured with PKH-26-stained NK cells isolated form healthy donor at 5/1 effector/target ratio. Images generated from time lapse movies showed that normoxic tumor cells were efficiently eliminated by NK cells **(Figure 10)**, as compared to hypoxic tumor cells displaying excessive autophagy**.**

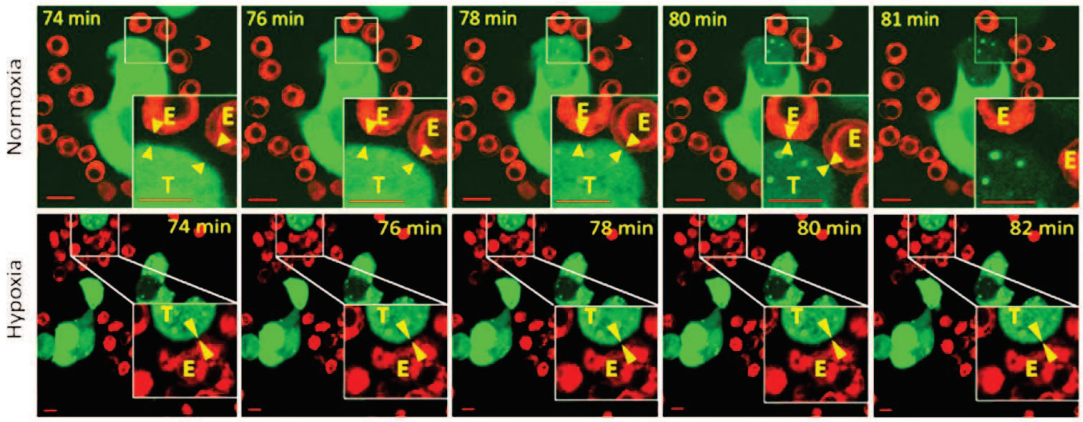

Figure 10: Movies of NK-mediated lysis of normoxic and hypoxic MCF-7 cells.
GFP-LC3-expressing MCF-7 cells (green) were pre-cultured onto glass coverslips under normoxic or hypoxic conditions. The cells were incubated with PKH-26-stained NK cells (red) at a 5/1 E/T ratio. Interactions between NK and normoxic or hypoxic MCF-7 cells were recorded by time-lapse video microscopy (presented as Movie S1 and Movie S2). At the indicated times, representative images were extracted from the movies. Bar: 10µm. Images of enlarged regions (boxes) show the interaction between NK cells (E) with both normoxic and hypoxic tumor cells (T). Bar: 10 µm.

To confirm that the elimination of normoxic cells by NK corresponds to the induction of cell death, we have repeated time lapse experiments by including the propidium iodide (PI) as a cell death indicator. Briefly, GFP-LC3 expressing MCF-7 cells were incubated under normoxia and hypoxia and co-cultured with NK cells isolated form healthy donor at 5/1 effector/target ratio. PI was added to the culture medium and time lapse was recorded as described above. Our results clearly show that normoxic cells were killed by NK cells as indicated by the uptake of PI **(Figure 11)**. Hypoxic tumor cells, however, remained resistant to NK-mediated killing as demonstrated by the absence of PI uptake. These results provided compelling evidence that, in contrast to hypoxic cells, normoxic cells underwent NK-mediated cell death.

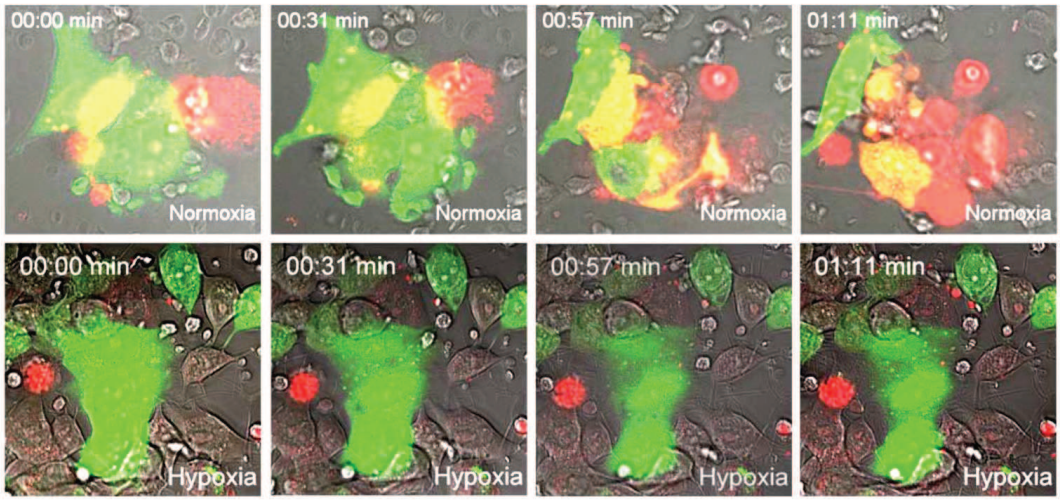

Figure 11: PI uptake by MCF-7 cells after co-culture with NK cells.
GFP-LC3 expressing MCF-7 cells (green) were pre-cultured anto µ-slide 8 wells under normoxic or hypoxic condition. Cells were next co-cultured with NK cells isolated from healthy donor at 5/1 ratio in the presence of propidium iodide (1.5 µM). Tumor cell killing was recorded by time lapse video microscopy using AxioVert 200M microscope (Carl Zeiss Micro-Imaging), and images were captured using X40 oil objective lens at intervals of 1 frame / 20 sec.

To determine whether the impairment of breast tumor cell susceptibility to NK-mediated lysis during hypoxia is not restricted to the MCF-7 cell line, we used T47D breast cancer cell line. The T-47 line was isolated from a pleural effusion obtained from a patient with an infiltrating ductal carcinoma of the breast. We showed that, similar to MCF-7, autophagy was induced in these cells under hypoxic condition, **(Figure 12)** and that this induction is correlated with the resistance of these cells to NK-mediated lysis.

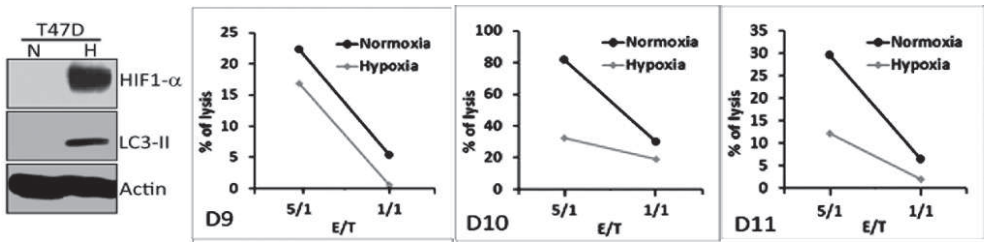

Figure 12: Hypoxia-induced autophagy decreases the susceptibility of T47D cells to NK-mediated lysis.
Upper panel: T47D cells were cultured under normoxia (N) or hypoxia (H) for 16 h. Expression of HIF-1α (hypoxia marker) and LC3 (autophagy marker) was assessed by immunoblot. Lower panels: NK cells were isolated from 3 different healthy donors (D9 to D11).

In addition we demonstrated that independently of hypoxia, autophagy induction by other stimuli also impairs NK-mediated lysis. We cultured MCF7 in starvation medium Earle's Balanced Salt Solution (EBSS), known to induce autophagy, and then subjected cells to NK lysis. We showed that induction of autophagy independently of hypoxia decrease NK-mediated lysis **(Figure 13)**.

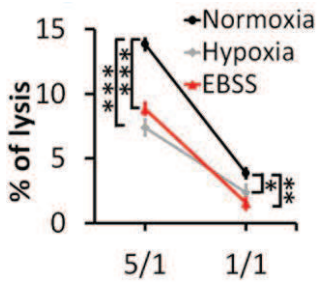

Figure 13: Starvation-induced autophagy decreases the susceptibility of MCF-7 cells to NK-mediated lysis. MCF-7 cells were cultured under normoxia, hypoxia or starvation (EBSS). Cytotoxicity assays were performed using NK cells isolated from healthy donor (D12). The percentage of tumor cell lysis is reported as an average (± SEM) of 3 experiments performed. Statistically significant differences are indicated by asterisks (*, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$).

To determine to what extent the induction of autophagy is implicated in the impairment of NK-cell mediated lysis, we used MCF-7 cells stably transfected expressing Beclin1 (MCF-7BECN1) under the control of a tetracycline-responsive promoter (tet-off). Thus, autophagy-competent (MCF-7BECN1+) or autophagy-defective (MCF-7BECN1-) MCF-7 cells can be generated by culturing cells in the absence (-) or presence (+) of doxycycline, respectively. **Figure 14** showed the effectiveness of Beclin-1 depletion and the inhibition of autophagosomes formation in MCF-7BECN1- under hypoxia.

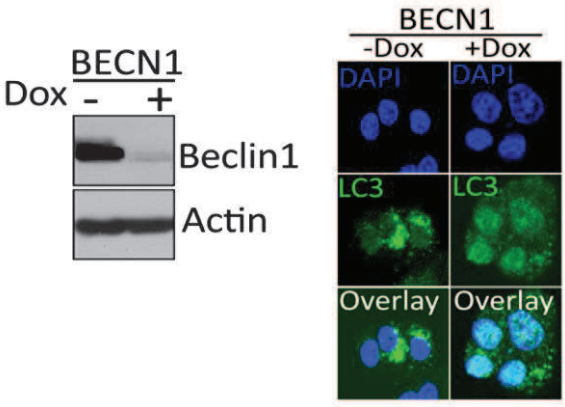

Figure 14: Targeting beclin 1 suppresses autophagy in MCF-7 cells.
Left panel: expression of Beclin1 was analyzed in control MCF-7 and MCF-7/tet-off clone (MCF-7BECN1) before and after doxycycline removal. Actin was used as a loading control. Autophagy competent or defective cells were generated by culturing MCF-7 expressing BECN1 under the control of a tetracycline-responsive promoter (MCF-7BECN1) in the absence (-Dox) or presence (+Dox) of doxycycline (100 ng/ml), respectively. The formation of autophagosomes (dot like structures) in hypoxic cells was assessed by immunofluorescence using Alexa-Fluor-488-coupled LC3 antibody. Nuclei were stained with DAPI. Bar: 10µm

We next evaluated the efficacy of NK cells to kill Beclin-1 defficient hypoxic cells. Results of the cytotoxic assay **(Figure 15)** clearly demonstrated that NK cells kill autophagy-deficient cells (MCF-7BECN1-) more efficiently than autophagy-competent cells (MCF-7BECN1+) under hypoxic (H) conditions. These results suggest that autophagy induction impairs NK-mediated lysis.

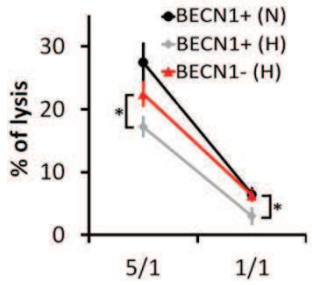

Figure 15: Inhibition of autophagy increases NK-mediated lysis of hypoxic MCF-7 cells.
Cytotoxicity assays were performed on autophagy competent (BECN1+) or defective (BECN1-) MCF-7 cells cultured under normoxic (N) or hypoxic (H) conditions with NK cells isolated from healthy donor (D13). The percentage of tumor cell lysis is reported as an average (± SEM) of 3 experiments and statistically significant differences are indicated by asterisks (*, $p < 0.05$).

As BECN1 modulation can affect the endocytic pathway, we have targeted ATG5 in MCF-7 cells and performed NK mediated cytotoxicity experiment. **Figure 16** (left panel) shows the effectiveness of ATG5 knockdown in MCF-7 cells and its impact on the inhibition of the autophagy flux under hypoxia (expression of LC3-II). Similar to targeting BECN1, ATG5 knockdown in hypoxic cells significantly restores NK cell-mediated killing (Figure. 16 right Panel). These data highlight that autophagy is specific determinant in the impairment of NK-mediated lysis of MCF-7 tumor cells.

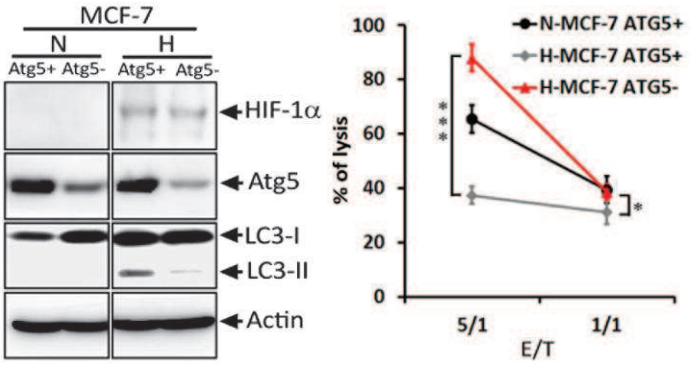

Figure 16: Targeting ATG5 restores NK-mediated cytotoxicity against hypoxic MCF-7 cells.
Upper panel: Control (Atg5+) and ATG5-defective (Atg5-) MCF-7 cells were cultured under normoxic (N) or hypoxic (H) conditions. The expression of Atg5, HIF-1α and LC3 was assessed by western blot. Lower panel: A cytotoxicity assay was performed at 5/1 and 1/1 E/T ratios using NK cells isolated from healthy donors as effector cells and control or ATG5- defective MCF-7 cells cultured under normoxic or hypoxic conditions as target cells. MCF-7 cell death was assessed using flow cytometry with TO-PRO®-3. Statistically significant differences are indicated by asterisks (*, $p < 0.05$; and ***, $p < 0.0005$).

2.2 Impaired NK-mediated lysis of hypoxic tumor cells is not related to a defect in tumor cell recognition by NK cells

To investigate whether hypoxia influences activating and inhibitory receptors at the surface of NK cells, we isolated NK cells from 2 healthy donors (D1 and D2) and exposed them overnight to hypoxia. Higher level of HIF1-α and LC3-II in hypoxic compared to normoxic NK cells was detected, indicating the activation of autophagy. We showed that despite the activation of autophagy in hypoxic NK cells, no differences were observed in the expression of Granzyme B and perforin **(Figure 17)**.

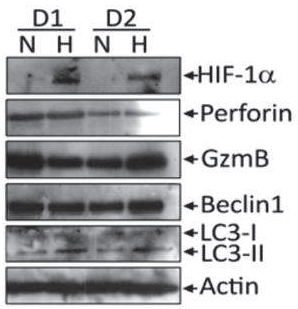

Figure 17: Effect of hypoxia on the expression level of Granzyme B and Perforin in NK cells.
NK cells isolated from two different healthy donors (D1 and D2) were cultured under normoxia (N) or hypoxia (H) for 16h (pO2 0.1%) and further subjected to immunoblot analysis for the expression of HIF-1α, Perforin, Granzyme B, Beclin1 and LC3.

We next investigated if prolonged hypoxia modulates the expression of activating and inhibitory receptors on the surface of NK cells. To determine whether such modulation occurs

in our experimental condition, we analyzed the expression of activating and inhibitory receptors on the surface of NK cells isolated from two healthy donors (D1 and D2) and cultured under normoxia and hypoxia. No significant differences were detected in expression of NKG2A, NKG2D, CD85j, CD158a, CD158b and CD159a at the surface of hypoxic compared to normoxic NK cells **(Figure 18)**.

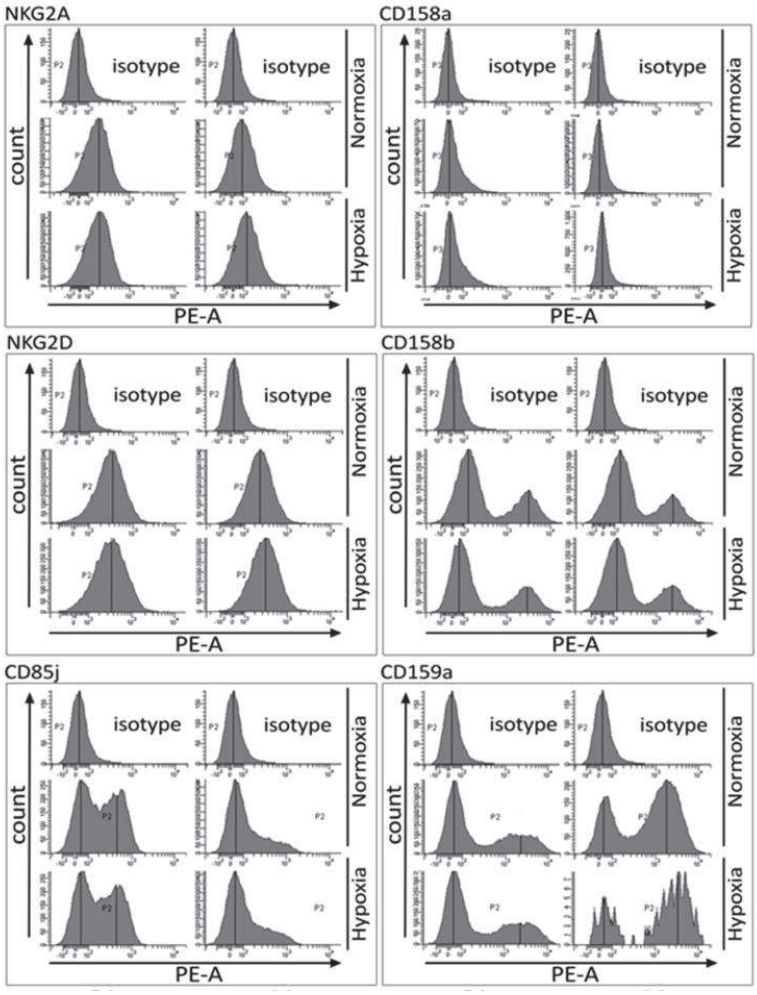

Figure 18: Effect of hypoxia on the expression of activating and inhibitory receptors on the surface of NK cells.
NK cells isolated from two different healthy donors (D1 and D2) were cultured under normoxia (N) or hypoxia (H) for 16h (pO2 0.1%). The expression of indicated NK cell surface receptors was assessed by flow cytometry (FACS Canto).

We next investigated whether hypoxic stress affects cytolytic activity of NK cells. Cytotoxicity assay was performed under experimental condition where effectors and target cells were under hypoxia. In line with our result showing that hypoxia do not modulate the expression of

activating and inhibitory receptors at the surface of NK cells, we did not observed any effect of hypoxia on NK cytolytic activity under these experimental conditions (**Figure 19**).

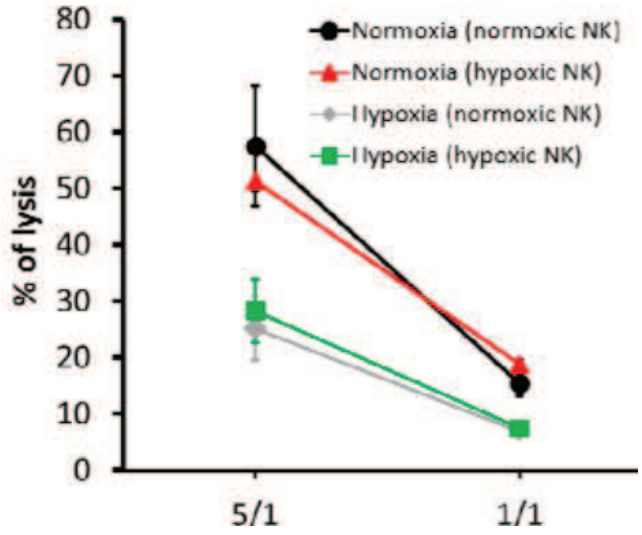

Figure 19: effect of hypoxia on NK cell activity.
Normoxic or Hypoxic tumor cells were co-cultured with NK cells previously incubated under normoxia (normoxic NK) or hypoxia (hypoxic NK) at 5/1 and 1/1 effector to target ratio. The % of lysis was reported. No statistically significant differences in target cell lysis are observed between normoxic and hypoxic NK.

We investigated whether the resistance of hypoxic tumor cells to NK-mediated lysis is related to an increase in NK-inhibitory MHC Class I molecules or a decrease in NK-activating NKG2D ligands on the cell surface. Among all analyzed molecules **(Figure 20)**, only HLA-A, B and C were found to be significantly upregulated on the surface of hypoxic cells (H-MCF-7BECN1+) and downregulated on autophagy-defective cells cultured under the same conditions (H-MCF-7BECN1-).

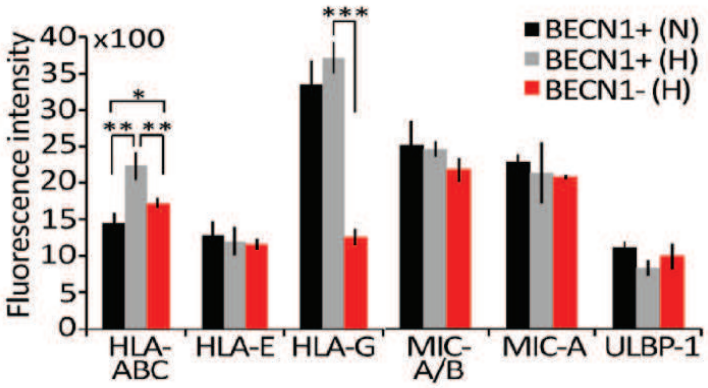

Figure 20: The expression of NK-inhibitory and activating receptors at the surface target cells.
Autophagy-competent (BECN1+) and -defective (BECN1-) MCF-7 cells cultured under normoxia (N) or hypoxia (H) were assessed by flow cytometry for the expression of MHC Class I molecules and NK cell-activating NKG2D ligands . Fluorescence intensity is reported as an average (± SEM) of 3 experiments. Statistically significant differences are indicated by asterisks (*, $p < 0.05$; **, $p < 0.005$; and ***, $p < 0.0005$).

Based on this result we investigated in more details the relative involvement of hypoxia-dependent up-regulation of HLA Class I molecules. Our data demonstrated that even when HLA Class I molecules are blocked, the lysis of hypoxic MCF-7BECN1- cells is significantly improved compared to hypoxic MCF-7BECN1+ cells **(Figure 21)**. We therefore suggest that independently of HLA Class I expression level, the autophagic status of target cells plays a key role in the resistance to NK-mediated lysis in our model.

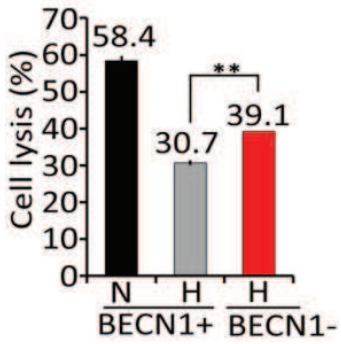

Figure 21: Effect of HLA class I blocking antibody on NK-mediated lysis of target cells
BECN1+ or BECN1- MCF-7 cells were pre-treated with mouse IgM anti-pan HLA class I A6-136 mAb and incubated under normoxic (N) or hypoxic (H) conditions before presentation to NK cells at a 5/1 E/T ratio. The percentage of target cell lysis is reported. Statistically significant differences are indicated by asterisks (**, $p < 0.005$).

Consistent with this result, data presented in **Figure 22** demonstrated that although a time-dependent increase in the percentage of conjugates between NK and tumor cells was observed, no significant difference in conjugate formation was observed between autophagy-competent (MCF-7 BECN1+) and -defective (MCF-7 BECN1-) cells cultured under normoxic (N) or hypoxic (H) conditions. Overall, these results suggest that the resistance to NK-mediated lysis under hypoxia is not exclusively depending on the regulation of activating and inhibitory ligands on hypoxic MCF-7 cells.

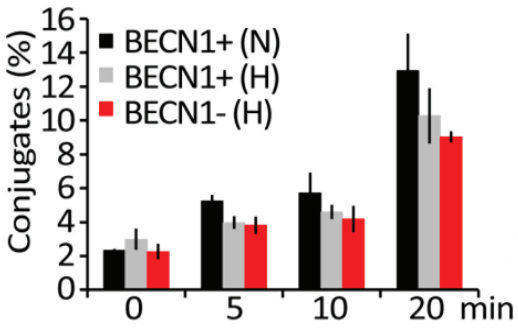

Figure 22: Conjugate formation between NK and target cells.
BECN1+ orBECN1- MCF-7 cells cultured under normoxia (N) or hypoxia (H) were incubated with NK cells. The percentage of conjugate formation at indicated time was determined by flow cytometry. Results are reported as an average (± SEM) of 3 independent experiments. No statistically significant differences were observed.

We next evaluated whether the degranulation activity of NK cells was affected by hypoxic tumor cells. **Figure 23** showed a basal level of CD107a on the surface of NK cells cultured alone (E), but a significantly higher level was detected when NK cells were co-cultured with normoxic or hypoxic tumor cells (E/T). As no difference in the level of CD107a was observed when NK cells were co-cultured with normoxic and hypoxic tumor cells, we conclude that the resistance of hypoxic tumor cells to NK-mediated lysis does not appear to be related to a defect in NK activity.

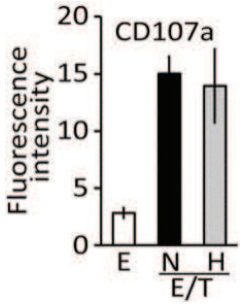

Figure 23: Expression of CD107a on the surface of NK cells
NK cells were cultured alone (E) or with normoxic (N) or hypoxic (H) MCF-7 cells at a 5/1 E/T ratio. The level of CD107a (a degranulation marker) on the surface of the NK cells was assessed by flow cytometry. Fluorescence intensity is reported as an average (± SEM) of 5 experiments performed with NK cells from different donors.

Therefore, our results further suggest that this resistance depends on an intrinsic mechanism makes tumor cells less sensitive to the cytotoxic granules released by NK cells. This hypothesis was further supported by data **(Figure 24)** demonstrating that under conditions where normoxic

and hypoxic tumor cells were treated with the pore-forming protein streptolysin-O, Granzyme B only kill normoxic cells.

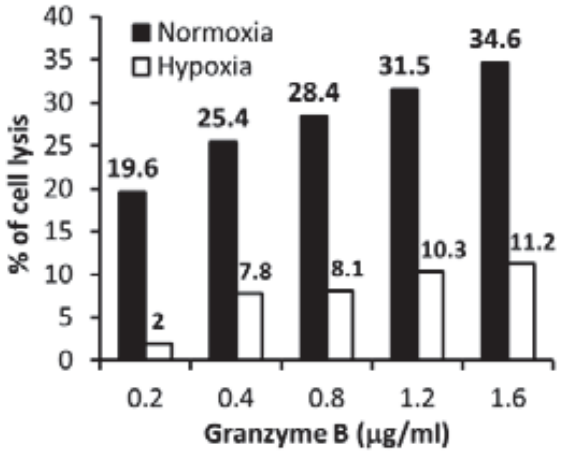

Figure 24: Effect of exogenous Granzyme B on tumor cell killing.
Normoxic and hypoxic MCF-7 cells were loaded with the indicated concentrations of exogenous, activated Granzyme B after treatment with the pore-forming protein streptolysin-O. The percentage of cell death was determined by flow cytometry.

2.3 Hypoxic tumor cells degrade NK cell-derived Granzyme B in lysosomes via autophagy

To investigate the mechanism underlying the observed reduction of NK-mediated killing of hypoxic cells, we analyzed the intracellular content of Granzyme B in target cells cultured under normoxia and hypoxia. PKH-26-stained normoxic or hypoxic MCF-7 cells were co-cultured with YT-Indy-NK cells expressing GFP-Granzyme B. Significantly lower levels of GFP-Granzyme B were detected in hypoxic cells compared to normoxic cells **(Figure 25).**

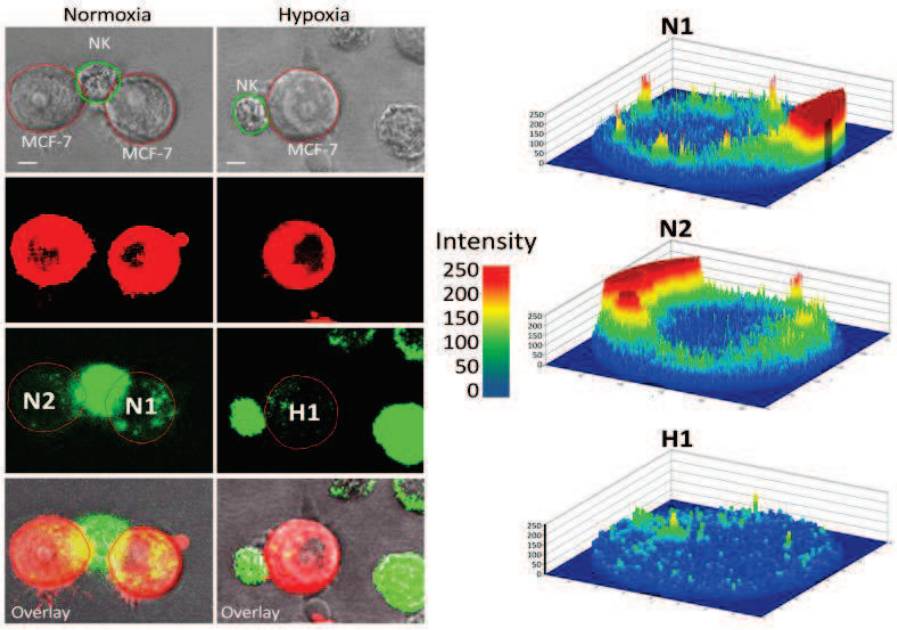

Figure 25: Hypoxia affects Granzyme B content in MCF-7 cells.
Left panel: PKH-26-stained normoxic or hypoxic MCF-7 cells (red) were co-cultured with YT-Indy-NK cells expressing GFP-Granzyme B (green) at 5/1 E/T ratio. Right panel: The green fluorescence corresponding to the Granzyme B transfer to normoxic (N1 and N2) and hypoxic (H1) target cell was quantified using Zeiss LSM Image Examiner and reported in right panels as an arbitrary unit.

We therefore evaluated whether the inhibition of autophagy restores Granzyme B level in hypoxic cells. The expression level of NK-derived Granzyme B in target cells was analyzed under normoxia or hypoxia on BECN1+ and BECN1- cells. Our results demonstrated that blocking autophagy restores Granzyme B content in hypoxic cells **(Figure 26).** These results indicate that the resistance of hypoxic tumor cells to NK-mediated killing is most likely related to the impairment of the Granzyme B level

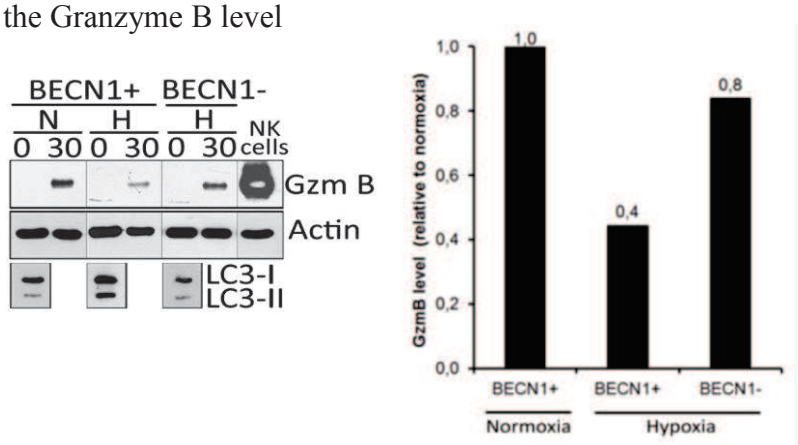

Figure 26: Analyze the level of Granzyme B in target cells
Left panel: Autophagy competent (BECN1+) or defective (BECN1-) MCF-7 cells were incubated under normoxia (N) or hypoxia (H) and co-cultured with NK cells at 5/1 E/T ratio for 0 and 30 min. Following separation, tumor cells were lysed and subjected to immunoblot for the intracellular Granzyme B content. Untreated NK cell lysate was used as a control for Granzyme B detection. The expression of LC3 was reported as a marker for autophagy. **Right panel:** Quantification of Granzyme B intracellular content of autophagy competent (BECN1+) and autophagy defective (BECN1-) MCF-7 cells cultured under normoxia or hypoxia was performed with Image J software.

NK-mediated killing of target cells is a multi-step process involves a directional secretion and localization of lytic granules containing Granzyme B and Perforin at the immunological synapse. Upon delivery to a target cell, perforin generates pores in the membranes, allowing granzymes to access target cell cytoplasm and induce apoptosis. Thus, we next investigate whether autophagy selectively degrades Granzyme B without affecting perforin in target cells. The expression level of NK-derived Granzyme B and perforin in target cells was analyzed under normoxia or hypoxia on untreated (-) or chloroquine-treated (+) cells. While no difference in the expression of perforin was observed, our results demonstrated that blocking autophagy by CQ specifically restores Granzyme B content in hypoxic cells **(Figure 27).**

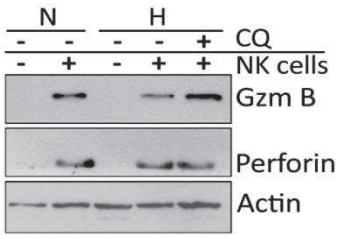

Figure 27: Restoration of Granzyme B level in autophagy in cells treated with CQ.
Normoxic (N) or hypoxic (H) MCF-7 cells were cultured alone (-) or with NK cells (+) at 5/1 ratio for 30 min in the presence (+) or absence (-) of chloroquine (CQ). Tumor cells separated from NK were subjected to immunoblot analysis to evaluate the intracellular Granzyme B and perforin content.

We next assessed whether the degradation of Granzyme B occurs by autophagy in lysosomes compartment. We therefore analyzed the effect the lysosomal hydrolase inhibitors E64d/ pepstatin **(Figure 28)**. Inhibition of lysosomal hydrolases by e64d/Pep restored Granzyme B content in hypoxic cells, suggesting that following its delivery to hypoxic cells, Granzyme B is degraded in lysosomes

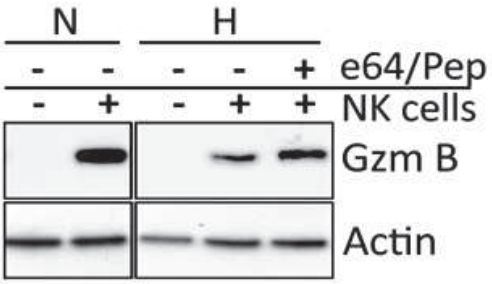

Figure 28: Restoration of Granzyme B level in cells treated with lysosomal hydrolases inhibitor e64d/pep
Normoxic (N) or hypoxic (H) MCF-7 cells were cultured alone (-) or with NK cells (+) at 5/1 ratio for 30 min in the presence (+) or absence (-) of e64d/pepstatin. Tumor cells separated from NK were subjected to immunoblot analysis to evaluate the intracellular Granzyme B content.

To further confirm this hypothesis, we analyzed the subcellular distribution of NK-derived Granzyme B by fractionation of autophagy-competent (BECN1+) or -defective (BECN1-) MCF-7 cells cultured under normoxic or hypoxic conditions. **Figure 29** showed a dramatic difference in the distribution pattern of Granzyme B between normoxic and hypoxic (BECN1+) cells. Granzyme B is mostly present in fractions 4 to 11 in normoxic cells; however, it is exclusively detected in fraction 2 and to a lesser extent in fraction 3 in hypoxic cells. Interestingly, Granzyme B-containing fractions 2 and 3 are positive for LC3 (autophagosomes) and Rab5 (endosomes), suggesting that these fractions may correspond to amphisomes (structures generated from the fusion of autophagosomes and late endosomes). We also analyzed the subcellular distribution of perforin in BECN1+ and BECN1- cells cultured under normoxia and hypoxia. Our result shows that, in hypoxic cells, perforin is detected with Granzyme B, LC3, and Rab in the same fractions (Fraction 2 and 3). Taken together, these results suggest that endosomes containing Granzyme B and perforin fuse with autophagosomes upon activation of autophagy in hypoxic cells, leading to the specific degradation of Granzyme B. Importantly, targeting autophagy in hypoxic cells dramatically changes the subcellular distribution of Granzyme B to a profile similar to that observed in normoxic cells.

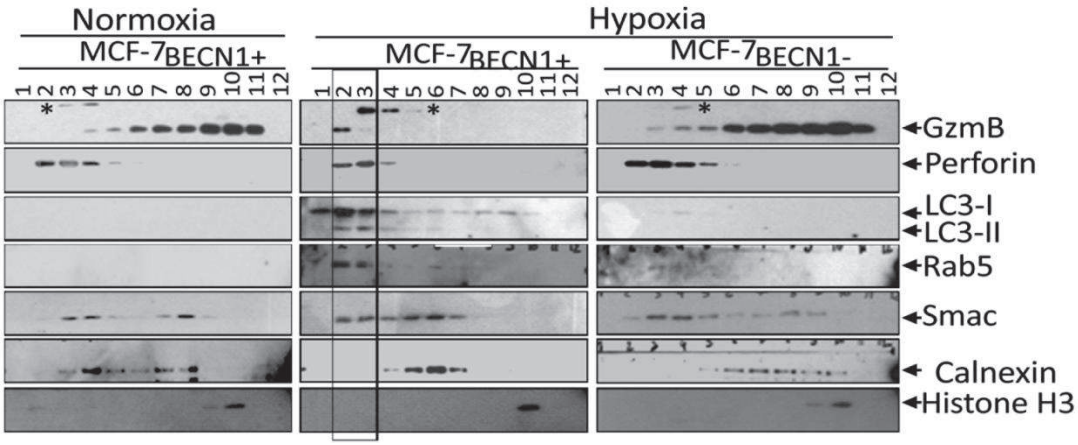

Figure 29: Subcellular distribution of Granzyme B in target cells
NK cells were cocultured with autophagy-competent (BECN1+) or -defective (BECN1-) normoxic or hypoxic MCF-7 cells. Cell lysates of separated tumor cells were subjected to subcellular fractionation. Fractions (1 to 12) were characterized by western blot using the indicated antibodies. * indicates an unspecific band.

Taken together, these results demonstrate that although both perforin and Granzyme B are transferred from NK to hypoxic cells, only Granzyme B is degraded in these cells by autophagy. This result strongly argues that autophagy selectively degrades Granzyme B. Mounting evidence suggests that the mechanistic basis of selective autophagy requires an interaction between the adaptor protein p62 and the protein to be degraded by autophagy. To investigate the involvement of p62 in selective degradation of Granzyme B by autophagy we targeted p62 by siRNA and analyzed its impact on the level of Granzyme B in hypoxic cells. Our results **(Figure 30)** show that targeting p62 in hypoxic cells restores the level of Granzyme B. This data suggests that Granzyme B is not "just an innocent victim" which is degraded by non-selective bulk autophagy but its degradation occurs by a p62- dependent selective autophagy.

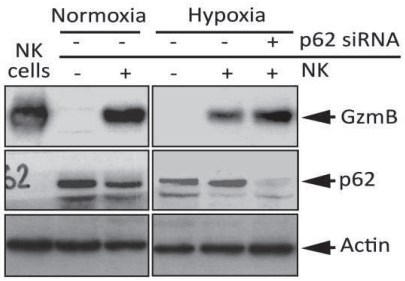

Figure 30: Effect of targeting p62 on Granzyme B level of target cells
Control (-) or p62 siRNA transfected-MCF-7 (+) cells were cultured under normoxia or hypoxia for 16 h alone. Tumor cells were presented to NK cells (+) at 5/1 E/T ratio for 30 min. Following separation from NK cells, lysates of tumor cells were subjected to immunoblot analysis in order to evaluate the Granzyme B intracellular content. Untreated NK cells lysate (NK cells) was used as control for Granzyme B detection.

The localization of NK-derived Granzyme B in autophagosomes of hypoxic cells was further supported by immunofluorescence data showing co-localization of Granzyme B-GFP with autophagosomes (LC3-stained structures) **(Figure 31)**. Those LC3-positive autophagosomes were also positive for the marker of endosomes EEA1 **(Figure 32).**

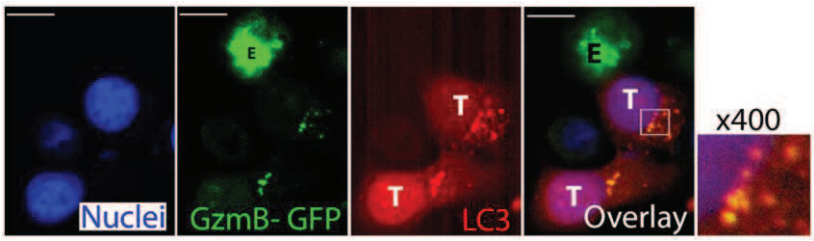

Figure 31: Colocalization of Granzyme B and LC3 in hypoxic MCF-7
Chloroquine-treated hypoxic MCF-7 cells (T) were co-cultured with Granzyme B-GFP-expressing NK cells (E). Target cells were stained with an Alexa-Fluor-568-coupled rabbit anti-LC3 antibody to visualize autophagosomes (red) and with DAPI to stain nuclei (blue). Co-localization of Granzyme B with LC3 in autophagosomes was visualized by confocal microscopy using a 100X oil immersion objective. Bar: 10 µm. An enlarged image (400X) of the overlay (box) is shown.

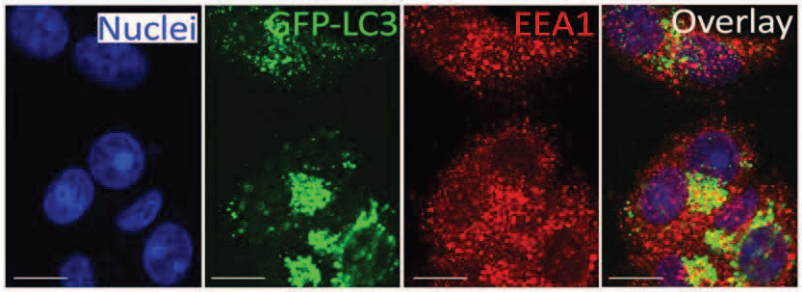

Figure 32: Staining of amphisomes in hypoxic MCF-7
Chloroquine treated GFP-LC3 expressing MCF-7 cell were cultured under hypoxic stained with anti-EEA1 Alexa-Fluor-568-coupled anti-rabbit IgG antibody (red) and DAPI for nuclei. Cells were analyzed by confocal microscopy. The overlay image shows co-localization of LC3 and EEA1 (yellow dots). Bar: 10 µm.

2.4 Targeting autophagy increased NK-mediated tumor regression in vivo

To assess whether inhibition of autophagy improves NK-mediated hypoxic tumor regression in vivo, we used two aggressive syngeneic murine models: B16-F10 melanoma tumors and 4T1

breast carcinoma tumors. B16-F10 tumors have been previously reported as highly hypoxic, with the activation of autophagy in hypoxic zones. 4T1 tumors have been reported hypoxic. Moreover this model is described as suitable experimental animal model for human mammary cancer[257].

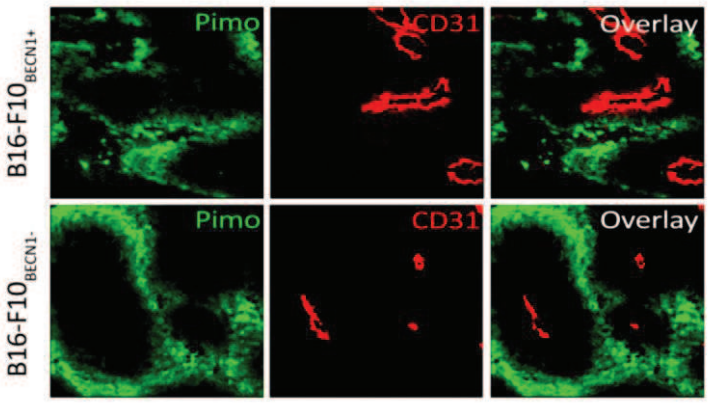

Figure 33: Hypoxic area in B16-F10 tumors
Fluorescence microscopy analysis of hypoxic area in B16-F10BECN1+ and BECN1- tumors stained with pimonidazol (Pimo). CD31 antibody was used to label blood vessels (Kindly provided by Dr M Z Noman, INSERM U753, Institut Gustave Roussy).

We established autophagy deficient 4T1 and B16F10 cell line, by knocking down BECN1. We evaluated the efficacy of BECN1 depletion and the doubling time to demonstrate that depletion of BECN1 does not affect tumor cell proliferation for both cell lines **(Figure 34)**

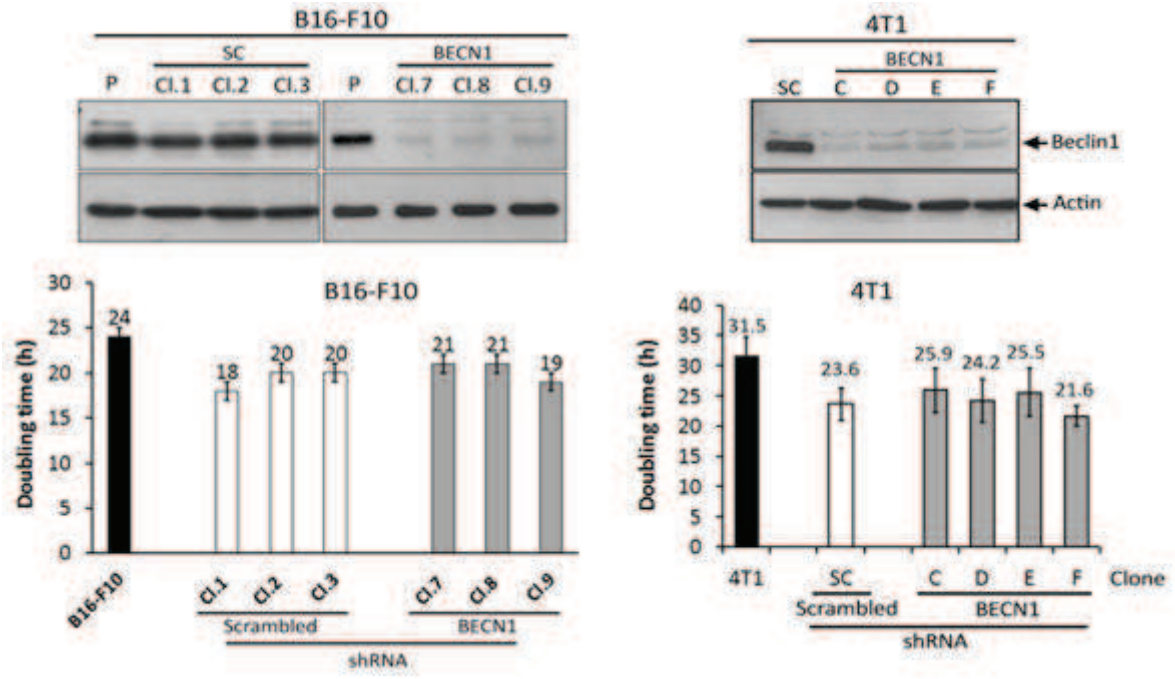

Figure 34: Characterization of autophagy-defective B16-F10 melanoma and4T1 breast tumor cells.

Cells were transduced with lentiviral particles containing scrambled (SC) or Beclin1 (BECN1) shRNA sequences. SC and BECN1 clones were selected and subjected to immunoblot (upper panel) to analyze the expression of Beclin1. The doubling time of each clones was evaluated by XTT assay (n=6) as described in materials and methods section (lower panel).

Next, we evaluated whether host NK cells are able to affect B16-F10 and 4T1 tumor growth in C57BL/6 and BALB/c mice, respectively. To address this issue, control (NK+) and NK-depleted (NK-) mice, which were generated by repeated injection of normal rabbit IgG and anti-asialo GM1, respectively, and engrafted with B16-F10 or 4T1 cells. The efficiency and selectivity of anti-asialo GM1 in depleting murine NK cells in C57BL/6 and BALB/c mice are showed in **Figure 35**.

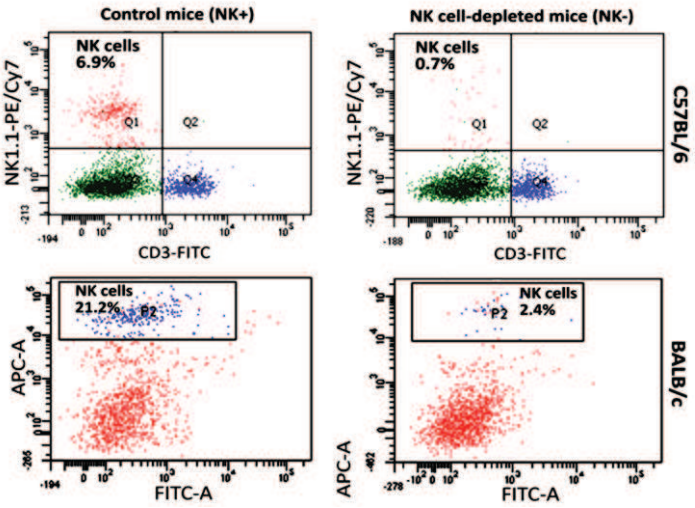

Figure 35: Analysis the depletion of NK cells in mice.
Effectiveness of NK cells depletion by anti-asialo GM1 injection in C57BL/6 and BALB/c mice was determined by flow cytometry analysis. Representative dot-plots of NK1.1-PE/Cy7 and Cd49b-APC for expression at the surface of PBMC from control (NK+) or NK-depleted (NK-) C57BL/6 and BALB/c mice respectively are shown.

Our results in **Figure 36** demonstrated a significant increase in B16-F10 and 4T1 tumor volume in NK- mice when compared to NK+ mice, indicating that NK cells play a role in B16-F10 and 4T1 tumor regression in vivo.

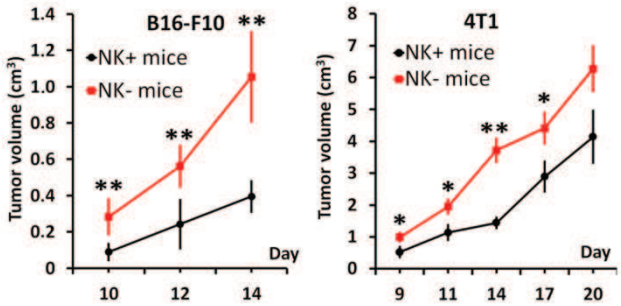

Figure 36: Role of NK cells in B16-F10 and 4T1 tumor development.
Control (NK+) and NK-depleted (NK-) C57BL/6 (left) or BALB/c (right) mice were engrafted with B16-F10 murine melanoma cells and 4T1 mammary carcinoma cells, respectively. Tumor growth in NK+ and NK- C57BL/6 (n=7) and BALB/c (n=10) mice was monitored using caliper measurements on the indicated days. Statistically significant differences in tumor volume are indicated by asterisks (*, $p < 0.05$, **, $p < 0.005$; and ***, $p < 0.0005$).

To determine the impact of autophagy on NK-mediated lysis in vivo, we analyzed the growth of autophagy-defective (BECN1-) B16-F10 and 4T1 tumor cells in both NK+ and NK- mice.

B16-F10BECN1- and 4T1BECN1- cells were generated using BECN1 shRNA lentiviral particles. B16-F10 and 4T1 cells infected with scrambled shRNA-expressing vectors (B16-F10BECN1+ and 4T1BECN1+) were used as autophagy-competent control cells. **Figure 37** demonstrated that in NK+ mice, the volume of B16-F10BECN1- and 4T1BECN1- tumors (red curves) was significantly reduced compared to that of BECN1+ tumors (black curves). This reduction is most likely due to the ability of NK cells to eliminate autophagy-defective cells more efficiently than autophagy-competent cells. Consistent with this hypothesis, in NK depleted mice (NK-), the regression of BECN1- tumors was no longer observed (grey vs. red curves). Taken together, these results suggest that blocking autophagy in hypoxic tumors facilitates and improves their elimination by NK cells in vivo.

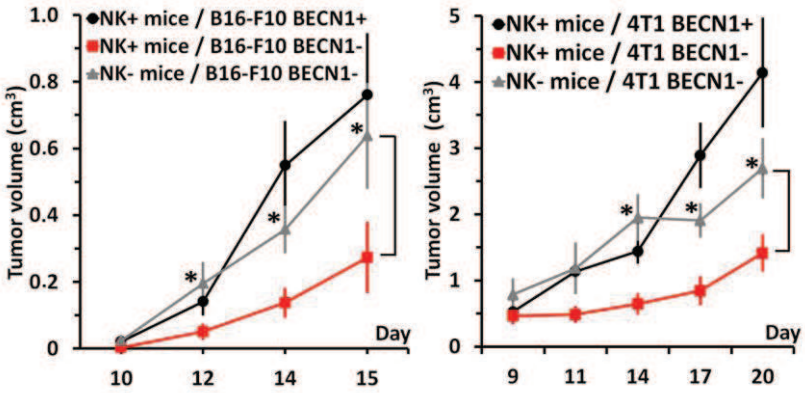

Figure 37: Effect of autophagy inhibition on NK cell-mediated immune response in mice
Autophagy-competent (BECN1+) or -defective (BECN1-) B16-F10 or 4T1 cells were injected subcutaneously or in the mammary fat pad, respectively, in control (NK+) and NK-depleted (NK-) C57BL/6 (n=7) and BALB/c (n =10) mice. Tumor growth was monitored using caliper measurements on the indicated days. Statistically significant differences are indicated by asterisks (*, $p < 0.05$).

To gain insight into how targeting autophagy improve tumor elimination by NK cells, we analyzed the expression of MHC class I at the cell surface of the murine melanoma B16-F10. Due to the weak expression of MHC class I molecules at the surface of these cells [258], we performed a quantitative and more sensitive FACS analysis, rather than immunohistochemistry, to address this issue and we included breast carcinoma 4T1 cells. Our data **(Figure 39)** show that targeting autophagy in hypoxic cells has no effect on the cell surface expression of MHC

Class I. These results suggest that the improvement of tumor elimination by NK in vivo occurs independently of MHC class I modulation.

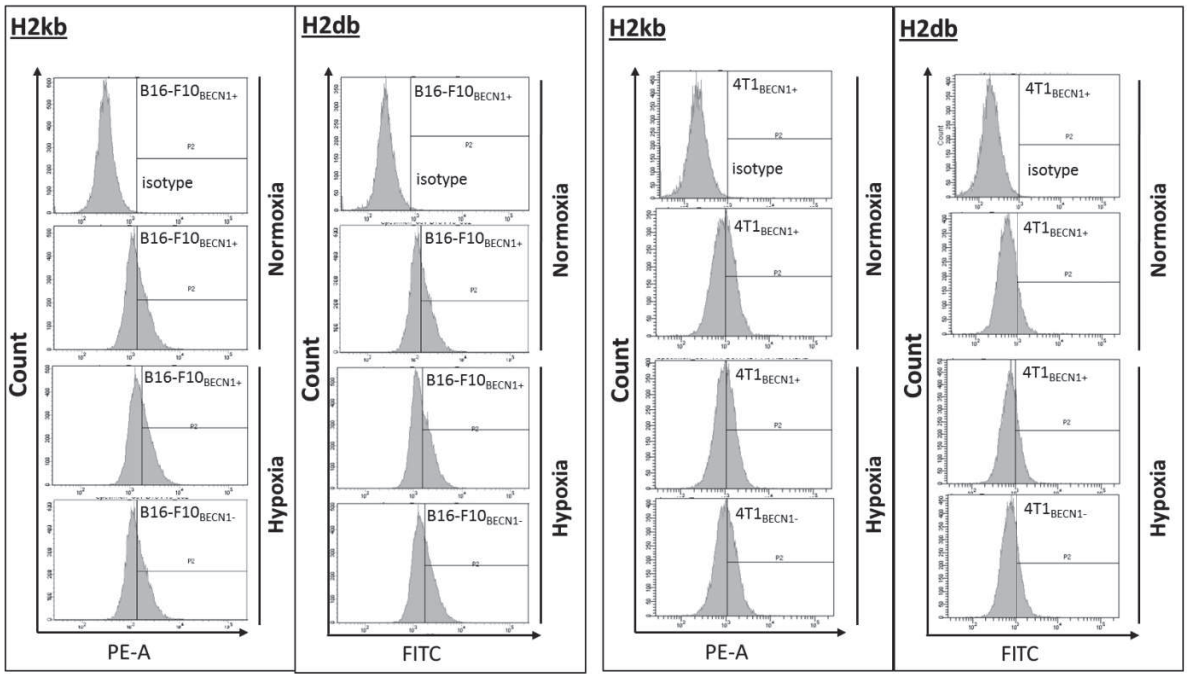

Figure 38: Analyze the expression of MHC class I on the surface of tumor cells.
Autophagy-competent (BECN1+) and –defective (BECN1-) cells were cultured under normoxia (N) or hypoxia (H) for 16h (pO2 0.1%). The expression of indicated MHC class I (H2kb and H2db) molecules was assessed by flow cytometry (FACSCanto) for B16-F10 (left panel) and 4T1 (right panel) cells.

3 DISCUSSION

Results presented in this thesis identify autophagy as a major player in the resistance of breast cancer cells to NK-mediated lysis. Our in vivo data validate this concept and highlight the inhibition of autophagy as a potential therapeutic approach to improve NK-based cancer immunotherapy. In addition, we elucidated the mechanism by which autophagy impairs the susceptibility of tumors to NK-mediated killing. To our knowledge, this study is the first to demonstrate that the activation of autophagy under hypoxic conditions causes the degradation of NK-derived Granzyme B leading to tumor escape form effective NK-mediated killing.

NK-based immunotherapy is a promising therapy for solid and hematologic cancers, and it can potentially be combined with chemotherapy, radiation, or monoclonal antibody therapy [259]. For example, the proteasome inhibitor bortezomib, which is clinically approved for the treatment of refractory/relapsed myeloma, down-regulates cells surface expression of HLA class I on target cells and enhances NK-mediated lysis of tumor cells without affecting NK-cell function [260]. Therfore, such combination therapy has important therapeutic implications for multiple myeloma and other NK cell-related malignancies in the context of adoptively-transferred allogeneic and autologous NK cells [261]. Another example is the combination of NK cell therapy with radiation therapy because irradiation-induced tissue injury that is known to increase the expression of NK activating ligands (e.g., NKG2D ligands) on malignant cells, thus rendering tumors more susceptible to NK cell cytotoxic activity. It has been recently showed that Ewing's sarcoma cells are highly sensitive to expanded allogeneic NK cells. Radiation therapy has been shown to significantly enhance the NK cell-mediated killing of Ewing’s sarcoma cell lines in an orthotopic murine model [262]. Another approach is to combine NK cell-based therapy with mAb therapy because NK cells express an activating Fc receptor that recognizes the constant region of tumor-bound antibodies. This allows NK cells to kill mAb-coated target cells through antibody-dependent cellular cytotoxicity (ADCC). Clinical examples of ADCC include patients with CD20+ lymphoma who have been treated

with rituximab (Rituxan™) or patients with HER2/neu expressing breast cancers who have been treated with trastuzumab (Herceptin™) [263] [264]. It has also been shown that co-administration of immunomodulatory cytokines (e.g., IL-12) can enhance the effects of anti-tumor mAbs via the activation of NK cells in vitro. This effect has been shown for patients with HER2 overexpressing cancers in a phase I trial of IL-12 and trastuzumab [265]. Based on the fact that NK cells can eliminate cancer cells in experimental conditions, it has been proposed that NK cells can be used clinically in therapeutic settings against cancer. Importantly, data from Haploidentical hematopoietic Stem Cell Transplantation (HSCT) and NK-cell-based adoptive immunotherapy support the clinical effects of NK cells, although adverse side effects of adoptive NK-cells transferred have not been reported [266]. With the current knowledge about the molecular specificities that regulate NK-cell function, it is tempting to speculate a possible design of tailored NK-cell-based immunotherapeutic strategies against human cancer.

However, one of the major obstacles for defining successful NK-based therapeutic protocols is the ability of tumor cells to activate several mechanisms that lead to tumor escape from NK-mediated lysis. Extensive efforts have been made in recent years to identify these mechanisms by focusing on the tumor microenvironment as a major factor influencing disease progression and response to therapy in cancer [267]. The cross-talk between tumor cells and the TME largely influences cancer progression and contributes to almost all of the hallmarks of cancer [268]. As our understanding of the pathways activated following tumor cell–stroma interactions has increased, significant efforts have been made over the last decade to identify and develop therapeutic agents that interfere with such interactions, block activated pathways and/or influence the recruitment of stromal cells into the TME [269].

Several lines of evidence indicate that the TME may impair the cytotoxic activity of NK cells. During tumor progression, tumor cells develop several mechanisms to impair NK cell function or to escape either from NK cell recognition and attack. These mechanisms include: i) a loss of

adhesion molecules, costimulatory ligands and ligands for activating receptors; ii) upregulation of MHC class I, soluble MIC, FasL or NO expression; iii) secretion of immunosuppressive factors such as IL-10 and TGF-β and resisting TO Fas- or perforin-mediated apoptosis [270]. As hypoxia is a common feature of the tumor microenvironment, it is now well established that hypoxia contributes to malignant progression in cancer by inducing invasive and metastatic phenotype of tumor cell and by activating resistance mechanisms to radiation and chemotherapy [271]. Under hypoxia, the activation of autophagy has been reported as a mechanism of tumor cell adaptation to stress in order to maintain their survival. Several preclinical evidences indicate that stress-induced autophagy in tumor cells is predominantly cytoprotective and that inhibition of autophagy can enhance tumor cell death by diverse anticancer therapies, highlighting autophagy as a novel therapeutic target in cancer treatment [234].

Through the degradation of cytoplasmic organelles, proteins, and macromolecules, and the recycling of the breakdown products, autophagy plays important roles in cell survival and homeostasis [272]. For example, autophagy is involved in the control of cell development, tissue homeostasis and the lifespan of organisms. Autophagy is also involved in diverse aspects of immunity, such as the elimination of intracellular pathogens, the increase of MHC class II presentation, the production of type I interferons (IFNs) and the regulation of T-cell homeostasis [203].

In this work we show that autophagy is induced in hypoxic tumors, and that such induction correlated with a decrease in NK-mediated lysis. It is now well known that exposure to low levels of oxygen leads to tumor escape from immune surveillance [73]. Indeed, inefficient killing of tumor cells by NK cells under hypoxia may be due, at least in part, to the down-regulation of NKG2D-activating ligands on the surface of tumor cells [273]. Consistent with this hypothesis, hypoxia has been reported to increase the shedding of MIC from the surface of prostate cancer cells [52]. Moreover, inhibition of NK cytotoxicity toward liver cell lines was

reported when the effector cells were cultured in hypoxic conditions [274]. While the effect of hypoxia on the function of immune cells has been extensively reported [275] [276] [277] , no data related to how hypoxia induces tumor cell resistance to NK cell-mediated killing. In this context we did not detect any decrease in the expression of MIC on the surface of hypoxic tumor cells, which suggests that, under our experimental conditions, the resistance to NK-mediated killing is not related to modulation in the expression of this specific NKG2D ligand. Similarly, we did not observed any defect in tumor cell recognition by NK-cells. However, our data demonstrate a significant up-regulation of the expression of NK cell-inhibiting MHC Class I molecules on the surface of hypoxic cells. Although the causal mechanisms underlying the increase in MHC class I on the surface of hypoxic are still unknown, we showed that an important part of this resistance occurs independently of MHC Class I expression levels but strikingly depends on autophagy activation.

NK cell activating receptors accumulate at the cytotoxic immune synapse in an actin-dependent manner where they provide synergistic signals to trigger NK cell effector functions. In contrast, NK cell inhibitory receptors, including members of the KIR family, accumulate at inhibitory immune synapses [278]. The formation of inhibitory immune synapses blocks actin dynamics and thereby prevent actin-dependent phosphorylation of activation receptors [279]. It has been reported that the balance between activating and inhibitory receptors is critically important for the formation of lytic immune synapses between NK and target cells. Actin filaments accumulates at the synapse, followed by the polarization of the microtubule organizing center (MTOC). These events lead to directional secretion of lytic granules towards the target cells [113]. In our model we did not observed any difference in conjugates formation between NK cells and normoxic or hypoxic cells, ruling out any effect of autophagy on the immunological synapse destabilization of hypoxic tumor cells, as recently described [280]. Furthermore, our results provide evidence that there is no difference in the degranulation level of NK cells when

co-cultured with normoxic or hypoxic cells. This result exclude a possible defect in the cytotoxic potential of NK cells toward hypoxic tumor cells as a mechanism of resistance.

We next investigated whether culturing NK cells under hypoxia affects their cytolytic activity. It has been shown that the escape of tumor cells from NK-mediated lysis in TME seems to be caused by local immunosuppression that compromises the lytic activity of the NK cells and enhances the resistance of the tumor cells [281]. The low interstitial pH and/or low partial pressure of oxygen are the two most prominent features of tumor microenvironment playing a role in such suppression [282]. It has been reported that hypoxia may increase the resistance of tumor cells to NK-mediated lysis and suppress NK cytotoxicity. In our experimental conditions we showed no significant differences in the expression of NKG2A, NKG2D, CD85j, CD158a, CD158b and CD159a at the surface of hypoxic compared to normoxic NK cells and no defect in NK cytolytic activity. The discrepancy between our data and those from the literature may be related to the time of exposure to hypoxia and to IL-2 stimulation.

Having demonstrated that NK cells fulfill their cytotoxic functions toward both hypoxic and normoxic cells, and that exogenous Granzyme B was unable to kill hypoxic tumor cells; we propose that autophagy operates as an intrinsic resistance mechanism in tumor cells.

Granzyme B is a key effector of cytotoxic lymphocyte-mediated cell death. NK-derived Granzyme B is delivered to target cells and reached its substrates by a mechanism which is not fully understood. It is proposed that the delivery of the lytic effector proteins perforin and granzymes to target cells occurs by at least three mechanisms. Cytotoxic granules containing Granzyme B may bound to the chondroitin proteoglycan protein serglycin. Granzyme B-serglycin complex is secreted from NK cells and delivered to target cells. Perforin binds in a calcium dependent manner to membranes and multimerizes to form pores. In target cells perforin release Granzyme B and free Granzyme B will therefore induced apoptosis via

degradation of specific substrates such as Bid or procaspase 3.Although perforin was originally proposed to provide a channel through the plasma membrane for granzyme transfert, it is now well documented that granzymes are first internalized via receptor-mediated endocytosis into an undefined intracellular compartment [131]. Subsequently, the exposure of target cells to perforin releases granzymes form this intracellular compartment into the cytoplasm [128]. The first Granzyme B receptor identified on target cells was the cation-independent mannose-6-phosphate receptor (M6PR), which internalizes Granzyme B via clathrin-dependent endocytosis [124]. Target cells lacking M6PR remain sensitive to Granzyme B killing, and Granzyme B internalization continues in the absence of the key endocytic pathway component, dynamin, suggesting that other receptors and routes of Granzyme B uptake must exist.

Other mechanism of Granzyme B uptake involves heat shock protein 70 (HSP70) bound to Granzyme B at the cell surface [129]. Hsp70 is frequently overexpressed in tumors, and cytosolic Hsp70 mediates the protection of tumor cells against environmental stress [283]. Membrane Hsp70 serves as a tumor-specific recognition structure for pre-activated natural killer (NK) cells and Granzyme B-induced apoptosis in Hsp70-positive tumor cells occurs in the absence of perforin [129]. We did not detect any differences in the cell surface expression of HSP70 or M6PR of normoxic and hypoxic cells. These results rule out a defect in the transfer of the lytic effector proteins, perforin and granzymes, to hypoxic target cells.

It has been reported that early endosomes can fuse with autophagic vacuoles to form amphisomes. This structure represents a pre-lysosomal compartment in which both the autophagic and endocytic pathways converge. Amphisome seems to be a prerequisite for the maturation of autophagic vacuoles, their subsequent fusion with lysosomes and for the formation of autophagolysosome [201]. During killer cell–mediated lysis, perforin at physiologically relevant sublytic concentration, creates small pores in the target-cell membrane, allowing calcium entry to the target cell. Calcium influx induces a damaged membrane–repair

response via calcium-dependent exocytosis of lysosomes and other vesicles [284]. Moreover, the damaged-membrane response induces endocytosis for the removal of the damaged membrane from the cell surface to preserve the integrity of the cell membrane. Following their rapid clathrin- and dynamin-dependent endocytosis, perforin and granzyme B are transferred to enlarged vesicles, called gigantosomes, which are positive for Rab5 [131].

Based on these findings, we propose that during its intracellular trafficking, Granzyme B could be exposed to a high to be degraded in hypoxic cells by excessive autophagy. Several data reported in this study support such a mechanism: i) the level of NK-derived Granzyme B detected in hypoxic cells is significantly lower than that in normoxic cells; ii) inhibition of autophagy restores the level of Granzyme B and subsequently restores NK-mediated lysis of hypoxic cells; and iii) NK-derived Granzyme B is detected in LC3- and Rab5-positive cellular compartments, suggesting its presence within amphisomes in hypoxic cells. Together, our data identify for the first time a potential mechanism by which autophagy impairs the susceptibility of tumor cells to NK-mediated lysis.

Recent evidence highlighted the selective elimination of aberrant protein aggregates, lipid droplets, dysfunctional organelles and invading pathogens by autophagy [285]. Molecular components involved in selective autophagy have been identified, implying that we are beginning to understand how selectivity is achieved in this process. Specific autophagy receptors are responsible for selective autophagy by tethering cargo to the site of autophagosomal engulfment [286]. In addition, it is becoming clear that post-translational modifications have an important role in substrate recognition and selectivity in vertebrates. For example, besides its role in proteasome-mediated degradation, protein ubiquitination, can constitute a modification that targets proteins to turnover by autophagy [287]. The recognition of ubiquitinated substrates is provided by molecular adaptors including p62, NBR1, NDP52, VCP and optineurin, which bind on one side to ubiquitin and, on the other end, to

autophagosome-specific proteins, such as the members of the LC3/GABARAP/Gate16 family [285]. However, the physiological roles of selective autophagy are not yet fully understood. Several reports highlighted the role of p62/sequestosome1 (p62/SQSTM1), targeting other proteins for proteasome degradation or autophagic digestion, at the crossroads of autophagy, apoptosis and cancer. Although p62/SQSTM1 was initially identified as an essential mediator of NFκB signaling, several recent studies point to its important role in selective autophagy and in the cross talk between the mTOR or MAPK signaling pathways [288]. p62/Sequestosome 1 is a scaffold protein involved in the regulation of autophagy, trafficking of proteins to the proteasome, and activation of NF-kappaB. p62 encodes an N-terminal PB1 domain in addition to the ZZ domain, TRAF6-binding domain, LC3 interaction region, and ubiquitin-associated domain. Each of them plays a critical role for the physiological function of p62 [289]. p62 mediates the interaction between ubiquitinated proteins and autophagosomes, leading to their degradation by autophagy-lysosomal pathway. This ubiquitin-mediated selective autophagy is thought to recognize the ubiquitinated proteins by the C-terminal ubiquitin-associated (UBA) domain of p62 [290].Our data suggests that Granzyme B is not "just an innocent victim" which is degraded by non-selective bulk autophagy but its degradation occurs by a p62-dependent selective autophagy. In this context, it remains to be determined whether anti-cancer strategies inhibiting p62 can be used as new anti-cancer therapy.

To evaluate the impact of autophagy on the regulation of NK-mediated anti-tumor immune responses in vivo, we used C57BL/6 and BALB/c mice transplanted with syngeneic murine 4T1 breast adenocarcinoma tumor cells and B16-F10 melanoma. The use of highly metastatic 4T1 mouse mammary tumor to evaluate the role of autophagy in vivo is based on data showing that these tumors are poorly vascularized and contain large regions of hypoxia [291]. In addition, the used of B16-F10 melanoma cells is based on previous reports showing that these tumor are also highly hypoxic and that autophagy was induced in hypoxic areas of tumor [253].

The use of melanoma model allows us to demonstrate that the improvement of NK-mediated tumor elimination was not restricted to breast tumors.

Our results clearly demonstrate that NK cells control both B16-F10 and 4T1 tumor development in vivo, as the depletion of NK cells significantly increases tumor growth. Having demonstrated the role of NK cells in the control of both B16-F10 and 4T1 tumor growth, we further showed that the growth of genetically engineered autophagy-defective B16-F10 and 4T1 cells was substantially inhibited in immunocompetent mice. As this inhibition was no longer observed when NK cells were depleted, our results highlight a critical role of autophagy in the impairment of NK-mediated tumor cell killing in vivo.

The TME plays a crucial role in the control of immune protection by a number of overlapping mechanisms, which ultimately lead to tumor evasion from immunosurveillance. Tumor and stromal cells including immune effectors, mediate tumor progression by secreting factors promoting angiogenesis and tumor cell proliferation and survival. In addition, through cytokine production and release, stromal cells create an immunosuppressive TME that facilitates tumor cell escape from clearance by the immune system. In TME, hypoxic stress is used by tumors to their own advantage by activating key biochemical and cellular pathways that are important for progression, survival, and metastasis. It has been reported that hypoxia stimulates the release of immunosuppressive molecules such as TGF-β, by tumor cells. More recently, hypoxia has been shown to increase lung cancer cell resistance to cytotoxic T-cell–mediated lysis [253] . Other studies have focused on assessing the direct effect of hypoxia on the activity of innate immune effectors inducing resistance to immunosurveillance. However, how hypoxia-induced i regulates NK-mediated response in the tumor microenvironment has long been unknown.

Autophagy has dual roles in cancer, acting as tumor suppressor by preventing the accumulation of damaged proteins and organelles and as a mechanism of cell survival that can promote the

growth of established tumors [203]. Tumor cells activate autophagy in response to cellular stress and/or increased metabolic demands due to rapid cell proliferation. Autophagy-related stress tolerance can enable cell survival by maintaining energy production that can lead to tumor growth and therapy resistance [234]. In this context, it has been shown that inhibition of autophagy restored chemosensitivity and enhanced tumor cell death highlighted autophagy as autophagy as a therapeutic target and led to multiple early phase clinical trials using hydroxychloroquine in combination with chemotherapy [292]. However, therapies that impair autophagy must also take into account as their effect may compromise activated lymphocytes in the immune response to cancer. Given that a strong antitumor immune response is a positive prognostic factor in overall patient survival, identifying ways to specifically block autophagy in tumor cells could be critically important for a robust anti-tumor immune response [293]. Despite significant data implicating autophagy in immune metabolism and function, numerous clinical trials are initiated to combine anti-cancer agents with the autophagy inhibitors CQ and HCQ. The proof of concept of this approach is to block the survival advantage provided by autophagy activation in tumor cells to abrogate resistance to therapy. While preclinical and clinical studies have shown efficacy in combining CQ and HCQ with conventional therapies, improved outcomes may be achieved by finding strategies to selectively disable the protective effects of autophagy in tumor cells and suppressive immune cells without affecting the essential functions of autophagy in the anti-tumor function of immune cells. This represents a considerable challenge due to the heterogeneity between immune systems from patient to patient and to the fact that some therapies exhibit immune activating effects in some circumstances, but not in others. Selectively targeting tumor cells may require improving the targeted delivery of anti-cancer agents and blocking unique components in the autophagy pathway and/or metabolic processes supported by autophagy. As targeted therapies become more specific, the negative impacts of clinical treatments on the immune system may be

avoided. Furthermore, administration of drugs specifically targeting selective forms of autophagy that are differentially upregulated in tumor cells may decrease tumor cell survival without compromising antitumor immunity. The selective autophagy of misfolded protein aggregates has recently been reported to be upregulated in tumor cells treated with the proteasome inhibitor bortezomib suggesting that this processes may contribute to reprograming tumor cell metabolism and be exploited therapeutically [294].

NK cell-based immunotherapy holds great confident for cancer treatment. However, only modest clinical success has been achieved using NK cell-based therapies in cancer patients. Progress in the field of understanding NK cell biology and function is therefore needed to assist in developing novel approaches to effectively manipulate NK cells for the ultimate benefit of treating cancer patients. While there are several ongoing clinical trials using NK cells for cancer treatment, this study highlights the importance of integrating specific autophagy inhibitors as an innovative strategy in NK-based cancer immunotherapy.

4 MATERIALS AND METHODS

4.1.1 Cell culture and transfection

Human breast cancer cells MCF-7 and T47D were obtained from ATCC and cultured in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza). MCF-7BECN1 tet-off cells, kindly provided by Dr B. Levine (University of Texas Southwest Medical Center, Dallas), were maintained in RPMI-1640 with 200 µg/ml hygromycin and 100 ng/ml doxycycline . Murine melanoma B16-F10 cells were obtained from ATCC and grown in DMEM supplemented with 10% FCS. YT-Indy-NK cells, expressing eGFP-Granzyme B, were used for specific experiments and were grown as previously described. Unless otherwise indicated, hypoxia was generated by incubating cells in a 0.1% pO2 for 16 h.

Cell transfections were performed using Lipofectamine® 2000 reagent (Invitrogen) according to manufacturer's instructions. MCF-7 cells were stably transfected with GFP-LC3 encoding vector containing geneticin resistance gene for selection (kindly provided by Dr N. Mizushima, Tokyo Medical and Dental University, Tokyo, Japan). B16-F10 cells were stably infected with either scrambled or Beclin1 shRNA lentiviral particles (Santa Cruz Biotechnology) containing puromycin resistance gene for selection.

4.1.2 NK cell isolation

Human Peripheral Blood Mononuclear Cells (PBMC) were obtained from healthy donors. The NK Cell Isolation Kit (Miltenyi Biotec) was used to isolate NK cells from PBMC according to the manufacturer's instructions. The purity and viability of isolated NK cells were evaluated by flow cytometry (≥ 98% and 98%, respectively). NK cells were activated for 20 h in RPMI-1640 medium supplemented with 300 U/ml of human recombinant Interleukin-2 (R&D Systems).

4.1.3 Subcellular fractionation

Cells were harvested and homogenized in buffer containing 0.25M sucrose, 1mM EDTA, 10mM acetic acid, 10mM triethanolamine, 20mM N-ethylmaleimide, and 2mM PMSF, pH 7.4.

Cells were disrupted by passing 5 times through a 25G needle and a clear homogenate was generated by two consecutive centrifugations at 1,000×g for 5 min at 4°C. The supernatant (500µl) was loaded on the top of a discontinuous OptiPrep™ (Axis-Shield) gradient prepared as follows: 0.75 ml of 30%, 26%, 22%, 18%, 14% and 10%, and submitted to ultracentrifugation at 268,000×g for 310 min in a MLS-50 rotor (k factor=71, Optima™ MAX-XP centrifuge, Beckman). Twelve fractions (400 µl) were collected from the top of the gradient, lysed in 1X Laemmli buffer and analyzed by immunoblot.

4.1.4 Granzyme B loading

Cells (1.5 x 105 cells) were loaded with exogenous Granzyme B (Granzyme B) from human lymphocytes (Enzo Life Sciences) using sublytic doses of pre-activated pore-forming protein streptolysin-O (SLO) (Sigma). Cells were washed in Hank's Balanced Salt Solution (HBSS) followed by the addition of 100µl of HBSS/0.5% fetal bovine serum and 100nM of both Granzyme B and SLO and incubated for 2 h at 37°C prior to analysis by multicolor flow cytometry (FACSCanto). Results are reported as a % of death of normoxic and hypoxic cells respectively, normalized to cells treated with SLO alone.

4.1.5 Flow cytometry analysis

Cells were stained for 30 min at 4°C with the following antibodies: HLA-ABC-PE, NK1.1-PE/Cy7 (BioLegend); HLA-E-PE, HLA-G-APC (eBiosciences); ULBP-1-PE, MIC-A/B-PE, MIC-A-APC (R&D Systems); CD56-PE/Cy7, CD3-FITC (ImmunoTools); CD107a-APC (BD Biosciences). Surface expression level of each protein was assessed by flow cytometry (FACSCanto).

4.1.6 Cell lysates and immunoblot

Cells were lysed in RIPA Buffer (Sigma Aldrich) supplemented with proteases and phosphatases inhibitors cocktail (Roche). Lysates were centrifuged at 15,000×g for 20 min at

4°C, and supernatants were collected. Protein concentration was determined using BioRad protein assay kit. Proteins were separated by SDS-PAGE, transferred onto PVDF membranes and blocked with 5% skim milk in 0.1% Tween20/TBS. The following primary antibodies were used: HIF-1α (BD Biosciences); LC3B (Cell Signaling); actin (Sigma Aldrich); Beclin1 (Cell Signaling); Granzyme B (Cell Signaling); GFP (Chemicon); Atg5 (Cell Signaling); HSP70 (Cell Signaling); M6PR (Abcam). After incubation with primary antibodies, immuno-reactive bands were detected using horseradish peroxidase–conjugated secondary antibodies (Jackson Immuno Research Laboratories). Protein bands were revealed by enhanced chemiluminescence ECL (GE Healthcare).

4.1.7 Time-lapse live video microscopy

Cells were pre-cultured under normoxia or hypoxia for 24 h. Isolated NK cells were pre-activated as described above and stained with PKH-26 (red) (Invitrogen) according to the manufacturer's recommendations. GFP-LC3 expressing normoxic or hypoxic MCF-7 cells were incubated with labeled NK cells at 5/1 E/T ratio in µ-Slide 8 well chambered coverslip (IBIDI) and analyzed with a Zeiss laser scanning confocal microscope LSM-510 Meta (Carl Zeiss). Cells were maintained at 37°C in a CO2 incubator mounted on the microscope stage. Time lapse was performed using Axiovert 200M microscope (Carl Zeiss MicroImaging) and frames were taken with 40X oil objective lens in intervals of 1 frame/1-2 min during 86 min for hypoxic cells and 82 min for normoxic cells.

4.1.8 Assesment of cell doubling-time

Cell doubling was evaluated using a colorimetric cell viability assay (XTT). Briefly, cells were seeded in 96-well plates and cell viability was determined every 24 h for 3 days, by measuring absorbance at 450 and 630 nm after addition of 50 µl of 1 mg/ml XTT solution (Sigma Aldrich). Growth curves between 24-72 h were plotted and cell doubling time was calculated.

4.1.9 Formation of conjugates

NK cells isolated from healthy donor were stained with PKH-26 (red) and MCF-7 cells with CellTrace™ CFSE (green) according to the manufacturer's instructions. Effector and target cells (E/T ratio: 5/1) were co-cultured at 37°C for 0, 5, 10 and 20 min. At the end of each time point, samples were immediately fixed in ice-cold 0.5% paraformaldehyde. The double positive population (E/T conjugates) was assessed by flow cytometry (FACSAria, BD Biosciences).

4.1.10 Degranulation assay

Purified and activated NK cells were incubated for 5 h alone or with target tumor cells at 5/1 E/T ratio in the presence of anti-human CD107a antibody (BD Biosciences). Golgi Stop (BD Biosciences) was added after 1 h of incubation to block the intracellular protein transport processes. NK cells were stained for 30 min with anti-CD56 antibody (ImmunoTools) and LIVE/DEAD dye (Invitrogen) according to the manufacturer's recommendations. NK cells incubated without target cells for 5 h were used as negative control. Samples were analyzed by flow cytometry (FACSCanto).

4.1.11 Cytotoxicity assay

Target cells labeled with 10 μM of CellTrace™ CFSE (Invitrogen) were co-incubated with effectors at 1/1 and 5/1 E/T ratios, for 4 h at 37°C and analyzed by flow cytometry (FACSCanto, BD Biosciences. TO-PRO®-3 (Invitrogen) was used to assess cell death. HLA-Class I was blocked using mouse IgM *anti*-pan *HLA* Class I A6-136 mAb .

4.1.12 Granzyme B transfer

YT-INDY-NK cells (1.25×10^6) were co-incubated with target cells (E/T ratio: 5/1) at 37°C for 0 and 30 min, washed twice with PBS/1% FCS/2mM EDTA and submitted to high speed vortex to disrupt the E/T conjugates. NK cells were separated from target cells using CD56 MicroBeads kit (Miltenyi Biotec). The purity of the fraction containing target MCF-7 cells was

assessed by flow cytometry (FACSCanto). The presence of Granzyme B in target cells was evaluated by immunoblot. The transfer of Granzyme B to target cells was assessed by LSM-510-Meta confocal microscope (Carl Zeiss) after co-incubation with GFP-Granzyme B expressing YT-INDY-NK cells.

4.1.13 Subcellular fractionation

E64d/pepstatin treated cells were subjected to hypoxia for 16 h before co-incubation with YT-INDY-NK cells. Autophagosomes isolation was performed according to the protocol established by Dr F. Reggiori (Department of Cell Biology, University Medical Centre Utrecht, the Netherlands) and reported in supporting information.

4.1.14 *In vivo* experiments

10-week-old C57BL/6 female mice were injected subcutaneously with anti-asialo GM1 antibody (Cedarlane CL8955, 3.8 mg/mouse) every five days (4 times) to generate NK depleted mice (NK-). Control (NK+) mice were injected with normal rabbit IgG (Dako, 3.8 mg/mouse). Three days after the first NK cell depletion, NK+ and NK- mice were injected subcutaneously with control (B16-F10$_{BECN1-}$) or autophagy defective (B16-F10$_{BECN1-}$) cells (2.3 x 10^5 cells / mouse). Tumor growth was monitored by caliper measurement at day 10, 12, 14 and 15 and tumor volume was calculated as follow: Volume (cm^3) = (width x length x height) x 0.5236. Mice were sacrificed at day 15.

4.1.15 Statistical analysis

The statistically significant differences were determined using two-tailed Student's t test with at least $p<0.05$.

5 ANNEXES

5.1 Article 1

Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia.

Granzyme B degradation by autophagy decreases tumor cell susceptibility to natural killer-mediated lysis under hypoxia

Joanna Baginska[a], Elodie Viry[a,1], Guy Berchem[a,1], Aurélie Poli[b], Muhammad Zaeem Noman[c], Kris van Moer[a], Sandrine Medves[a], Jacques Zimmer[b], Anaïs Oudin[d], Simone P. Niclou[d], R. Chris Bleackley[e], Ing Swie Goping[e], Salem Chouaib[c,2], and Bassam Janji[a,2,3]

[a]Laboratory of Experimental Hemato-Oncology, Department of Oncology, and [b]Laboratory of Immunogenetics and Allergology, Public Research Center for Health, L-1526 Luxembourg City, Luxembourg; [c]Institut National de la Santé et de la Recherche Médicale Unit 753, Institut de Cancérologie Gustave Roussy, F-94805 Villejuif Cedex, France; [d]NorLux, Neuro-Oncology Laboratory, Department of Oncology, Public Research Center for Health, L-1526 Luxembourg City, Luxembourg; and [e]Department of Biochemistry, University of Alberta, T6G 2H7 Edmonton, AB, Canada

Edited by Noboru Mizushima, Graduate School and Faculty of Medicine, The University of Tokyo, Tokyo, Japan, and accepted by the Editorial Board September 1, 2013 (received for review March 18, 2013)

Recent studies demonstrated that autophagy is an important regulator of innate immune response. However, the mechanism by which autophagy regulates natural killer (NK) cell-mediated antitumor immune responses remains elusive. Here, we demonstrate that hypoxia impairs breast cancer cell susceptibility to NK-mediated lysis in vitro via the activation of autophagy. This impairment was not related to a defect in target cell recognition by NK cells but to the degradation of NK-derived granzyme B in autophagosomes of hypoxic cells. Inhibition of autophagy by targeting beclin1 (BECN1) restored granzyme B levels in hypoxic cells in vitro and induced tumor regression in vivo by facilitating NK-mediated tumor cell killing. Together, our data highlight autophagy as a mechanism underlying the resistance of hypoxic tumor cells to NK-mediated lysis. The work presented here provides a cutting-edge advance in our understanding of the mechanism by which hypoxia-induced autophagy impairs NK-mediated lysis in vitro and paves the way for the formulation of more effective NK cell-based antitumor therapies.

hypoxic tumor microenvironment | innate immunity | breast adenocarcinoma | immunotherapy

The major obstacle to defining an efficient immunotherapy protocol for the treatment of solid tumors is the capacity of the tumor microenvironment (TME) to inhibit the host immune response at both the local and systemic levels (1). The TME is a complex, dynamic structure composed of malignant cells and cells that influence cancer evolution, such as endothelial and immune cells (2). The TME is an important aspect of cancer biology, as it can promote neoplastic transformation, support tumor growth and invasion, protect tumors from host immunity, foster therapeutic resistance, and provide niches for dormant metastases (3). Natural killer (NK) cells are large, granular CD3−/T-cell receptor−/CD56+ lymphocytes that play a fundamental role in antitumor innate immunity (4) and participate in tumor immunosurveillance. They can kill tumor cells directly via the engagement of death receptors [Fas and TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand)] and the release of perforin- and granzyme-containing granules; or indirectly via antibody-dependent cell-mediated cytotoxicity (5, 6). The role of NK cells in tumor regression was investigated by several studies showing that tumor infiltration by NK cells predicts a favorable outcome for several cancers (7–10). The activity of NK cells is regulated by the expression of activating and inhibitory receptors that together dictate the fate of target tumor cells. The function of NK cells is inhibited by the interaction between HLA class I molecules, expressed on the surface of target cells, and killer cell Ig-like receptors (KIR) and/or CD94/NKG2A, expressed on the surface of NK cells (11). The activation of NK cells is mediated by NKp46, NKp30, and NKp44, as well as CD226 and NKG2D. The up-regulation of stress-inducible proteins on the surface of cancer cells, such as MHC class I-related chain (MIC) A and B ligands and UL16-binding proteins (ULBPs), leads to the activation of NK cells through the engagement of NKG2D (12). The loss of HLA class I expression and the up-regulation of MICA/B and ULBP1 is a common characteristic of tumors (13), which makes them targets for NK-mediated lysis (14).

Accumulating data suggest that a hypoxic microenvironment protects cancer cells from the antitumor immune response by multiple overlapping mechanisms (reviewed in ref. 15). We recently reported that under hypoxic conditions, tumor cells resist antigen-specific cytotoxic T-lymphocytes (CTL)-mediated lysis in a signal transducer and activator of transcription 3 (STAT3)-dependent manner (16). Hypoxia also contributes to the reprogramming of dendritic cells (DCs) and perpetuates inflammation via the induction of a proinflammatory DC phenotype (17). Under hypoxia, tumor cells release a number of chemokines, such as endothelial monocyte-activating polypeptide II (EMAP-II) (18), vascular endothelial growth factor (VEGF) (19), endothelin 2 (20), and monocyte chemotactic

Significance

Natural killer (NK) cells are effectors of the antitumor immunity, able to kill cancer cells through the release of the cytotoxic protease granzyme B. NK-based therapies have recently emerged as promising anticancer strategies. It is well established that hypoxic microenvironment interferes with the function of antitumor immune cells and constitutes a major obstacle for cancer immunotherapies. We showed that breast cancer cells evade effective NK-mediated killing under hypoxia by activating autophagy that we have identified to be responsible for the degradation of NK-derived granzyme B. We demonstrated that blocking autophagy restored NK-mediated lysis in vitro, and facilitated breast tumor elimination by NK cells in mice. We provided evidence that targeting autophagy may pave the way to achieve more effective NK-based anticancer immunotherapy.

Author contributions: J.B., E.V., and B.J. designed research; J.B., E.V., G.B., A.P., M.Z.N., K.v.M., S.M., and A.O. performed research; R.C.B. and I.S.G. contributed new reagents/analytic tools; J.B., E.V., G.B., A.P., M.Z.N., K.v.M., S.M., J.Z., S.P.N., S.C., and B.J. analyzed data; and J.B., E.V., and B.J. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. N.M. is a guest editor invited by the Editorial Board.

[1]E.V. and G.B. contributed equally to this work.

[2]S.C. and B.J. were co-principal investigators.

[3]To whom correspondence should be addressed. E-mail: bassam.janji@crp-sante.lu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1304790110/-/DCSupplemental.

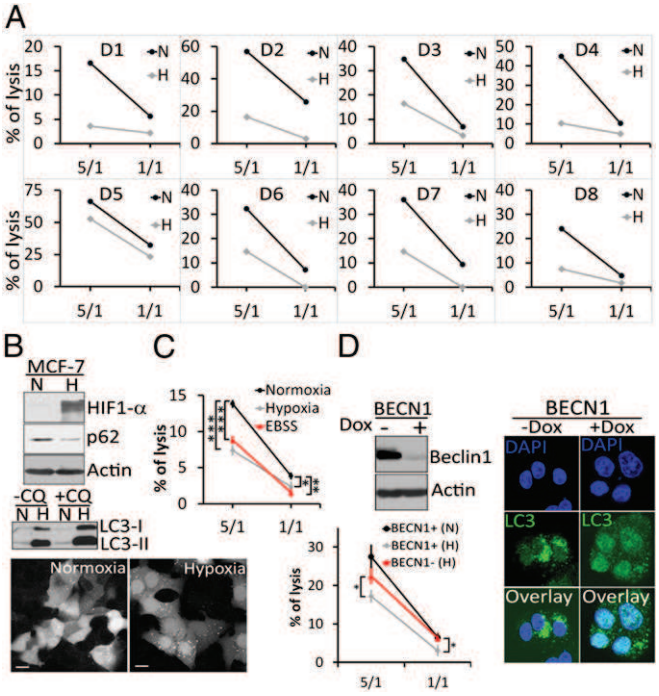

Fig. 1. Hypoxia-induced autophagy decreases the susceptibility of breast cancer cells to NK-mediated lysis in vitro. (*A*) Cytotoxicity assays were performed in duplicate using NK cells isolated from eight healthy donors (D1 to D8) at 5/1 or 1/1 E/T (Effector/Target) ratios on normoxic (N) or hypoxic (H) MCF-7 cells. Cell death was assessed by flow cytometry using TO-PRO-3. (*B*, *Upper*) Cells were cultured under normoxia (N) or hypoxia (H) conditions for 16 h. The expression levels of hypoxia inducible factor 1α, p62, and LC3 were assessed by immunoblot. (*B*, *Middle*) Untreated (–CQ) or chloroquine (60 μM) treated (+CQ) cells cultured under normoxia (N) or hypoxia (H) were assessed by immunoblot for the expression of LC3. (*B*, *Lower*) GFP–LC3 expressing normoxic or hypoxic MCF-7 cells were analyzed by fluorescence microscopy for the autophagosome formation (dots). (Scale bar, 10 μm.) (*C*) MCF-7 cells were cultured under normoxia, hypoxia, or starvation [Earle's balanced salt solution (EBSS)]. Cytotoxicity assays were performed using NK cells isolated from a healthy donor (D12). The percentage of tumor cell lysis is reported as an average (±SEM) of three experiments performed. Statistically significant differences are indicated by asterisks (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). (*D*, *Upper Left*) Autophagy-competent and -defective MCF-7 cells were generated by culturing cells expressing BECN1 under the control of a tetracycline-responsive promoter (BECN1) in the absence (–) or presence (+) of doxycycline (100 ng/mL), respectively. Beclin1 expression was assessed by immunoblot. (*D*, *Right*) The formation of autophagosomes (dot-like structures) in autophagy-competent (–Dox) and autophagy-defective (+Dox) hypoxic cells was assessed using immunofluorescence microscopy with an Alexa-Fluor-488–coupled LC3 antibody. Nuclei were stained with DAPI. (Scale bar, 10 μm.) (*D*, *Lower Left*) Cytotoxicity assays were performed on autophagy competent (BECN1+) or defective (BECN1–) MCF-7 cells cultured under normoxic (N) or hypoxic (H) conditions with NK cells isolated from a healthy donor (D13). The percentage of tumor cell lysis is reported as an average (±SEM) of three experiments, and statistically significant differences are indicated by asterisks (*$P < 0.05$).

protein-1 (MCP-1/CCL2) (21). The secretion of such cytokines leads to the accumulation of tumor-associated macrophages (TAM) and regulatory T-cells (Treg). TAM and Treg cells produce transforming growth factor-β and suppress the proliferation and activity of NK cells (22). In addition, it has been proposed that hypoxia contributes to the escape of prostate tumor cells from NK-mediated immunosurveillance by increasing the shedding of MIC molecules (23).

It is well established that the adaptation of cancer cells to hypoxic stress can also occur through the activation of autophagy, which is a process responsible for the degradation and recycling of long-lived proteins and cytoplasmic organelles in well-characterized structures named autophagosomes. Recently, the role of autophagy was expanded to include immunological functions, including interactions with the immune system in the TME (16, 24, 25). Although these studies highlight autophagy as an important player in the regulation of cancer immunity, no data are currently available that address the mechanisms by which autophagy regulates NK-dependent immune responses during hypoxia.

While clinical trials using NK-based immunotherapy have shown promising results for the treatment of hematological malignancies, solid tumors are frequently resistant to this therapy. Understanding the molecular mechanisms that underlie tumor escape from NK-mediated surveillance remains an issue of major interest. In this report, we demonstrate that hypoxia reduces breast cancer cell susceptibility to NK-mediated lysis by a mechanism involving the activation of autophagy and the subsequent degradation of NK-derived granzyme B (GzmB) in vitro. We provide in vivo evidence that blocking autophagy in hypoxic tumors induces tumor regression by facilitating NK-mediated elimination. This study highlights autophagy as a unique mechanism of tumor cell resistance to NK-mediated lysis.

Results

Hypoxia-Induced Autophagy in Breast Cancer Cells Decreases Susceptibility to NK-Mediated Lysis. We investigated whether hypoxia impairs NK-mediated lysis of MCF-7 breast adenocarcinoma cells. Using NK cells isolated from the peripheral blood monocellular cells (PBMC) of eight healthy donors (D1 to D8), we demonstrated (Fig. 1*A*) that despite the interindividual variability of NK cell cytolytic potential, the percentage of hypoxic (H) MCF-7 cell lysis by NK cells was decreased compared with that of normoxic (N) MCF-7 cells in all cases.

This impairment correlated with the induction of the autophagic flux as indicated by the degradation of p62/Sequestosome 1 (SQSTM1), the accumulation of microtubule-associated protein light chain-3 II (LC3-II) in chloroquine (CQ)-treated cells and the formation of autophagosomes in hypoxic cells (Fig. 1*B* and Fig. S1*A*). Time-lapse video microscopy of GFP–LC3-expressing normoxic or hypoxic MCF-7 cells (Fig. S1*B*) cocultured with PKH-26–stained NK cells confirmed that normoxic tumor cells were efficiently lysed by NK cells (Movie S1) compared with hypoxic tumor cells, which displayed several autophagosomes (Movie S2). In addition, time-lapse video microscopy performed under the same conditions using propidium iodide provided compelling evidence that normoxic tumor cells underwent NK-mediated cell death (Movie S3) compared with hypoxic tumor cells (Movie S4). Similar results were obtained using the T47D breast cancer cell line (Fig. S1*C*), which confirms that the impairment of breast tumor cell susceptibility to NK-mediated lysis during hypoxia is not restricted to the MCF-7 cell line. In addition, we demonstrated that, independently of hypoxia, autophagy induction by other stimuli (e.g., starvation) also impairs NK-mediated lysis (Fig. 1*C*). To determine the extent to which the induction of autophagy is implicated in the impairment of NK-cell–mediated lysis, we used the MCF-7 cell line expressing Beclin1 (MCF-7_{BECN1}) under the control of a tetracycline-responsive promoter (tet-off). Using this cell line, autophagy-competent (MCF-7_{BECN1+}) or autophagy-defective (MCF-7_{BECN1-}) MCF-7 cells can be generated by culturing cells in the absence (–) or presence (+) of doxycycline, respectively (Fig. 1*D*, *Upper Left* and *Right*). Cytotoxic assay (Fig. 1*D*, *Lower Left*) clearly demonstrated that NK cells kill autophagy-deficient (BECN1–) MCF-7 cells more efficiently than autophagy-competent (BECN1+) MCF-7 cells under hypoxic conditions. Similar results were obtained in ATG5-deficient MCF-7 cells (Fig. S1*D*). These data indicate that autophagy itself is a key determinant in the impairment of NK-mediated lysis of MCF-7 tumor cells.

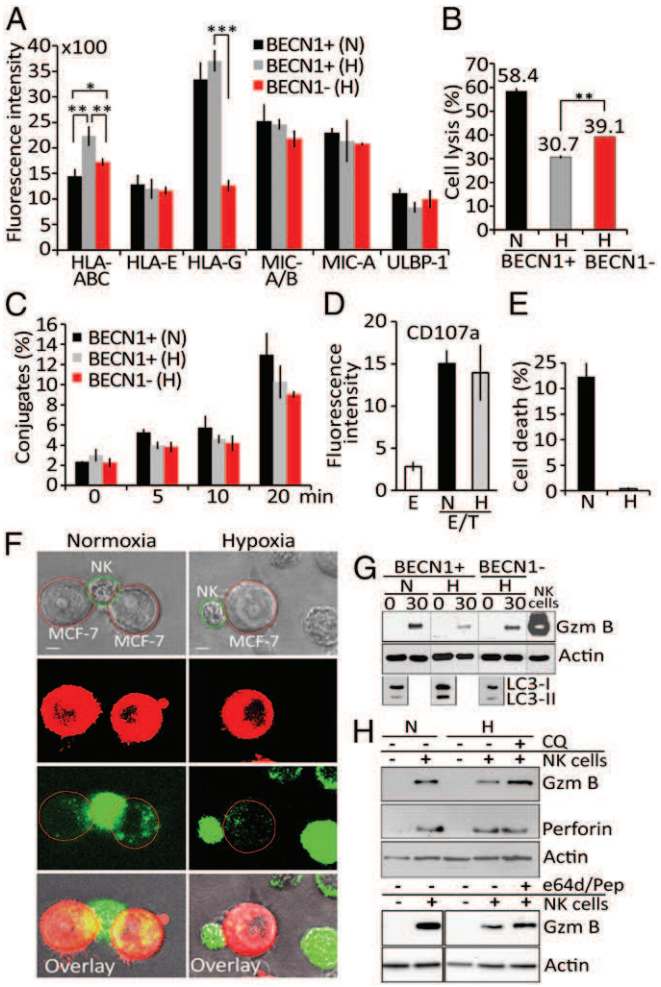

Fig. 2. Hypoxia-induced autophagy impairs tumor cell susceptibility to NK-mediated lysis without affecting NK cell function. (*A*) Autophagy-competent (BECN1+) and -defective (BECN1−) MCF-7 cells cultured under normoxia (N) or hypoxia (H) were assessed by flow cytometry for the expression of MHC class I molecules and NK cell-activating NKG2D ligands . Fluorescence intensity is reported as an average (±SEM) of three experiments. Statistically significant differences are indicated by asterisks (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$). (*B*) BECN1+ or BECN1− MCF-7 cells were pretreated with mouse IgM anti-pan HLA class I A6-136 mAb and incubated under normoxic (N) or hypoxic (H) conditions before presentation to NK cells at a 5/1 E/T ratio. The percentage of target cell lysis is reported. Statistically significant differences are indicated by asterisks (**$P < 0.005$). (*C*) BECN1+ or BECN1− MCF-7 cells cultured under normoxia (N) or hypoxia (H) were incubated with NK cells. The percentage of conjugate formation at indicated time was determined by flow cytometry. Results are reported as an average (±SEM) of three independent experiments. No statistically significant differences were observed. (*D*) NK cells as effectors (E) were cultured alone or with normoxic (N) or hypoxic (H) MCF-7 cells at a 5/1 E/T ratio. The level of CD107a (a degranulation marker) on the surface of the NK cells was assessed by flow cytometry. Fluorescence intensity is reported as an average (±SEM) of five experiments performed with NK cells from different donors. (*E*) Normoxic (N) or hypoxic (H) MCF-7 cells were loaded with exogenous, activated granzyme B (0.8 μg/mL) using the pore-forming protein streptolysin-O. The percentage of cell death was determined by flow cytometry. Results are reported as an average (±SEM) of three experiments. (*F*) PKH-26–stained normoxic or hypoxic MCF-7 cells (red) were cocultured with YT–Indy–NK cells expressing GFP–GzmB (green) at 5/1 E/T ratio. The content of NK-derived GFP–GzmB in target cells was monitored after 30 min of coculture by a Zeiss laser-scanning confocal microscope (LSM-510 Meta) with a 60× oil immersion objective. (Scale bar, 10 μm.) (*G*) Autophagy-competent (BECN1+) or -defective (BECN1−) MCF-7 cells were incubated under normoxia (N) or hypoxia (H) and cocultured with NK cells at 5/1 E/T ratio for 0 and 30 min. Following separation, tumor cells were lysed and subjected to immunoblot for the intracellular GzmB content. NK cell lysate was used as a control for GzmB detection. The expression of LC3 was reported as a marker for autophagy. (*H*) Normoxic (N) or hypoxic (H) MCF-7 cells were cultured alone (−) or with NK cells (+) at 5/1 ratio for 30 min in the presence (+) or absence (−) of chloroquine (CQ) or e64d/pepstatin. Tumor cells separated from NK were subjected to immunoblot analysis to evaluate the intracellular GzmB and perforin content.

Impaired NK-Mediated Lysis of Hypoxic Tumor Cells Is Not Related to a Defect in Tumor Cell Recognition by NK Cells. We investigated whether the resistance of hypoxic tumor cells to NK-mediated lysis is related to an increase in NK-inhibitory MHC class I molecules or a decrease in NK-activating NKG2D ligands on the cell surface. Among all analyzed molecules (Fig. 2*A*), only HLA-A, B, and C were found to be significantly up-regulated on the surface of autophagy competent hypoxic MCF-7 cells [BECN1+ (H)] and down-regulated on autophagy-defective cells cultured under the same conditions [BECN1− (H)]. We next investigated whether the resistance of hypoxic MCF-7 cells to NK-mediated lysis was related to an increase in HLA class I molecules. Our data demonstrated that even when HLA class I molecules are blocked, the lysis of hypoxic (BECN1−) MCF-7 cells is significantly improved compared with hypoxic (BECN1+) MCF-7 cells (Fig. 2*B*). We therefore suggest that independently of HLA class I expression level, the autophagic status of target cells plays a key role in the resistance to NK-mediated lysis in our model. Similarly, the data presented in Fig. 2*C* demonstrated a time-dependent increase in the percentage of conjugates between NK and tumor cells, but no significant difference in conjugate formation was observed between autophagy-competent (BECN1+) and -defective (BECN1−) cells cultured under normoxic or hypoxic conditions. Representative images from time-lapse experiments support the conclusion that NK cells maintain their ability to interact with hypoxic cells in our model (Fig. S2). We also addressed whether the degranulation activity of NK cells was affected by hypoxic tumor cells. Fig. 2*D* showed a basal level of CD107a on the surface of NK cells cultured alone (E), but a significantly higher level was detected when NK cells were cocultured with normoxic or hypoxic tumor cells (E/T). As no difference in the level of CD107a was observed when NK cells were cocultured with normoxic and hypoxic tumor cells, the resistance of hypoxic tumor cells to NK-mediated lysis does not appear to be related to a defect in NK activity. Our results further suggest that resistance is dependent on an intrinsic mechanism that makes tumor cells less sensitive to the cytotoxic granules released by NK cells. This hypothesis was supported by data (Fig. 2*E*) demonstrating that under conditions where normoxic and hypoxic tumor cells were treated with the pore-forming protein streptolysin-O, GzmB only killed normoxic cells.

In Vitro Hypoxic Tumor Cells Degrade NK Cell-Derived Granzyme B in Lysosomes via Autophagy. To investigate the mechanism underlying the observed reduction of GzmB-mediated killing of H cells, we analyzed the intracellular GzmB content of target cells. PKH-26–stained normoxic or hypoxic MCF-7 cells were cocultured with YT–Indy–NK cells expressing GFP–GzmB. Significantly lower levels of GFP–GzmB were detected in hypoxic cells than in normoxic (N) cells (Fig. 2*F*). We next evaluated whether targeting autophagy by knocking down BECN1 restores GzmB level in hypoxic cells. Our results demonstrated that the level of GzmB is restored in BECN1-defective cells (Fig. 2*G*). As the NK-mediated lysis of hypoxic cells was also restored by targeting ATG5, it remains to be determined whether the level of GzmB was affected in ATG5-defective cells. Nevertheless, these results suggest that following its delivery to hypoxic cells, GzmB is loaded into autophagosomes and subsequently degraded in lysosomes. This was further supported by our data (Fig. 2*H*) showing that the level of GzmB was restored by autophagy and lysosomal hydrolases inhibitors chloroquine and e64d/Pepstatin,

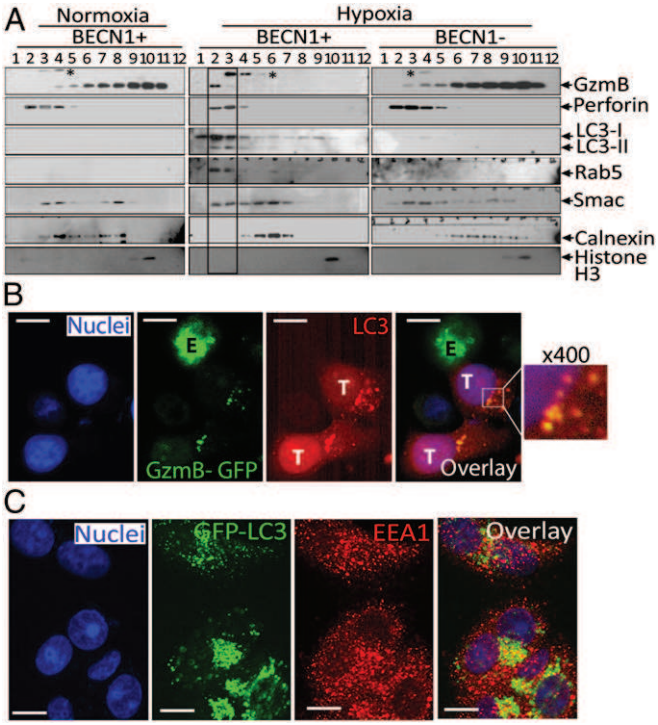

Fig. 3. In vitro tumor cells degrade NK cell-derived granzyme B in lysosomes via autophagy. (*A*) NK cells were cocultured with autophagy-competent (BECN1+) or -defective (BECN1−) normoxic or hypoxic MCF-7 cells. Cell lysates of separated tumor cells were subjected to subcellular fractionation. Fractions 1 to 12 were characterized by Western blot using the indicated antibodies. * indicates an unspecific band. (*B*) Chloroquine-treated hypoxic MCF-7 cells (T) were cocultured with GzmB–GFP–expressing NK cells (E). Target cells were stained with an Alexa-Fluor-568–coupled rabbit anti-LC3 antibody to visualize autophagosomes (red) and with DAPI to stain nuclei (blue). Colocalization of GzmB with LC3 in autophagosomes was visualized by confocal microscopy using a 100× oil immersion objective. An enlarged image (400×) of the overlay (box) is shown. (Scale bar, 10 μm.) (*C*) Chloroquine treated GFP–LC3 –expressing MCF-7 cell were cultured under hypoxia stained with anti-EEA1 Alexa-Fluor-568–coupled anti-rabbit IgG antibody (red) and DAPI for nuclei. Cells were analyzed by confocal microscopy. The overlay image shows colocalization of LC3 and EEA1 (yellow dots). (Scale bar, 10 μm.)

respectively. In contrast, the level of perforin in target cells was not affected by hypoxia or autophagy inhibition (Fig. 2*H*). The subcellular distribution of NK-derived GzmB was further analyzed by fractionation of autophagy-competent (BECN1+) or -defective (BECN1−) MCF-7 cells cultured under normoxic or hypoxic conditions. Fig. 3*A* showed a dramatic difference in the distribution pattern of GzmB between normoxic and hypoxic (BECN1+) cells. GzmB is mostly present in fractions 4 to 11 in normoxic cells; however, it is exclusively detected in fraction 2 and to a lesser extent in fraction 3 in hypoxic cells. Interestingly, the GzmB-containing fractions 2 and 3 are positive for LC3 (autophagosomes) and Rab5 (endosomes), suggesting that these fractions may correspond to amphisomes (structures generated from the fusion of autophagosomes and late endosomes). Taken together, these results suggest that endosomes containing GzmB and perforin fuse with autophagosomes upon activation of autophagy in hypoxic cells, leading to the specific degradation of GzmB. The selectivity of GzmB degradation by autophagy was further supported by our data demonstrating that inhibition of the autophagy cargo protein p62 restores GzmB level in hypoxic targets (Fig. S3). Importantly, targeting autophagy in hypoxic cells dramatically changes the subcellular distribution of GzmB to a profile similar to that observed in normoxic cells. The presence of NK-derived GzmB in autophagosomes of hypoxic cells was further confirmed by immunofluorescence data showing colocalization of GzmB–GFP with autophagosomes (LC3-stained structures) (Fig. 3*B*); those autophagosomes also contained the endosomal marker EEA1 (Fig. 3*C*).

Targeting Autophagy Increased NK-Mediated Tumor Regression in Vivo. To assess whether inhibition of autophagy improves NK-mediated tumor regression in vivo, we used two aggressive syngeneic murine models: B16–F10 melanoma tumors and 4T1 breast carcinoma tumors. B16–F10 tumors have been previously reported as highly hypoxic, with a selective activation of autophagy in hypoxic zones (16), and 4T1 tumors have been reported as a suitable experimental animal model for human mammary cancer (26). We first evaluated whether host NK cells affect B16–F10 and 4T1 tumor growth in C57BL/6 and BALB/c mice, respectively. To address this issue, control (NK+) and NK-depleted (NK−) mice, which were generated by repeated injection of normal rabbit IgG and antiasialo GM1, respectively, were engrafted with B16–F10 or 4T1 cells. The efficiency and selectivity of antiasialo GM1 in depleting murine NK cells in C57BL/6 and BALB/c mice are shown in Fig. S4*A*. The data presented in Fig. 4*A* demonstrated a significant increase in B16–F10 and 4T1 tumor volume in NK− mice compared with NK+ mice, indicating that NK cells play a role in B16–F10 and 4T1 tumor regression in vivo. To determine the impact of autophagy on NK-mediated lysis in vivo, we analyzed the growth of autophagy-defective (BECN1−) B16–F10 and 4T1 tumor cells in both NK+ and NK− mice. B16–F10$_{\mathrm{BECN1-}}$ and 4T1$_{\mathrm{BECN1-}}$ cells were generated using BECN1 shRNA lentiviral particles. B16–F10 and 4T1 cells infected with scrambled shRNA-expressing vectors (B16–F10$_{\mathrm{BECN1+}}$ and 4T1$_{\mathrm{BECN1+}}$) were

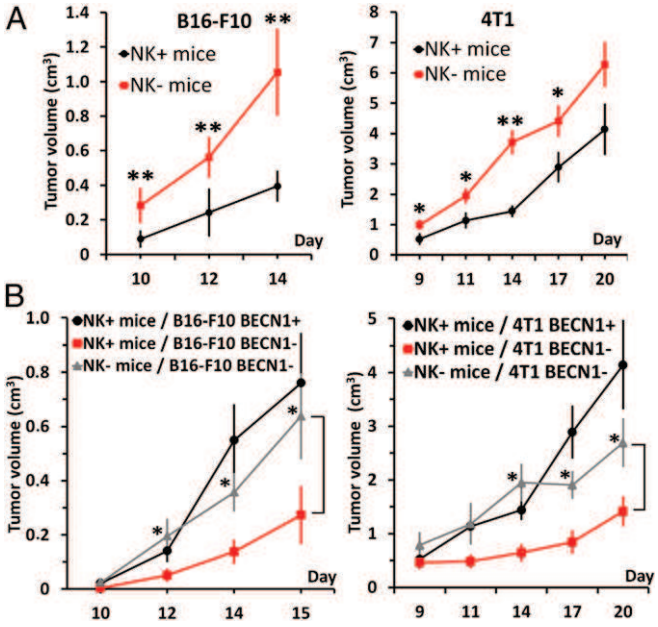

Fig. 4. Targeting autophagy in vivo improves tumor elimination by NK cells. (*A*) Control (NK+) and NK-depleted (NK−) C57BL/6 (*Left*) or BALB/c (*Right*) mice were engrafted with B16–F10 murine melanoma cells and 4T1 mammary carcinoma cells, respectively. Tumor growth in NK+ and NK− C57BL/6 (n = 7) and BALB/c (n = 10) mice was monitored using caliper measurements on the indicated days. Statistically significant differences in tumor volume are indicated by asterisks (*P < 0.05; **P < 0.005). (*B*) Autophagy-competent (BECN1+) or -defective (BECN1−) B16–F10 or 4T1 cells were injected s.c. (*Left*) or in the mammary fat pad (*Right*), respectively, in control (NK+) and NK-depleted (NK−) C57BL/6 (n = 7) and BALB/c (n = 10) mice. Tumor growth was monitored using caliper measurements on the indicated days. Statistically significant differences are indicated by asterisks (*P < 0.05).

used as autophagy-competent control cells. Stable clones of B16–$F10_{BECN1-}$ and $4T1_{BECN1-}$ cells were selected, and their in vitro growth was determined (Fig. S4*B*). The in vivo experimental design is shown in Fig. S4*C*. Fig. 4*B* demonstrated that in NK+ mice, the volume of B16–$F10_{BECN1-}$ and $4T1_{BECN1-}$ tumors (red curves) was significantly reduced compared with that of BECN1+ tumors (black curves). This reduction is most likely due to the ability of NK cells to eliminate autophagy-defective cells more efficiently than autophagy-competent cells. Consistent with this hypothesis, in NK-depleted mice (NK–), the regression of BECN1– tumors was no longer observed (gray vs. red curves). Taken together, these results suggest that blocking autophagy in tumors facilitates and improves their elimination by NK cells in vivo.

Discussion

In this report, we identify autophagy as a major player in the resistance of breast cancer cells to NK-mediated lysis. Our in vivo data validate this concept and highlight the inhibition of autophagy as a potential therapeutic approach to improve NK-based cancer immunotherapy. In addition, this report elucidates the mechanism by which autophagy impairs the susceptibility of tumor cells to NK-mediated killing. This study demonstrates that the activation of autophagy under hypoxic conditions causes the degradation of NK-derived GzmB in vitro, which compromises the ability of NK cells to eliminate tumor cells. As the impairment of NK-mediated lysis of tumor cells was also observed under starvation conditions, it remains important to determine whether the degradation of GzmB occurs independently of the nature of stimuli involved in the activation of autophagy in target tumor cells.

NK-based immunotherapy is a promising therapy for solid and hematologic cancers, and it can potentially be combined with chemotherapy, radiation, or monoclonal antibody therapy (27). One of the major obstacles for defining successful NK-based therapeutic protocols is the ability of tumor cells to activate several mechanisms that lead to tumor escape from NK-mediated lysis. Extensive efforts have been made in recent years to identify these mechanisms (28). Inefficient killing of tumor cells by NK cells may be due, in part, to the down-regulation of NKG2D-activating ligands on the surface of tumor cells. Consistent with this hypothesis, hypoxia has been reported to increase the shedding of MIC from the surface of prostate cancer cells (23). In MCF-7 cells, however, we did not detect any decrease in the expression of MIC on the surface of H cells, which suggests that in our model, resistance to NK-mediated killing is not related to modulation in the expression of this NKG2D ligand. Interestingly, our data demonstrate a significant up-regulation of the expression of NK cell-inhibiting MHC class I molecules on the surface of hypoxic cells. Although the causal mechanisms underlying this increase remain unknown, our result indicated that resistance to NK-mediated lysis under hypoxia occurs independently of MHC class I expression levels. In addition, it is unlikely that resistance is related to a defect in NK/target cell interaction, as no difference in the formation of conjugates between NK cells and normoxic or hypoxic cells was observed. Based on these data, we exclude the possibility of autophagy-induced destabilization of the immunological synapse in hypoxic tumor cells, as recently described (29). Our results provide evidence that there is no difference in the degranulation level of NK cells when cocultured with normoxic or hypoxic cells. This eliminates the possibility of a defect in the cytotoxic potential of NK cells toward hypoxic tumor cells as a mechanism of resistance. Having demonstrated that NK cells fulfill their cytotoxic functions toward both hypoxic (H) and normoxic (N) cells, and that exogenous GzmB was unable to kill hypoxic tumor cells, we propose that autophagy operates as an intrinsic resistance mechanism in tumor cells.

The delivery of the lytic effector proteins perforin and granzymes to target cells occurs by at least three mechanisms. GzmB can be taken up by endosomes within the target cell by fluid-phase endocytosis or by mannose 6–phosphate receptor (M6PR)-mediated endocytosis (30). The third mechanism involves heat shock protein 70 (HSP70) bound to GzmB at the cell surface (31). We did not detect any differences in the cell surface expression of HSP70 or M6PR of normoxic and hypoxic cells (Fig. S5). These results rule out a defect in the transfer of the lytic effector proteins, perforin and granzymes, to hypoxic target cells. It is still debated whether granzymes enter via perforin pores formed at the plasma membrane or whether perforin and granzymes are endocytosed with subsequent release of granzymes from endosomes into the cytoplasm (32). Recent studies indicated that perforin activates clathrin- and dynamin-dependent endocytosis, which removes perforin and granzymes from the plasma membrane to enlarged early endosomes called "gigantosomes." Subsequently, perforin forms pores in the gigantosome membrane, allowing for the gradual release of GzmB (33). Once released inside target cells, GzmB initiates apoptotic cell death. It has been reported that early endosomes can fuse with autophagic vacuoles to form amphisomes. This seems to be a prerequisite for the maturation of autophagic vacuoles, their subsequent fusion with lysosomes, and the formation of autophagolysosome (34). Based on these findings, we propose that during the intracellular trafficking, GzmB is subjected to degradation in autophagosomes under hypoxic conditions in vitro. Several data reported in this study support such a mechanism: *i*) the level of NK-derived GzmB detected in hypoxic cells is significantly lower than that in normoxic cells; *ii*) inhibition of autophagy by targeting BECN1 restores the level of GzmB and subsequently restores NK-mediated lysis of hypoxic cells; and *iii*) NK-derived GzmB is detected in LC3- and Rab5-positive cellular compartments, suggesting its presence within amphisomes in hypoxic cells. Together, our data identify a potential mechanism by which autophagy impairs the susceptibility of tumor cells to NK-mediated lysis in vitro. A schematic representation of this mechanism is provided in Fig. S6.

To evaluate the impact of autophagy on the regulation of NK-mediated immune responses to tumors in vivo, we used C57BL/6 and BALB/c mice transplanted with syngeneic murine B16–F10 melanoma and 4T1 breast adenocarcinoma tumor cells. The use of B16–F10 transplantation in mice to evaluate the role of autophagy in vivo provides several advantages, as previously reported (16). Our results clearly demonstrate that NK cells control B16–F10 and 4T1 tumor development in vivo, as the depletion of NK cells dramatically increases tumor growth. After establishing the role of NK cells in the control of both B16–F10 and 4T1 tumor growth, we further demonstrated that the growth of genetically engineered autophagy-defective B16–F10 and 4T1 cells was substantially inhibited in immunocompetent mice. Our data, demonstrating that an inhibition of tumor growth was no longer observed when NK cells were depleted, highlight the key role of autophagy in the impairment of NK-mediated tumor cell killing in vivo.

The TME plays a critical role in the control of immune protection by a number of overlapping mechanisms, which ultimately lead to tumor evasion from immunosurveillance. Tumors have evolved to use hypoxic stress to their own advantage by activating key biochemical and cellular pathways that are important for progression, survival, and metastasis. In this regard, our study underlines the inhibition of autophagy as a cutting-edge approach for the formulation of more effective NK-based cancer immunotherapies. Although there are several ongoing clinical trials using NK cells for cancer treatment, this study highlights the importance of integrating autophagy inhibitors as an innovative strategy in NK-based cancer immunotherapy.

Materials and Methods

Cytotoxicity Assay. Target cells were labeled with 10 μM of CellTrace carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen), coincubated with effector cells at 1/1 and 5/1 effector to target cell (E/T) ratios for 4 h at

37 °C and analyzed by flow cytometry (FACSCanto). TO-PRO-3 (Invitrogen) was used to assess cell death. HLA-class I was blocked using a mouse IgM anti-pan HLA class I A6-136 mAb (35).

Granzyme B Transfer. YT–INDY–NK cells (1.25×10^6) were coincubated with target cells (E/T ratio: 5/1) at 37 °C for 0 and 30 min, washed twice with a solution of 1% (vol/vol) FCS and 2 mM EDTA in PBS, and submitted to high-speed vortexing to disrupt E/T conjugates. Target cells were separated from NK cells using a CD56 MicroBeads kit (Miltenyi Biotec). The purity of the fraction containing the target MCF-7 cells was assessed by flow cytometry (FACSCanto). The presence of granzyme B in target cells was evaluated by immunoblot. The transfer of granzyme B to target cells was also assessed using a Laser scanning microscopy (LSM)-510-Meta (Carl Zeiss) after coincubation with GFP–granzyme B-expressing YT–INDY–NK cells.

Subcellular Fractionation. Normoxic or hypoxic MCF-7 cells were treated with e64d and pepstatin before coincubation with YT–INDY–NK cells for 30 min at 37 °C. Following separation from NK cells, subcellular fractionation of the target cells was performed according the protocol established by Fulvio Reggiori (Department of Cell Biology, University Medical Centre Utrecht, Utrecht, The Netherlands) to isolate autophagosomes. Details of the experimental procedure are described in the *SI Materials and Methods*.

In Vivo Experiments. Ten-week-old C57BL/6 or 7-wk-old BALB/c female mice were injected s.c. with anti-asialo GM1 antibody (3.8 mg per mouse) (Cedarlane CL8955) at indicated days to generate NK-depleted mice (NK−). The experimental design is presented in Fig. 4*B*, *Upper*. Control (NK+) mice were injected with normal rabbit IgG (3.8 mg per mouse) (Dako). Three days after the first NK cell depletion, mice were injected with tumor cells. C57BL/6 mice were injected s.c. with control (B16–$F10_{BECN1+}$) or autophagy-defective (B16–$F10_{BECN1-}$) B16–F10 cells (2.3×10^5 cells per mouse). BALB/c mice were injected orthotopically in the mammary fat pad with control ($4T1_{BECN1+}$) or autophagy-defective ($4T1_{BECN1-}$) 4T1 cells (5×10^4 cells per mouse). Tumor growth was monitored using caliper measurements on the indicated days, and tumor volume was calculated as follows: Volume (cm^3) = (width × length × height) × 0.5236. C57BL/6 and BALB/c mice were killed on days 15 and 20, respectively. Animal experiments have been approved by the national and institutional (CRP-Sante) review boards responsible for animal studies in Luxembourg.

ACKNOWLEDGMENTS. We thank Prof. Rolf Bjerkvig (University of Bergen) for helpful discussions and critical reading of the manuscript; Dr. Beth Levine (University of Texas Southwest Medical Center) for providing MCF-7_{BECN1} tet-off cells; Prof. Alessandro Moretta (Department of Experimental Medicine, University of Genova) for providing anti-pan HLA class I A6-136 mAb; and Dr. Fulvio Reggiori (University Medical Centre Utrecht). We also thank Dr. Etienne Moussay, Dr. Jérome Paggetti, Hakim Boulenouar, Loredana Jacobs, Virginie Baus, and Nicolaas H. C. Brons for technical assistance. This work was supported by grants from Public Research Center for Health, Luxembourg (REC-LHCE-2009-0201) and Fondation Cancer, Luxembourg (FC/2012/02) (to B.J.); grants from the Ligue Contre le Cancer (Comité de Val de Marne), Institut National du Cancer (10128), and Association de Recherche sur le Cancer (R01354) (to S.C.); and by the Canadian Institutes of Health Research (to R.C.B. and I.S.G.). J.B. is a recipient of an Aide à la Formation-Recherche (AFR) grant (2009-1201) from the Fonds National de la Recherche, Luxembourg. S.C. and B.J. were coprincipal investigators of this work.

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Supporting Information

Baginska et al. 10.1073/pnas.1304790110

SI Materials and Methods

Cell Culture, Treatment, and Transfection. Human MCF-7 and T47D breast cancer cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 supplemented with 10% (vol/vol) FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza). MCF–7beclin1 (BECN1) tetracycline-responsive promoter (tet-off) cells, which were kindly provided by B. Levine (University of Texas Southwest Medical Center, Dallas), were maintained in RPMI-1640 supplemented with 200 μg/mL hygromycin and 100 ng/mL doxycycline (1).

Cell transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. MCF-7 cells were stably transfected with a GFP–LC3–encoding vector containing a geneticin resistance gene for selection (kindly provided by N. Mizushima, Tokyo Medical and Dental University, Tokyo). ATG5-defective MCF-7 cells (ATG5−) were generated by stable infection with ATG5 shRNA lentiviral particles (Santa Cruz Biotechnology) containing a puromycin resistance gene for selection. Control MCF-7 cells (ATG5+) were infected with scrambled shRNA. Targeting p62/SQSTM1 was performed by transfection of cells with p62 siRNA (Qiagen). Unless otherwise indicated, hypoxia was generated by incubating cells in 0.1% (vol/vol) pO2 for 16 h. Alternatively, autophagy was induced by cultivating cells in Earle's Balanced Salt Solution (EBSS) for 16 h. Lysosomal hydrolases were inhibited by treatment of cells with e64d/pepstatin (200 ng/mL) for 16 h. Inhibition of autophagic flux was performed by incubating cells with chloroquine (60 μM) for 1 h. YT–Indy–natural killer (NK) cells, expressing eGFP–granzyme B, were used for specific experiments and were grown as previously described (2). Murine melanoma B16–F10 cells were obtained from the ATCC and grown in DMEM supplemented with 10% (vol/vol) FCS. YT–Indy–NK cells, expressing eGFP–granzyme B, were used for specific experiments and were grown as previously described (2). Murine mammary carcinoma 4T1 cells were obtained from the ATCC and grown in RPMI-1640 supplemented with 10% (vol/vol) FCS. Cells were stably infected with either scrambled or Beclin1 shRNA lentiviral particles (Santa Cruz Biotechnology) containing a puromycin resistance gene for selection.

NK Cell Isolation. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors. An NK Cell Isolation Kit (Miltenyi Biotec) was used to isolate NK cells from the PBMC according to the manufacturer's instructions. The purity and viability of isolated NK cells were evaluated by flow cytometry (≥98% and 98%, respectively). NK cells were activated for 20 h in RPMI-1640 medium supplemented with 300 U/mL of human recombinant Interleukin-2 (R&D Systems).

Subcellular Fractionation. Cells were harvested and homogenized in buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine, 20 mM *N*-ethylmaleimide, and 2 mM PMSF, pH 7.4. Cells were disrupted by passing them through a 25G needle five times, and a clear homogenate was generated by two consecutive centrifugations at 1,000 × *g* for 5 min at 4 °C. The supernatant (500 μL) was loaded onto the top of a discontinuous OptiPrep (Axis-Shield) gradient [0.75 mL each of 30, 26, 22, 18, 14, and 10% (vol/vol) solutions] and submitted to ultracentrifugation at 268,000 × *g* for 310 min in a MLS-50 rotor (k factor = 71, Optima MAX-XP centrifuge, Beckman). Twelve fractions (400 μL) were collected from the top of the gradient, lysed in 1× Laemmli buffer, and analyzed by immunoblot.

Granzyme B Loading. Cells (1.5×10^5 cells) were loaded with exogenous granzyme B (GzmB) from human lymphocytes (Enzo Life Sciences) using sublytic doses of preactivated pore-forming protein streptolysin-O (SLO) (Sigma). Cells were washed in Hank's Balanced Salt Solution (HBSS), followed by the addition of 100 μL of HBSS with 0.5% (vol/vol) FBS and 100 nM of both GzmB and SLO. The cells were incubated for 2 h at 37 °C before analysis using multicolor flow cytometry (FACSCanto). Results are reported as a percentage of death for normoxic (N) and hypoxic (H) cells and normalized to cells treated with SLO alone.

Flow Cytometry Analysis. Cells were stained for 30 min at 4 °C with the following antibodies: HLA–ABC–phycoerythrin (PE) and NK1.1–PE/Cy7 (BioLegend); HLA–E–PE and HLA–G–APC (eBiosciences); ULBP-1–PE, MIC–A/B–PE, and MIC–A–APC (R&D Systems); CD56–PE/Cy7 and CD3–FITC (ImmunoTools); and CD107a–APC and CD49b–APC (BD Biosciences). Surface expression levels of each protein were assessed using flow cytometry (FACSCanto).

Cell Lysates and Immunoblot. Cells were lysed in RIPA Buffer (Sigma Aldrich) supplemented with a protease and phosphatase inhibitor mixture (Roche). Lysates were centrifuged at 15,000 × *g* for 20 min at 4 °C, and supernatants were collected. Protein concentration was determined using a BioRad protein assay kit. Proteins were separated by SDS/PAGE, transferred onto PVDF membranes, and blocked with 5% (vol/vol) skim milk in 0.1% (vol/vol) Tween 20 in TBS. The following primary antibodies were used: HIF-1α and p62 (BD Biosciences); LC3B, Beclin1, granzyme B, Atg5, Smac, Calnexin, heat shock protein (HSP) 70, and Histone H3 (Cell Signaling); actin (Sigma Aldrich); perforin (Biolegend); EEA1 Rab5A (Santa Cruz Biotechnology); GFP (Chemicon); and M6PR (Abcam). After incubation with primary antibodies, immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories). Protein bands were revealed by enhanced chemiluminescence ECL (GE Healthcare).

Time-Lapse Live Video Microscopy and Confocal Imaging. Cells were precultured under normoxic or hypoxic conditions for 24 h. Isolated NK cells were preactivated as described above and stained with PKH-26 (red) (Invitrogen) according to the manufacturer's recommendations. GFP–LC3–expressing normoxic or hypoxic MCF-7 cells were incubated with labeled NK cells at a 5/1 E/T ratio in μ-Slide eight-well chambered coverslips (IBIDI) and analyzed with a Zeiss LSM-510 Meta laser scanning confocal microscope (Carl Zeiss). In specific time-lapse experiments, propidium iodide (1.5 μM) was added to the cell culture medium as a cell death indicator. Cells were maintained at 37 °C in a CO_2 incubator mounted on the microscope stage. Time-lapse microscopy was performed using an Axiovert 200M microscope (Carl Zeiss MicroImaging), and images were captured with a 40× oil objective lens at intervals of one frame per 1–2 min during 82 min for normoxic cells and 86 min for hypoxic cells.

For specific experiments, autophagosomes and endosomes were visualized using an LSM-510-Meta confocal microscope (Carl Zeiss). These cellular structures were detected after staining with anti-LC3 (Cell Signaling) and anti-EEA1 (Santa Cruz Biotechnology) primary antibodies, respectively, followed by incubation with Alexa-Fluor-488/568–coupled secondary antibodies. DAPI was used to stain nuclei (blue).

Cell Doubling-Time Evaluation. Cell doubling was evaluated using a colorimetric cell viability assay (XTT). Briefly, cells were seeded in 96-well plates, and cell viability was determined every 24 h for 3 d by measuring absorbance at 450 and 630 nm after the addition of 50 μL of 1 mg/mL XTT solution (Sigma Aldrich). Growth curves were plotted between 24 and 72 h, and the cell-doubling time was calculated.

Formation of Conjugates. NK cells isolated from healthy donors were stained with PKH-26 (red), and MCF-7 cells were stained with CellTrace CFSE (green) according to the manufacturer's instructions. Effector and target cells (E/T ratio: 5/1) were co-cultured at 37 °C for 0, 5, 10, and 20 min. At each time point, the samples were immediately fixed in ice-cold 0.5% (vol/vol) paraformaldehyde. The double-positive population (E/T conjugates) was assessed using flow cytometry (FACSAria).

Degranulation Assay. Purified and activated NK cells were incubated for 5 h alone or with target tumor cells at a 5/1 E/T ratio in the presence of anti-human CD107a antibody (BD Biosciences). Golgi Stop (BD Biosciences) was added after 1 h of incubation to block intracellular protein transport processes. The NK cells were stained for 30 min with anti-CD56 antibody (ImmunoTools) and LIVE/DEAD dye (Invitrogen) according to the manufacturer's recommendations. NK cells incubated without target cells for 5 h were used as a negative control. Samples were analyzed using flow cytometry (FACSCanto).

Statistical Analysis. Statistical analyses were performed using a two-tailed Student *t* test. Differences were considered statistically significant when the *P* value was <0.05.

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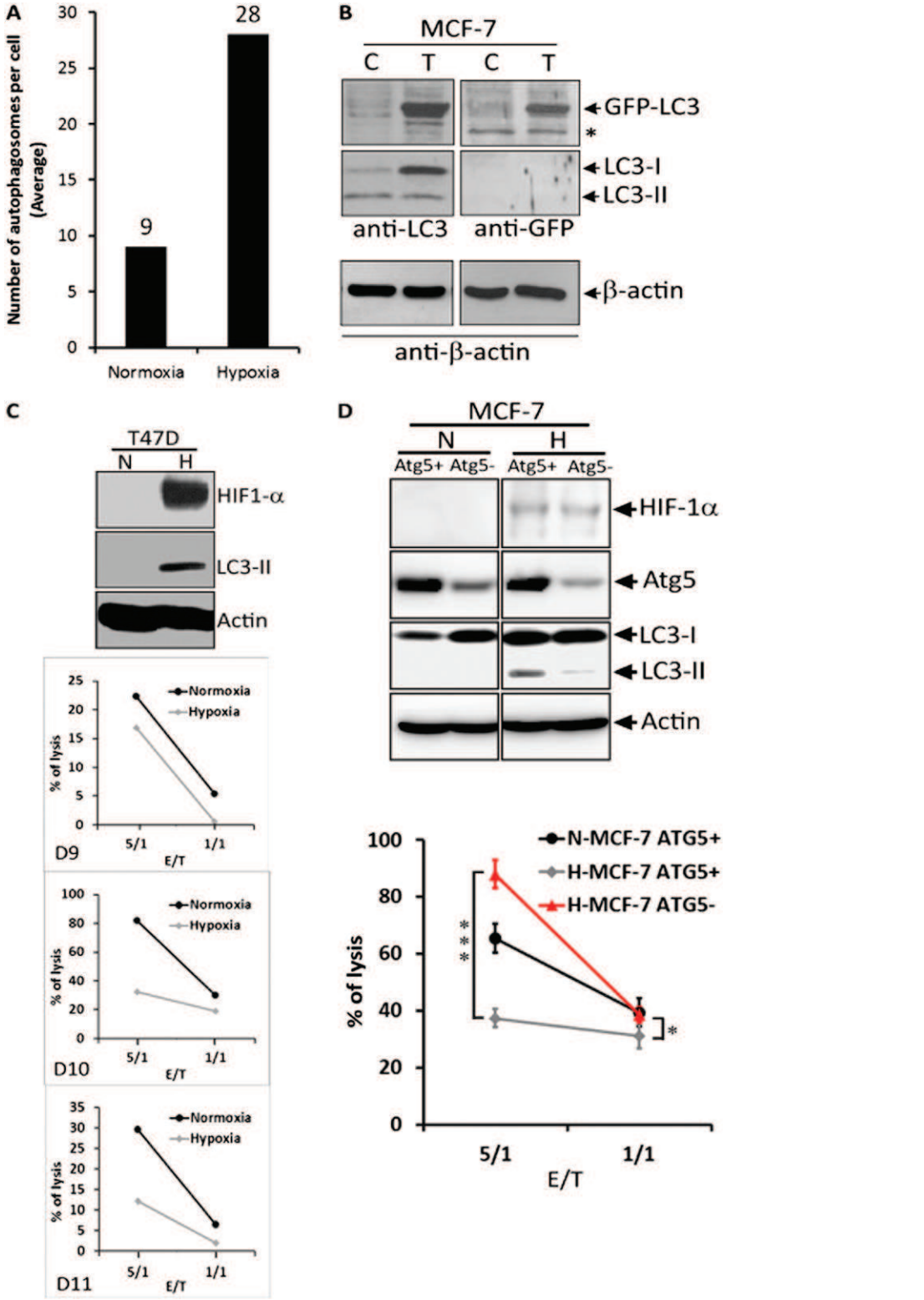


Fig. S1. (*A*) Quantification of the number of autophagosomes from representative images of normoxic and hypoxic MCF7 cells. The number of autophagosomes in 10 cells was counted and reported as an average number of autophagosomes per cell. (*B*) MCF-7 cells were stably transfected with GFP–LC3 cDNA and maintained in RPMI supplemented with 10% (vol/vol) FBS and geneticin (0.5 μg/mL). The expression of exogenous GFP–LC3 was assessed by immunoblot using anti-LC3 or anti-GFP antibodies, as indicated. An actin antibody was used for the loading control. C, control untransfected cells; T, cells transfected with GFP–LC3-encoding vector. The asterisk indicates an unspecific band. (*C*) Hypoxia-induced autophagy decreases the susceptibility of T47D breast cancer cells to NK-mediated lysis. (*C*, *Upper*) T47D cells were cultured under normoxia (N) or hypoxia (H) for 16 h. Expression of HIF-1α (hypoxia marker) and LC3 (autophagy marker) was assessed by immunoblot. (*Right*) NK cells were isolated from three different healthy donors (D9 to D11). Cytotoxicity assays were performed as described in Fig. 1*A*. (*C*, *Lower*) Cytotoxicity assays were performed as described in Fig. 1*A*. (*D*) Targeting ATG5 restores NK-mediated cytotoxicity against H

Legend continued on following page

MCF-7 cells. (*D*, *Upper*) Control (Atg5+) and ATG5-defective (Atg5–) MCF-7 cells were cultured under normoxic or hypoxic conditions. The expression of Atg5, HIF-1α, and LC3 was assessed by Western blot. (*D*, *Lower*) A cytotoxicity assay was performed at 5/1 and 1/1 E/T ratios using NK cells isolated from healthy donors as effector cells and control or ATG5–MCF-7 cells cultured under normoxic or hypoxic conditions as target cells. MCF-7 cell death was assessed using flow cytometry with TO-PRO-3. Statistically significant differences are indicated by asterisks (*$P < 0.05$; ***$P < 0.0005$).

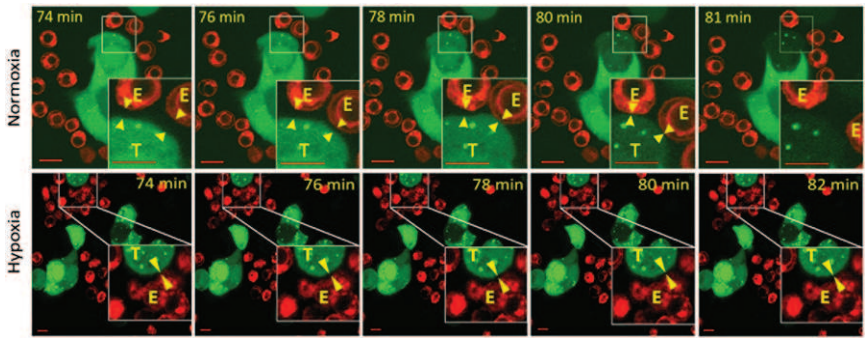


Fig. S2. GFP–LC3-expressing MCF-7 cells (green) were precultured onto glass coverslips under N or H conditions. The cells were incubated with PKH-26–stained NK cells (red) at a 5/1 E/T ratio. Interactions between NK and normoxic or hypoxic MCF-7 cells were recorded by time-lapse video microscopy (presented as Movies S1 and S2). At the indicated times, representative images were extracted from the movies. (Scale bar, 10 μm.) Images of enlarged regions (boxes) show the interaction between NK cells (E) with both N and H tumor cells (T). (Scale bar, 10 μm.)

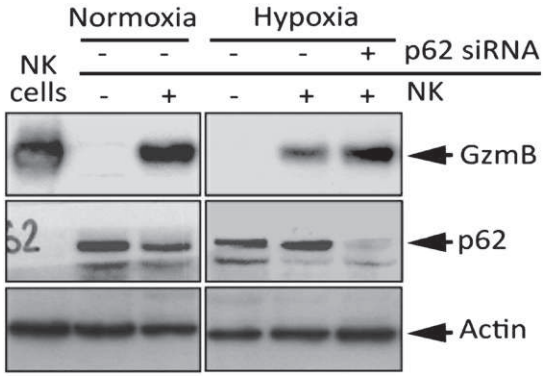


Fig. S3. Control (–) or p62/SQSTM1 siRNA transfected–MCF-7 (+) cells were cultured under normoxia or hypoxia for 16 h alone. Tumor cells were presented to NK cells (+) at 5/1 E/T ratio for 30 min. Following separation from NK cells, lysates of tumor cells were subjected to immunoblot analysis to evaluate the GzmB intracellular content. Untreated NK cells lysate (NK cells) was used as control for GzmB detection.

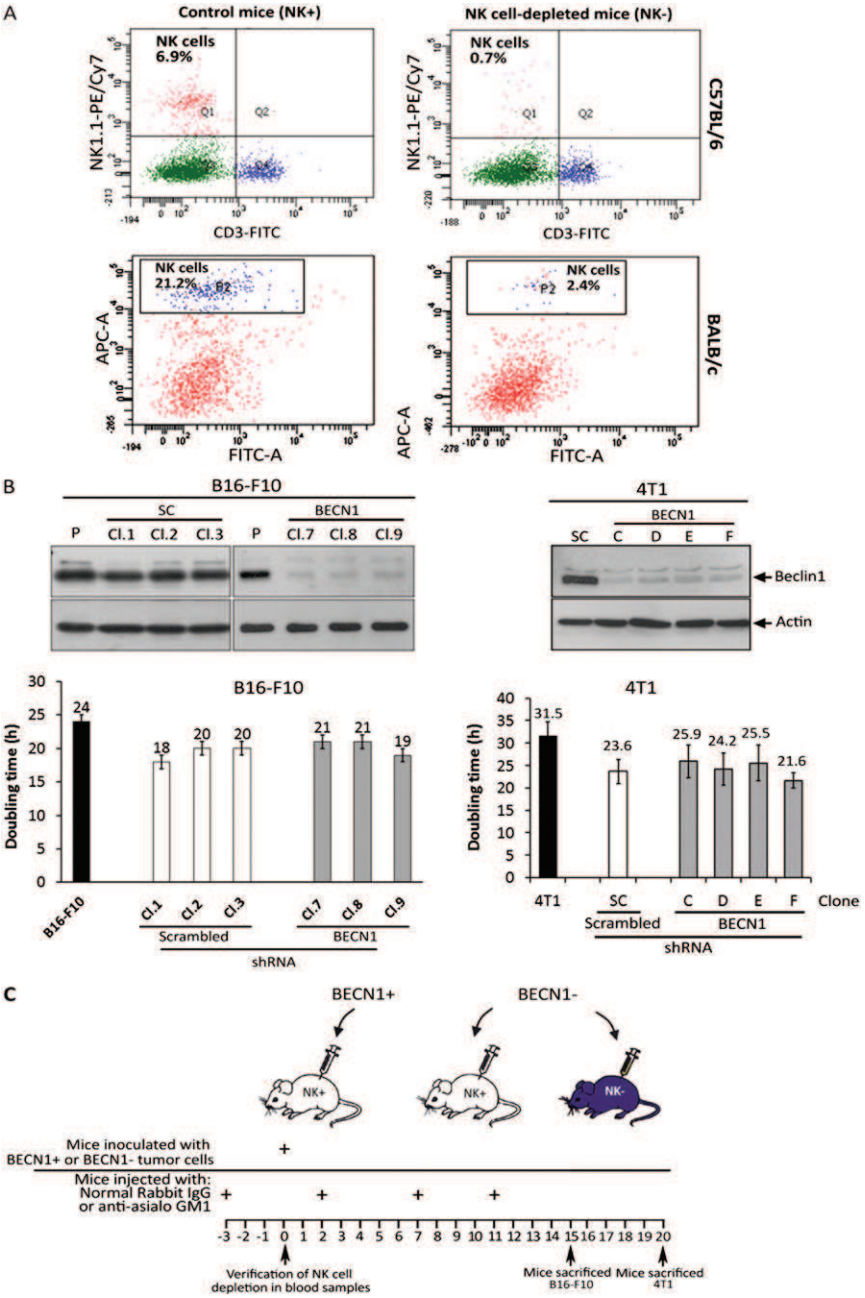


Fig. S4. (*A*) Validation of NK cell depletion in C57BL/6 and BALB/c mice. The effectiveness of NK cell depletion by antiasialo GM1 injection in C57BL/6 and BALB/c mice was determined using flow cytometric analysis. Representative dot-plots of NK1.1 and CD3 (C57BL/6) and CD49b (BALB/c) surface expression on PBMC from control (NK+) or NK-depleted (NK−) mice are shown. (*B*) Generation of autophagy-defective B16–F10 and 4T1 cells. Parental cells (P) were transduced with lentiviral particles containing scrambled (SC) or Beclin1 (BECN1) shRNA sequences. (*B*, *Upper*) SC and BECN1 B16–F10 and 4T1 clones were subjected to immunoblot analysis to evaluate the expression of Beclin1. (*B*, *Lower*) The doubling time of each clone was evaluated using the XTT assay ($n = 6$). (*C*) A schematic diagram of the experimental setup for in vivo experiments.

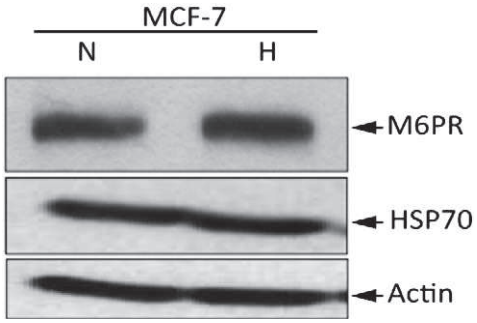


Fig. S5. Immunoblot analysis of the expression of M6PR and HSP70 in H and N MCF-7 cells. MCF-7 cells were cultured under normoxia (N) or hypoxia (H) for 16 h. The expression of M6PR and HSP70 was assessed by immunoblot.

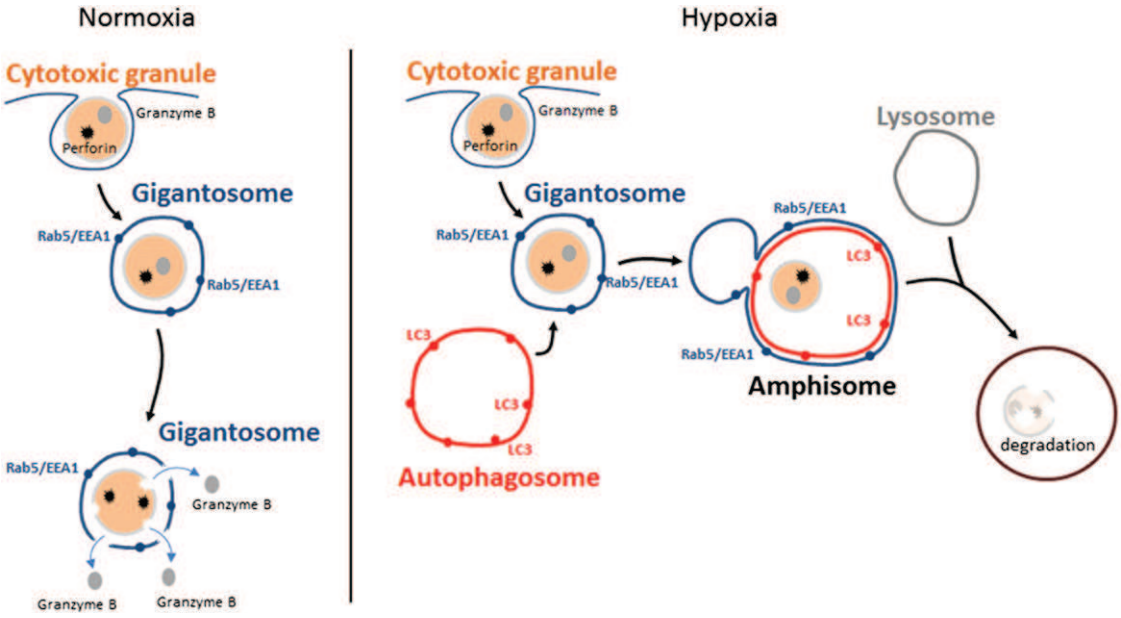
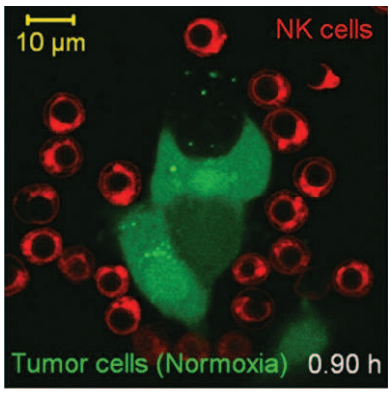
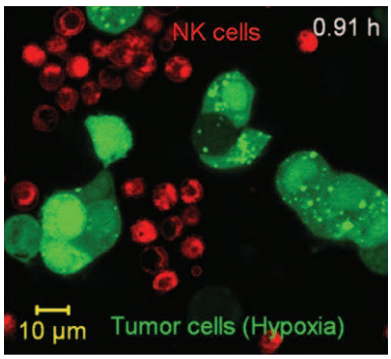


Fig. S6. Schematic representation of the mechanism underlying the degradation of GzmB by autophagy under hypoxia. Cytotoxic granules containing perforin and granzyme B are endocytosed into enlarged endosomes called "gigantosomes" in target cells. Under normoxia, perforin forms pores in the gigantosome membrane allowing granzyme B to be gradually released in the cytosol. In hypoxic cells the activation of autophagy leads to the formation of autophagosomes which will fuse with gigantosomes to form amphisomes. The fusion between amphisomes and lysosomes selectively degrades granzyme B in this compartment.



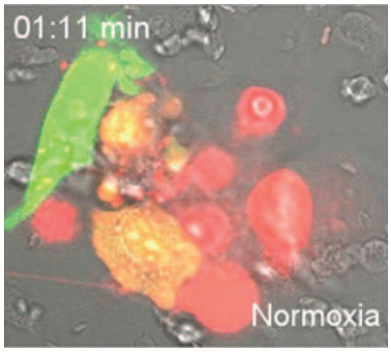
Movie S1. Time-lapse video microscopy of NK-mediated lysis of normoxic GFP–LC3–expressing MCF-7 cells. The movie was performed using N cells cultured on µ-Slide eight-well chambered coverslips (IBIDI) in the presence of PKH26-stained NK cells from a healthy donor (red) at a 5/1 E/T ratio. The movie was recorded for 1 h 22 min. Images were captured at intervals of one frame every 1–2 min with a Zeiss LSM-510 Meta laser-scanning confocal microscope (Carl Zeiss).

Movie S1



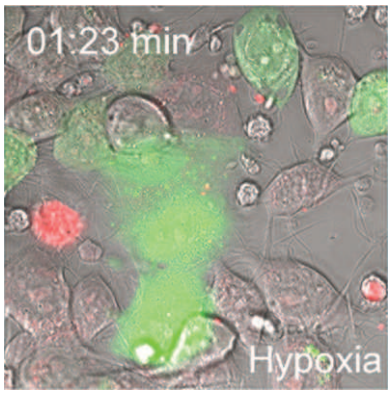
Movie S2. Time-lapse video microscopy of NK-mediated lysis of hypoxic GFP–LC3–expressing MCF-7 cells. The movie was performed using H cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of PKH26-stained NK cells from a healthy donor (red) at a 5/1 E/T ratio. The movie was recorded for 1 h 26 min. Images were captured at intervals of one frame every 1–2 min with a Zeiss LSM-510 Meta laser-scanning confocal microscope (Carl Zeiss).

Movie S2



Movie S3. Time-lapse video microscopy of NK-mediated lysis of normoxic GFP–LC3–expressing MCF-7 cells in the presence of cell death indicator. The movie was performed using normoxic cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of propidum iodide (PI) (1.5 μM) and NK cells from a healthy donor at a 5/1 E/T ratio. Tumor cell killing was recorded by time-lapse video microscopy using AxioVert 200M microscope (Carl Zeiss Micro-Imaging), and images were captured using ×40 oil objective lens at intervals of one frame per 20 s.

Movie S3



Movie S4. Time-lapse video microscopy of NK-mediated lysis of hypoxic GFP–LC3–expressing MCF-7 cells in the presence of cell death indicator. The movie was performed using hypoxic cells cultured on μ-Slide eight-well chambered coverslips (IBIDI) in the presence of propidum iodide (PI) (1.5 μM) and NK cells from a healthy donor at a 5/1 E/T ratio. Tumor cell killing was recorded by time-lapse video microscopy using AxioVert 200M microscope (Carl Zeiss Micro-Imaging), and images were captured using ×40 oil objective lens at intervals of one frame per 20 s.

Movie S4

5.1 Article 2

Autophagic degradation of GZMB/granzyme B: A new mechanism of hypoxic tumor cell escape from natural killer cell-mediated lysis.

Autophagic degradation of GZMB/granzyme B: A new mechanism of hypoxic tumor cell escape from natural killer cell-mediated lysis.

Elodie Viry,[1,a] Joanna Baginska,[1,a] Guy Berchem,[1] Muhammad Zaeem Noman,[2] Sandrine Medves,[1] Salem Chouaib,[2,b] and Bassam Janji,[1,b]

[1]Laboratory of Experimental Hemato-Oncology, Department of Oncology, Public Research Center for Health, 84, Val Fleuri, L-1526 Luxembourg City, Luxembourg

[2]INSERM U753, Institut de Cancérologie Gustave Roussy, 114, rue Edouard Vaillant, F-94805 Villejuif Cedex, France

[a] These authors contributed equally to this work.
[b] Co-senior authors

Corresponding author: Bassam Janji
Phone: +352 26 970 285
Fax: +352 26 970 290
Email: bassam.janji@crp-sante.lu

Keywords: Hypoxia, autophagy, Granzyme B, breast cancer, Natural Killer.

Punctum to:

Baginska J, Viry E, Berchem G, Poli A, Noman MZ, van Moer K, Medves S, Zimmer J, Oudin A, Niclou SP, Bleackley RC, Goping IS, Chouaib S and Janji B. Granzyme B degradation by autophagy decreases tumor cell susceptibility to Natural Killer-mediated lysis under hypoxia. **Proc Natl Acad Sci U S A. 2013 Oct 7. [Epub ahead of print]**

Abstract:

The crucial issue for defining successful natural killer (NK)-based anticancer therapy is the ability of tumor cells to activate resistance mechanisms leading to escape from NK-mediated killing. It is now well established that such mechanisms are likely evolved under hypoxia in the tumor microenvironment. Here, we show that hypoxia-induced autophagy impairs breast cancer cell susceptibility to NK-mediated lysis and that this impairment is reverted by targeting autophagy. We provide evidence that activation of autophagy in hypoxic cells is involved in selective degradation of the pro-apoptotic NK-derived serine protease GZMB/granzyme B, thereby blocking NK-mediated target cell apoptosis. Our *in vivo* data validate the concept that targeting autophagy in cancer cells promotes tumor regression by facilitating their elimination by NK cells. This study provides a cutting-edge advance in our understanding of how hypoxia-induced autophagy impairs NK-mediated lysis and might pave the way for formulating more effective NK-based antitumor therapy by combining autophagy inhibitors.

Tumor hypoxia is associated with tumor growth, malignant progression, and resistance to therapy, and becomes a central issue in cancer treatment. Hypoxic cells activate signaling pathways that regulate proliferation, angiogenesis and death. Cancer cells have adapted these pathways, allowing tumors to survive and grow under hypoxia. Recently, hypoxia in the tumor microenvironment has been reported to suppress the antitumor immune response and to enhance tumor escape from immune surveillance. In line with this concept, we showed that hypoxic breast cancer cells are less susceptible to NK-mediated lysis than normoxic cells. More interestingly, we demonstrated that the resistance of hypoxic cancer cells to NK-mediated killing is strikingly dependent on autophagy activation, as genetic inhibition of autophagy is sufficient to suppress this resistance and restore NK-mediated killing of hypoxic cells. Furthermore, we showed that hypoxia is not a prerequisite event for autophagy-dependent induction of tumor escape from NK. Indeed, we observed that, similar to hypoxia-induced autophagy, starvation-induced autophagy is also able to impair tumor susceptibility to NK-mediated killing. Our results highlight autophagy as a key determinant in tumor cell evasion from NK-mediated killing.

It is well established that a dynamic and precisely coordinated balance between activating and inhibitory receptors governs NK cell activation programs. In our model, no significant differences are observed in the expression of activating and inhibitory receptors on the surface of NK cells, and in the expression of their ligands (except HLA class I molecules) at the surface of normoxic and hypoxic target cells. While the causal mechanism underlying the increase in HLA class I in hypoxic cells remains elusive, we demonstrated, using an HLA class I blocking antibody, that the resistance of hypoxic tumor cells occurs independently of

upregulated-HLA class I molecules. Furthermore, we could not observe any defect in the ability of NK cells to secrete cytotoxic granules toward hypoxic or normoxic cells. Together, our results provide additional clues regarding the critical role of autophagy as an intrinsic mechanism that makes hypoxic tumor cells less sensitive to NK cell attack.

As cancer cells have evolved multiple mechanisms of resistance in order to outmaneuver an effective immune response and escape from immune cell killing, we next focused on autophagy as an intrinsic resistance mechanism operating in hypoxic cells. NK cells recognize and kill their targets by several mechanisms including the release of cytotoxic granules containing PRF1/perforin and GZMB. It has been recently proposed that PRF1 and GZMB enter target cells by endocytosis and traffic to enlarged endosomes called "gigantosomes". Subsequently, PRF1 forms pores in the gigantosome membrane, allowing for the gradual release of GZMB and the initiation of apoptotic cell death. The fusion between early endosomes and autophagic vacuoles to form amphisomes seems to be a prerequisite in some cases for the formation of autolysosomes. In keeping with this, we attempted to analyze GZMB content in hypoxic tumor cells. We hypothesized that during intracellular trafficking, GZMB could be exposed to a high risk of being targeted to amphisomes and thereby degraded by autophagy in the lysosomal compartment. Several lines of data reported in this study support such a mechanism: i) the level of NK-derived GZMB detected in hypoxic cells is significantly lower than that in normoxic cells; ii) inhibition of autophagy or lysosomal hydrolases restores the level of GZMB and subsequently restores NK-mediated lysis of hypoxic cells; and iii) NK-derived GZMB is detected in LC3- and RAB5-positive cellular compartments, suggesting its presence within amphisomes in hypoxic cells. Based on these findings, we proposed a

mechanism by which GZMB may be degraded by autophagy during its intracellular trafficking leading to cancer cell escape form NK cell attack (**Fig. 1**).

Autophagy has long been considered as a bulk degradation cell process. However, several studies have reported that autophagy can be a selective degradation process under stress conditions. The molecular basis of selective autophagy involves several cargo protein receptors such as SQSTM1/p62, NBR1, OPTN and CALCOCO2/NDP52, which are able to interact with ubiquitinated proteins and target them into phagophores in order to be degraded in lysosomes. Based on this, an important issue arises from our results: Is GZMB selectively degraded by autophagy or it is just an "innocent victim" which is subject to a bulk nonspecific degradation in hypoxic tumor cells under excessive autophagy? While much remains to be learned mechanistically, several lines of data reported in this study support the selective degradation of GZMB by autophagy: i) The level of GZMB in hypoxic cells is restored by targeting SQSTM1; and ii) even if PRF1 is detected in the same subcellular compartment as GZMB in hypoxic cells, we do not observe any difference in its expression level compared to normoxic cells.

In light of our *in vitro* observations, we investigated whether targeting autophagy enhances in vivo an NK-mediated antitumor immune response. We used BALB/c and C57BL/6 mice transplanted with syngeneic murine 4T1 breast adenocarcinoma and B16-F10 melanoma tumor cells, respectively. We first demonstrated that NK cells control in vivo B16-F10 and 4T1 tumor development, as the depletion of host NK cells significantly increases tumor growth. There is a significant reduction of tumor volume in autophagy-defective B16-F10 and 4T1 cells presumably as a consequence of potentiation of tumor cell killing by NK cells. Therefore, it

would be interesting to investigate whether this improvement is related to the increase in the infiltration/recruitment of different immune subsets in the tumor bed or to the decrease of immune suppressive cells such as T regulatory cells, myeloid-derived suppressor cells or tumor-associated macrophages. Whether targeting autophagy affects the ability of cancer cells to induce an immunosuppressive microenvironment, including the secretion of various immunosuppressive cytokines remains also an issue of great interest.

Overall, the present study underlines the inhibition of autophagy as a cutting-edge approach to overcome the suppressive effect of the hypoxic tumor microenvironment on the antitumor immune response. This study highlights the importance of integrating autophagy inhibitors as a potential therapeutic approach to improve NK-based cancer immunotherapy.

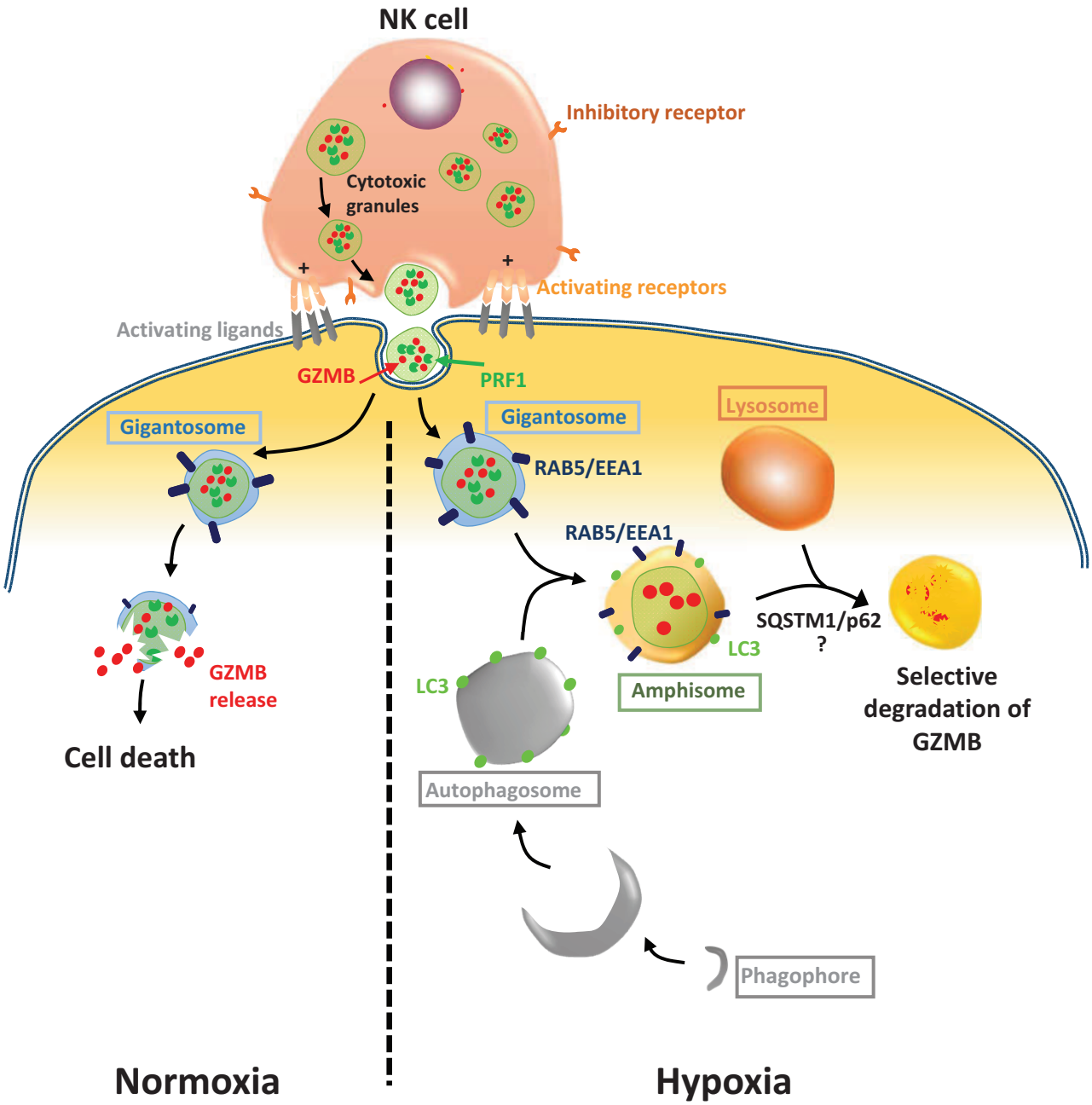
Acknowledgments

This work was supported by grants from Public Research Center for Health (REC-LHCE-2009-0201) and "Foundation Cancer" Luxembourg (FC/2012/02) to B.J. and "Association de Recherche sur le Cancer" to S.C. J.B is a recipient of an AFR grant (2009-1201) from the "Fonds National de la Recherche", Luxembourg.

Figure legend:

Figure 1. Selective degradation of NK-derived GZMB by autophagy in hypoxic tumor cells. Following the recognition of their targets NK cells secrete cytotoxic granules containing PRF1, GZMB, and other hydrolytic enzymes to the target cells. These granules enter target cells and traffic to enlarged endosomes marked by RAB5 or EEA1, which are called 'gigantosomes'. In normoxic cells, PRF1 forms pores in the gigantosome membrane, allowing the release of GZMB and the initiation of cell death. Under hypoxia, excessive autophagy in target cells leads to the fusion of autophagosomes with gigantosomes containing PRF1 and GZMB and the formation of structures marked with LC3 and RAB5-EEA1 called amphisomes. Although PRF1 is detected in the same subcellular compartment as GZMB, the fusion of amphisomes with lysosomes leads to specific degradation of GZMB in a SQSTM1/p62-dependent manner. The degradation of GZMB by autophagy leads to hypoxic tumor cell escape from NK-mediated killing.

Viry et al., Figure 1



5.1 Article 3

Critical role of tumor microenvironment in shaping the natural killer cell-mediated anti-tumor immunity

Critical role of tumor microenvironment in shaping the natural killer cell-mediated anti-tumor immunity

Joanna Baginska[1&], Elodie Viry[1&], Jérôme Paggetti[1], Sandrine Medves[1], Guy Berchem[1], Etienne Moussay[1,] and Bassam Janji[1,*]

[1] Laboratory of Experimental Hemato-Oncology, Department of Oncology, Public Research Center for Health. Luxembourg City, Luxembourg.

[*] Correspondence: Dr. Bassam Janji
Public Research Center for health (CRP-Santé)
Department of Oncology
84, Val Fleuri
L-1526 Luxembourg City, Luxembourg
Phone: +352 26 970 285
E. mail: bassam.janji@crp-sante.lu

[&] Equally contributed to this work

Running title: Tumor microenvironment shapes the anti-tumor response of NK cells

Keywords: Hypoxia, natural killer cells, autophagy, tumor-derived exosomes, tumor microenvironment

Abstract

Considerable evidence has been accumulated over the last ten years showing that the tumor microenvironment is not simply a passive recipient of immune cells, but an active participant in the establishment of immunosuppressive conditions. It is now well documented that hypoxia, within the tumor microenvironment, affects the functions of immune effectors including natural killer (NK) cells by multiple overlapping mechanisms. Indeed, each cell in the tumor microenvironment, irrespective of its transformation status, holds the capacity to adapt hostile tumor microenvironment and produce immune modulatory signals or mediators affecting the function of immune cells either directly or through the stimulation of other cells present in the tumor site. This observation has led to intense research efforts focused mainly on tumor-derived factors. Notably, it has become increasingly clear that tumor cells secrete a myriad of environmental factors such as cytokines, growth factors, exosomes, microRNAs impacting the immune cell response. Moreover, tumor cells in hostile microenvironment may activate their own intrinsic resistance mechanisms, such as autophagy to escape effective immune response. Such adaptive mechanisms may also include the ability of tumor cells to modify their metabolism and release several metabolites to impair the function of immune cells. In this review, we will summarize the different mechanisms evolved in the tumor microenvironment that affect the anti-tumor immune function of NK cells and discuss the importance of the tumor microenvironment and its targeting to awake or reawake an effective tumor immunity.

1 Introduction

Natural killer (NK) cells are potent cytolytic lymphocytes belonging to the innate immune system. NK cells comprise up to 15% of all circulating lymphocytes and are also found in peripheral tissues including liver, peritoneal cavity and placenta. Although resting NK cells circulate in the blood, they are capable to infiltrate most cancer tissues following activation by cytokines. NK cells can be rapidly activated in the periphery by NK cell stimulatory factors, such as interleukin (IL)-12, interferon (IFN)-α and -β, IL-15 or IL-2 (Smyth *et al.* 2002). Regulation of NK cells activity depends on repertoire of germline encoded activating and inhibitory receptors. The activating receptors recognize stress-induced, pathogen-derived or tumor specific ligands, whereas the inhibitory receptors bind self-molecules presented on normal cells. Owing to a diversified set of inhibitory and activating receptors, NK cells are capable to recognize and kill an array of tumor cells (Moretta *et al.* 2008). Beyond innate activity, NK cells are important for regulation of anti-tumor adaptive immunity (Zingoni *et al.* 2004, Paust *et al.* 2010).

Based on the fact that NK cells can eliminate cancer cells in experimental conditions, it has been proposed that NK cells can be used clinically in therapeutic settings against cancer. Importantly, data from haploidentical hematopoietic stem cell transplantation and NK cell-based adoptive immunotherapy support the clinical effects of NK cells (Passweg *et al.* 2006). Based on our current knowledge about the molecular specificities that regulate NK cell functions, it is tempting to speculate that a design of tailored NK cell-based immunotherapeutic strategies against cancer might be possible.

Recent data confirm that NK cells are required for the induction of potent anti-tumor specific cytotoxic T lymphocytes (T cells) responses, by a mechanism involving dendritic cell (DCs)-editing (Amakata *et al.* 2001, Walzer *et al.* 2005). Furthermore, NK cells can recognize tumors that might evade T cells mediated-killing by aberrant human leucocyte antigen (HLA) expression (Nielsen *et al.* 2012), indicating that tumor development is dependent on NK cell immune surveillance as well as other immune cells.

Significant correlation between high intratumoral levels of NK cells and increased survival was shown in several types of cancer (Senovilla *et al.* 2012). Indeed, high level of NK infiltrating tumors has been associated with a significant improvement of clinical outcome in patients with head and neck squamous carcinoma (HNSCC). It has been reported by Van Herpen *et al.* that CD56+ NK cells in IL-12-treated lymph nodes produced considerable amounts of IFN-γ which subsequently lead to tumor regression in HNSCC patients (van Herpen *et al.* 2005). A direct positive correlation between density of CD57+ NK cells and good prognosis has been reported for oral squamous carcinoma (Turkseven and Oygur 2010) and gastric carcinoma (GC) tumor (Ishigami *et al.* 2000). In addition, NK cell infiltration was found to correlate also with the depth of invasion, the clinical stage, and the venous invasion. Therefore, the 5-year survival rate of GC patients with a high rate of NK infiltration was significantly better than that of patients with a low level of NK infiltration (Ishigami *et al.* 2000).

NK-based immunotherapy is a promising strategy for solid and hematologic cancers, and it can potentially be combined with chemotherapy, radiation, or monoclonal antibody therapy. For example, the proteasome inhibitor bortezomib (Velcade®), which is clinically approved for the treatment of refractory/relapsed myeloma, down-regulates cell surface expression of HLA class I on target cells and enhances NK-mediated lysis of tumor cells without affecting NK cell function (Godfrey and Benson 2012). Therefore, such combination therapy has important therapeutic implications for multiple myeloma and other NK cell-related malignancies in the context of adoptively transferred allogeneic and autologous NK cells (Miller *et al.* 2005). NK cell-based

therapy can be combined with radiation therapy because irradiation-induced tissue injury has been found to increase the expression of NK activating ligands (e.g. NKG2D ligands) on malignant cells, thereby rendering tumors more susceptible to NK cell cytotoxic activity (Cho *et al.* 2011). Another NK cell-based approach used in therapy is the antibody-dependent cellular cytotoxicity (ADCC). This approach is based on the ability of NK cells, expressing an activating Fc receptor, to kill tumor cells by recognizing the constant region of tumor-bound monoclonal antibodies (mAbs). Clinically, ADCC strategy has been used in CD20+ lymphoma patients treated with rituximab (Rituxan™) (Clynes *et al.* 2000) or HER2/neu expressing breast cancers patients treated with trastuzumab (Herceptin™) (Bekaii-Saab *et al.* 2009). It is worthy to note that the co-administration of immunomodulatory cytokines (e.g. IL-12) can enhance the effects of anti-tumor mAbs *via* the activation of NK cells *in vitro*. This effect has been observed in breast cancer patients overexpressing HER2 and treated with IL-12 and trastuzumab in a phase I trial (Jaime-Ramirez *et al.* 2011).

Despite the progress made in the field of NK-based immunotherapy, there are still many obstacles in eliciting an effective immune response. One major impediment is the ability of tumor cells to activate several mechanisms that lead to tumor escape from NK-mediated killing. It has become increasingly clear that the tumor microenvironment (TME) plays a crucial role in the impairment of the immune response and in the development of many overlapping mechanisms that create an immunosuppressive microenvironment. It has been reported that tumor-associated NK cells displayed a modified phenotype, thereby supporting the notion that tumor-induced alterations of activating NK cell receptor expression may hamper immune surveillance and promote tumor progression (Stagg and Allard 2013). Decreased cytotoxic activity of NK cells infiltrating tumors was also observed in different types of human cancer such as lung carcinoma (Platonova *et al.* 2011), indicating that TME is a critical factor influencing NK-mediated killing of tumor cells. Hypoxia is a characteristic feature of locally advanced solid tumors resulting from an imbalance between oxygen supply and consumption and considered as one of the hallmarks of TME (Harris 2002). It is now well established that hypoxia contributes to malignant progression in cancer by inducing an invasive and metastatic phenotype of tumor cells, and by activating resistance mechanisms to different anti-cancer therapies. Extensive efforts have been made in recent years to identify these mechanisms. We review here how the local microenvironment, in the particular context of hypoxia, impacts NK cell responsiveness and shapes anti-tumor response (Figure 1).

2 Tumor-derived factors create immunosuppressive microenvironment for NK cell functions

2.1 Impairment of NK cell function by cells from the tumor microenvironment

Hypoxic tumor cells hold the ability to activate resistance mechanisms to create an immunosuppressive microenvironment. Indeed, through their ability to produce cytokines such as tumor necrosis factor (TNF)-α and stromal cell–derived factor 1 (SDF-1), hypoxic tumor cells induce homing of bone marrow–derived CD45+ myeloid cells to tumor areas (Mantovani *et al.* 2008). The invasion of myeloid cells in TME is reported to be a highly immunosuppressive factor for NK cells (Murdoch *et al.* 2004). Myeloid-derived suppressor cells ($MDSC_S$) are one of the major components of the immune-suppressive network responsible for the impairment of NK cell- and T cell-dependent anti-cancer immunity (Monu and Frey 2012). The immunosupressive function of MDSCs is related to their production of IL-10 that decreases the production by macrophages of IL-12, a pro-inflammatory cytokine involved in the activation of NK cells (Sinha *et al.* 2007). Furthermore, it has been demonstrated that hypoxia, *via* the induction of hypoxia-inducible factor (HIF)1-α in MDSCs is responsible for their differentiation to macrophages (Park *et al.* 2013). Although macrophages contribute to tumor cell death in the early immune response to neoplasia, their presence in TME correlates with a poor prognosis for patients in advanced stages.

Macrophages constitute a major component of the myeloid infiltrating tumors and can comprise up to 80% of the cell mass in breast carcinoma (Lewis and Murdoch 2005). In the TME, macrophages differentiate into CD206+ tumor associated macrophages (TAM) and accumulate in hypoxic areas of endometrial, breast, prostate and ovarian cancers (Wagner and Dudley 2013). This accumulation is related to the release of macrophage chemoattractants, such as endothelial monocyte-activating polypeptide (EMAP) II, endothelin 2 and vascular endothelial growth factor (VEGF) in the hypoxic TME, or to the inhibition of the C-C chemokine receptors (CCR) 5 and 2 expression (Owen and Mohamadzadeh 2013). Exposure of TAM to tumor-derived cytokines such as IL-4 and IL-10 is able to convert them into polarized type II or M2 macrophages owing immunosuppressive and pro-angiogenic activities. Subsequently, M2 macrophages establish an environment that skew CD4+ and CD8+ T cell immunity toward a tumor-promoting type 2 response (Webb *et al.* 2007). It has been also demonstrated that hypoxia upregulates the expression of the matrix metalloprotease (MMP)-7 protein on the TAM surface leading to cleavage of Fas ligand from neighbouring cancer cells, making them less responsive to NK cells and T cell-mediated lysis (Burke *et al.* 2003) (Figure 2A).

The impairment of NK cell function under hypoxic stress could also be related to the ability of cancer cells to secrete tumor-derived secreted factors (TDSFs) involved in the recruitment of a newly discovered type of myeloid cells CD11b(+)/Ly6C (med)/Ly6G(+) and the suppression of NK cell function in pre-metastatic niche in mice model. Interestingly, although NK cell abundance was increased in such condition, these NK cells were not fully mature and exerted limited cytotoxicity. One of the most consistent factor involved in such event is the monocyte chemotactic protein-1 (MCP-1/CCL2) (Sceneay *et al.* 2012, Sceneay *et al.* 2013).

Recently, a link between tumor hypoxia and immune tolerance to NK cells through the recruitment of regulatory T cells (Treg) has been established. Hypoxia induces secretion of the immunosuppressive cytokine transforming growth factor (TGF)-β1 which subsequently induces the proliferation and the accumulation of Treg in TME. Moreover, cancer-expanded MDSCs

induce anergy of NK cells through membrane-bound TGF-β and subsequently down-regulate the activating receptor NKG2D on the surface of NK cells (Deng *et al.* 2013).

The immunosuppressive microenvironment can also be created through the ability of cancer cells to exploit cancer-associated fibroblasts (CAFs), which sharply interfere with the NK cells cytotoxicity and its cytokine production. Notably, it has been reported that CAFs were able to inhibit the IL-2-dependent surface expression of NKp44, NKp30, and DNAX accessory molecule-1 (DNAM-1) receptors. Such inhibition results in impaired NK cell-mediated killing of melanoma target cells (Balsamo *et al.* 2009). Likewise, CAFs directly impact cells of the TME and/or attract additional cells to the tumor site by secreting numerous factors including IL-6, TGF-β, VEGF, SDF-1, CXCL1/2 and IL-1b (Pietras and Ostman 2010)(Figure 2A).

2.2 Inhibition of NK cells by tumor cell-derived factors

The MHC class I chain-related (MIC) molecules, MICA and MICB, as well as the UL16-binding proteins (ULBP), expressed on the surface of broad range of carcinomas and some hematopoietic malignancies, play important role in tumor surveillance by NK cells. The interaction of cell surface MIC molecules with NKG2D receptor on NK cells is critical to activate target cell killing. In this context, hypoxia has been reported to increase MICA shedding from the surface of cancer cells through the impairment of nitric oxide (NO) signaling and therefore to affect the NK-mediated killing of target cells (Figure 2B). Soluble MIC lead to a down-regulated expression of NKG2D and chemokine receptor (CXCR) 1 on NK cell surface (Barsoum *et al.* 2011). Another mechanism has been proposed to down-regulate the expression of MICA on the surface of tumor cells under hypoxia. This mechanism involved the HIF-1α-dependent upregulation of A Disintegrin and metalloproteinase domain-containing protein (ADAM) 10, which subsequently decreases the level of MICA on the tumor cell membrane (Siemens *et al.* 2008). In melanoma cells, hypoxic stress can induce the formation of non-classical HLA-G dimers at the surface of target cells, thereby protecting melanoma cells from NK-mediated killing. It appears that such induction is mediated by IFN-β and –γ secretion and by direct interaction of HLA-G with NK cells (Zilberman *et al.* 2012).

The inhibition of the expression of NKp30, NKp44, and NKG2D receptors on the surface of NK cells in melanoma model impairs NK cell-mediated cytolytic activity (Pietra *et al.* 2012). Although NK cells in the TME adapt and survive hypoxic stress by upregulating HIF-1α, they lose the ability to upregulate the surface expression of the major activating NK cell receptors (NKp46, NKp30, NKp44, and NKG2D) in response to IL-2 or other activating cytokines, including IL-15, IL-12, and IL-21. However, it is worthy to note that hypoxia does not significantly alter the surface density and the function of the Fc-γ receptor CD16, thus allowing NK cells to maintain their capability of killing target cells *via* antibody-dependent cellular cytotoxicity (Balsamo *et al.* 2013).

In addition to solid tumor, the immunosuppressive effect of hypoxic TME has been also described for multiple myeloma (MM) cells by showing that hypoxia reduced NK cell killing of MM cell lines despite an unchanged NK cell degranulation level. In addition, hypoxia did not alter surface expression of NK cell ligands (HLA-ABC and -E, MICA/B and ULBP1-2) and receptors (killer cell Ig-like receptors (KIR), NKG2A/C, DNAM-1, natural cytotoxicity receptors (NCR) and 2B4), but decreased the expression of the activating NKG2D receptor and intracellular level of perforin and granzyme B. Pre-activation of NK cells by IL-2 abrogated the detrimental effects of hypoxia and increased NKG2D expression (Sarkar *et al.* 2013).

It is now well documented that the killing capacity of NK cells can be potentiated by cytokines such as IFN-γ and IL-2 (Reiter 1993). Besides its effect on damping of the cytotoxic activity of

NK cells, hypoxia has been described to substantially decrease their ability to be activated by IFN-γ through a mechanism that is not fully understood (Fink *et al.* 2003). Overall, it appears that manipulation of the TME will be an important consideration for achieving optimal NK-mediated anti-tumor responses.

As NKG2D ligands mRNAs are expressed in normal tissues, it has been proposed that their expression might be regulated at post-transcriptional level by microRNAs (miRNAs) (Champsaur and Lanier 2010). Indeed, a subset of endogenous cellular miRNAs is proposed to repress MICA and MICB by targeting their 3' UTR regions (Stern-Ginossar *et al.* 2008). Upon stress induction, increase in MICA and MICB transcription might exceed the inhibitory function of miRNAs, whose expression remains constant, and results in overexpression of MICA and MICB. Interestingly, among this subset of miRNAs, miR-17-5p, miR-20a, miR-93, miR-106b, miR-372, miR-373, and miR-520 have been shown to be overexpressed in various tumors and to be involved in tumor progression and invasion. Therefore, a new function of these miRNAs has been proposed in the impairment of immune response through the regulation of MICA and MICB expression (Figure 1). Based on these observations, a "miRNAs-based immunoevasion" model has been described implicating intracellular cancer-associated miRNAs as important factors able to impair immune recognition through the targeting of NK ligands (Stern-Ginossar *et al.* 2008). Furthermore, miR-10b, an important "metastamir", has been described to down-regulate MICB and to decrease NKG2D-dependent cytotoxicity of NK cells (Tsukerman *et al.* 2012). MiR-520b, an IFN-γ induced miRNA, has been also described to regulate MICA expression at both transcriptional and post-transciptional levels (Yadav *et al.* 2009). It has been proposed that viruses take advantage of "microRNA-based immunoevasion". Indeed the hcmv-miR-UL112 encoded by the human cytomegalovirus impairs NK cell function during viral infection through the modulation of MICB expression (Stern-Ginossar *et al.* 2007). In addition, hcmv-miR-UL112 acts synergistically with the cellular miR-376a to induce escape from NK-mediated immune elimination (Nachmani *et al.* 2010). Together these studies highlight the importance of miRNAs in the regulation of NKG2D ligands expression and tumor immune surveillance. Whether the expression of such miRNAs is regulated by hypoxia in the TME remains to be investigated.

3 Tumor microenvironment-dependent modulation of cancer cell metabolism affects NK cell functions

Through the sensing of oxygen level and/or the transcriptional activity of HIF-1α, hypoxia plays a key role in the reprogramming of cancer cell metabolism. Indeed, reduced O_2 availability induces HIF-1α, which regulates the transcription of set of genes that encode proteins involved in various aspect of cancer biology (Mole *et al.* 2009). A well-known example is the shift of glucose and energy metabolism from oxidative to glycolytic metabolism that allows the maintenance of redox homeostasis under conditions of prolonged hypoxia (Semenza 2010). Effects of such metabolic adaptations evolved by hypoxic cancer cells have received a particular attention in the establishment of immune tolerance. In this section, we will focus on the mechanisms involved in tumor metabolism adaptation that participate in shaping NK cell anti-tumor response within hypoxic microenvironment (Figure 1).

3.1 Lactate

To adapt oxygen deprivation, hypoxic cancer cells undergo a dramatic alteration of cellular glucose metabolism characterized by a high glycolytic activity. HIF-1α plays a central role in this

metabolic switch by inducing the expression of multiple genes involved in glucose uptake (glucose transporters-1 and -3) and metabolism (i.e. hexokinases-1 and -2, lactate dehydrogenase A) (Brahimi-Horn *et al.* 2011). In addition, HIF-1α regulates the expression of monocarboxylate transporter 4 and pyruvate dehydrogenase kinase 1, thereby inhibiting the conversion of pyruvate to acetyl CoA (Kim *et al.* 2006). The accumulation of pyruvate in cell prevents its metabolism through the tricarboxylic acid cycle in mitochondria. Pyruvate is subsequently reduced to lactate and finally released from the tumor cells. It has been recently reported that cancer cells escape innate immune response through the release of lactate in the microenvironment and low extracellular pH, as a consequence of 'Warburg effect' induced under hypoxia. *In vivo* and *in vitro* evidence have been provided indicating that tumor-derived lactate directly and indirectly alters NK cell functions. The direct effect involves the impairment of the cytolytic activity of NK cells by down-regulating NKp46 expression and by reducing perforin/granzyme B production. Moreover, lactate affects the NK-mediated killing indirectly through the increased MDSCs generation from mouse bone marrow, thus creating an immunosuppressive microenvironment. Interestingly, these immunosuppressive effects were efficiently reverted in lactate dehydrogenase A-depleted cancer model (Husain *et al.* 2013).

3.2 Adenosine

Hypoxia-driven accumulation of adenosine in the TME has been identified as another mechanism for immune modulation (Sitkovsky and Ohta 2013). It has been reported that the concentration of adenosine in the extracellular fluid of solid carcinomas may be increased by 10 to 20-fold compared to normal tissues (Blay *et al.* 1997). The accumulation of adenosine is sustained, at least in part, by the hypoxia-mediated modulation of enzymes implicated in adenosine metabolism (i.e. adenosine kinase, endo-5'-nucleotidase). Moreover, additional generation of extracellular adenosine from extracellular ATP occurs through the sequential enzymatic activity of the membrane-bound nucleotidases CD39 and CD73. It has been shown that CD73, involved in the dephosphorylation of AMP to adenosine, is upregulated by HIF-1α (Stagg *et al.* 2010) (Synnestvedt *et al.* 2002). Once released in the extracellular environment, adenosine exerts various immunomodulatory effects *via* binding on adenosine receptors (i.e. A1, A2A, A2B and A3) expressed on multiple immune subsets including NK cells.

In contrast to other immune cells such as macrophages and neutrophils, the effect of extracellular adenosine on NK cells is not fully elucidated, but seem to occur through various mechanisms. Briefly, adenosine has been shown to inhibit tumor necrosis factor (TNF)-α release from IL-2-stimulated NK cells and suppress their proliferation (Miller *et al.* 1999). Another study reported that adenosine inhibits cytotoxic granules exocytosis from murine NK cells *via* binding to an unidentified adenosine receptor (Williams *et al.* 1997). More recently, data support the fact that adenosine and its stable analogue 2-chloroadenosine inhibit perforin- and Fas ligand-mediated cytotoxic activity as well as cytokines production (i.e. IFN-γ, macrophage inflammatory protein 1-α, TNF-α and granulocyte-macrophage colony-stimulating factor) from activated NK cells. These inhibitory effects occur through the stimulation of the cyclic AMP/Protein kinase A pathway following the binding of adenosine to A2A receptors on NK cells (Raskovalova *et al.* 2005) (Lokshin *et al.* 2006).In this context, targeting CD73-adenosine pathway has recently emerged as a potential clinical strategy for immunotherapy (Stagg *et al.* 2010). *In vitro* data revealed that the inhibition of CD39, CD73 or A2A adenosine receptor by siRNA, shRNA or specific inhibitors resulted in significant improvement of NK cell lytic activity against ovarian cancer cells (Hausler *et al.* 2011). Furthermore, *in vivo* blocking of A2A adenosine receptor enhanced NK cells activity

in a perforin-dependent manner and reduced metastasis of CD73-overexpressing breast cancer cells (Beavis *et al.* 2013).

As multiple immune competent cells express adenosine receptors, an additional level of immunomodulatory activity, *via* adenosine, needs to be considered. For example, it has been reported in several studies that adenosine interaction with other immune subsets impairs the cytotoxic activity, the pro-inflammatory cytokines production and the proliferation of T cells. In addition, adenosine impairs the recruitment and the immunosuppressive activity of MDSCs in tumor as well as the migration and the immunosuppressive function of Treg into the TME (Kumar 2013). Taken together, by sustaining the immunoregulatory activity of extracellular adenosine, all the mechanisms described above collaborate to impair the anti-tumor NK-mediated immunity.

3.3 Nitric oxide

Accumulating evidence suggests that the exposure of cells to low oxygen levels results in marked inhibition of nitric oxide (NO) production (McCormick *et al.* 2000). NO is produced from L-arginine in a reaction catalyzed by the nitric oxide synthase (NOS) enzymes, with oxygen as a required cofactor. Hypoxia has also been shown to increase arginase activity, thereby redirecting L-arginine into the urea cycle, away from the NO generation pathway (Louis *et al.* 1998). Siemens *et al.* provided evidence that hypoxia-mediated impairment of NO signaling in tumor cells contributes to tumor escape from NK immunosurveillance. They demonstrated that hypoxia-mediated shedding of MIC occurs through a mechanism involving impaired NO signaling in human prostate cancer, because such shedding can be blocked after reactivating NO signaling through administration of NO mimetic agents (Siemens *et al.* 2008). This work suggests that reactivation of NO can be a potential immunotherapy and help to overcome hypoxia-driven tumor escape.

3.4 Prostaglandin E2

Several lines of evidence suggest that the deregulation of cyclooxygenase-2 (COX-2)/ prostaglandin E2 (PGE2) pathway is key factor in tumor evasion of immune response (Kalinski 2012). COX enzymes catalyze the formation of prostaglandins from arachidonic acid following sequential oxidation. Interestingly, it has been shown that COX-2 can be overexpressed in both adenoma and carcinoma cells under hypoxia *via* a mechanism dependent on HIF-1α. This upregulation is associated with PGE2 overproduction and secretion in the microenvironment (Kaidi *et al.* 2006). Early studies showed that PGE2 suppresses the cytolytic activity of NK cells (Bankhurst 1982, Goto *et al.* 1983) by a mechanism related to the inhibition of IFN-γ production (Joshi *et al.* 2001, Walker and Rotondo 2004). Recently, Pietra *et al.* have shown that melanoma cells affect the function of NK cells by down-regulating the surface expression of activating receptors, including NKp30, NKp44, and NKG2D. This impairment appears to be related, at least in part, to PGE2 production by melanoma cells as the use of specific inhibitor of PGE2 restored NK cell functions (Pietra *et al.* 2012). In addition to its direct effect on NK cells, more recent data reported that PGE2 can indirectly affect the NK cell function by promoting the establishment of an immunosuppressive microenvironment. It has been shown that PGE2 promotes the development of Treg cells (Whiteside and Jackson 2013), macrophages (Heusinkveld *et al.* 2011) and MDSCs (Ochoa *et al.* 2007, Sinha *et al.* 2007).

3.5 Galectins

Galectins are proteins belonging to the lectins family which participate in the delivery of signals after binding to glycoproteins and glycolipids on the cell surface of target cells. Using a proteomic approach, Le *et al.* have identified galectin-1 as a novel hypoxia-regulated protein (Le *et al.* 2005). They proposed that tumor aggressiveness of HNSCC is dependent of hypoxia-mediated production and secretion of galectin-1, which in turn negatively regulates the anti-tumor immune response. Additional studies have supported the contribution of galectin-1 in creating immunosuppressive microenvironment at sites of tumor growth by several mechanisms (Rabinovich and Croci 2012). Thus, it has been reported that recombinant galectin-1 is able to promote the differentiation of CD4+CD25+ Treg cells *in vitro* (Juszczynski *et al.* 2007). Recently, Dalotto-Moreno *et al.* showed that tumor-derived galactosin-1 increases the abundance and/or the expansion of peripheral Treg cells *in vivo* and modulate their suppressive capacity. Conversely, attenuation of galectin-1 reduces the frequency of Treg within tumor, lymph nodes and spleen and abrogates the immunosuppressive function of Treg (Dalotto-Moreno *et al.* 2013). More recently, galectin-3, another member of galectin family regulated by HIF-1α (Zeng *et al.* 2007), has also been reported to exert an immunosuppressive function in TME. Tsuboi *et al.* provided evidence that cell-surface galectin-3 on bladder tumor cells modulates MICA-NKG2D interaction by binding MICA through poly-N-acetyllactosamine, thereby severely impairing the NK cell activation and degranulation (Tsuboi *et al.* 2011).

4 Regulation of NK cell-mediated killing by autophagy

It has become increasingly clear that under hypoxic stress tumor cells activate key biochemical and cellular pathways that are important for tumor progression, survival, and metastasis. Several recent reports highlight autophagy as a critical process that modulates the anti-tumor immune response. Briefly, autophagy is a catabolic process where cell self-digests its own components. Autophagy can be activated in response to multiple stressors including hypoxia, nutrient starvation, growth factor withdrawal and endoplasmic reticulum stress. Under stressful stimuli, autophagy activation serves as an adaptive response to provide nutrients and prevents accumulation of altered cell components (Mathew and White 2011).

To adapt hypoxia, cells activate autophagy through both HIF-1α-dependent and -independent pathways, depending on the sensor activated (i.e., low oxygen, unfolded protein response, energy depletion) (Schlie *et al.* 2011). The role of autophagy in cancer immunity seems to be complex as hypoxia-induced autophagy occurs in target cells and in tumor-infiltrating immune cells. Although the role of autophagy induction in target cells is well documented, relatively little attention has been given to its role in immune cells. Therefore, understanding how autophagy modulates tumor immune response represents a major challenge in the field of tumor immunotherapy. Recently, it has been reported that NK cells not only provide lytic signals to their target cancer cells, but also promote autophagy in the remaining un-killed target cells. Moreover, the NK-mediated autophagy induction in target cells was enhanced by provision of IL-2 and by cell-cell interaction between NK and tumor cells. This study highlights autophagy induction in target cells as a cell resistance mechanism to NK-mediated killing (Buchser *et al.* 2012). More recently we validated this concept *in vitro* and *in vivo* by showing that targeting autophagy under hypoxia restores NK-mediated lysis in breast cancer cells. In addition, we provided a mechanistic evidence that the activation of autophagy under hypoxia led to the degradation of NK-derived granzyme B making hypoxic tumor cells less sensitive to NK-mediated killing (Figure 3) (Baginska *et al.* 2013).

5 Tumor-derived extracellular vesicles influence NK cell activity

Recent advances have led to identify an additional mechanism elaborated by tumor cells to escape NK cell recognition and impair NK-mediated immune response (Bobrie *et al.* 2011). Indeed, tumor cells release vesicle-bound molecules (cytokines, NKG2D ligands, and microRNA) targeting and inhibiting NK cell functions (Clayton and Tabi 2005).

Exosomes are 50-150 nm membrane vesicles derived from the multi-vesicular bodies which are secreted by all cell types (Reviewed in (Kalra *et al.* 2012)). As a consequence, exosomes can be found in many biological fluids among which urine, plasma, and saliva. As their content reflects the cells from which they are derived, exosomes represent, therefore, attractive biomarkers (Kahlert and Kalluri 2013). Exosomes and other types of extracellular vesicles are well-known mediators of intercellular communication and play a crucial role in the development of aggressive and metastatic tumors (Park *et al.* 2010, Peinado *et al.* 2012).

5.1 Cancer cell-derived exosomes

The production of NKG2D ligand-bearing exosomes has been proposed as a mechanism for tumor cells escape from immune recognition (Clayton and Tabi 2005, Liu *et al.* 2006, Reiners *et al.* 2013). Indeed, it has been demonstrated that, in contrast to ULPB2, released ULBP3 is included into exosomes. Remarkably, ULBP3-containing exosomes have been shown as more potent down-regulators of NKG2D receptor than soluble form of ULBP2 protein release by metalloproteinase ADAM10 and 17. Pre-incubation of NK cells with ULBP3-containing exosomes induced a dramatic reduction of NKG2D-mediated lytic activity of NK cells against cells expressing MICA (Fernandez-Messina *et al.* 2010). Tumor-derived exosomes (TDE) are rapidly taken up by NK cells and remain stable for 48h (Liu *et al.* 2006, Keller *et al.* 2009). The transfer of TDE bearing membrane-anchored TGF-β, and MICA and MICB leads to down-regulation of NKG2D expression at the surface of NK cells and impairs their cytotoxic functions (Figure 4) (Clayton and Tabi 2005, Clayton *et al.* 2008). In addition, proteomic analyses on TDE have revealed the presence of classical MHC class-I molecules (Reviewed in (Kalra *et al.* 2012)). The systematic shedding of these molecules on exosome surface and their subsequent binding to KIR can, therefore, be considered as a potential inhibitory mechanism of NK cell functions and as a decoy to reduce the direct tumor-NK cell interactions. It has been shown that TDE can weakly impair the cell proliferation of NK cells compared to their strong negative effect on the proliferation CD8+ T cells (Clayton *et al.* 2007). However, numerous studies highlighted TGF-β as a major immunosuppressive molecule for NK cells (Lee *et al.* 2004, Clayton *et al.* 2008, Wilson *et al.* 2011). Indeed, elevated plasma level of TGF-β was detected in lung or colorectal cancer patients compared to healthy volunteers. This increase inversely correlated with NKG2D surface expression on NK cells in these patients (Lee *et al.* 2004). Recently, TGF-β was shown to block NK cell activation by repressing gene expression and antagonizing IL-15 induced proliferation (Wilson *et al.* 2011). A striking observation was also done by Clayton *et al.* who identified exosomal TGF-β1 as a more potent contributor to antiproliferative effects than the soluble form (Clayton *et al.* 2007).

Several cancer models have generated evidence supporting important roles of TDE. Indeed, mammary carcinoma exosomes promote tumor growth by suppressing NK cell function in mice (Liu *et al.* 2006). A decrease in splenic NK cell cytotoxicity was observed after *in vivo* injection of TDE. Moreover, a reduction in the number and the percentage of NK cells was observed in the lungs 3 days after exosomes injection, without reduction of the NK viability. Interestingly, TDE also reduced the expression of the NK pore-forming and cytolytic protein perforin (Figure 4) (Liu

et al. 2006, Wilson *et al.* 2011) whereas the level of granzyme B was left unaffected (Liu *et al.* 2006). A decrease in NK cells proliferation in response to IL-2 was also observed after treatment with exosomes derived from different tumor cell types (breast and melanoma) due to inhibition of the JAK-STAT signaling. However, TDE did not affect DCs maturation but hampered their ability to stimulate the immune response (Liu *et al.* 2006). The granzyme B-inhibitory serpin proteinase inhibitor-9 (PI-9) has also been identified inside exosomes (Buschow *et al.* 2010) and could also play an important role in the resistance of tumor cells to NK cells (Figure 4). Taken together, these data underline the crucial role that TDE may have on the tumor immunosurveillance not only by affecting the NK cell receptors but also by deeply affecting their fundamental cellular functions such as cell proliferation and release of cytotoxic molecules to impair effective anticancer immune response.

In addition, there are numerous studies supporting that hypoxic stress may influence the composition of TDE. Indeed, to substitute oxygen deprivation and lack of nutrients, tumor cells induce the expression of angiogenic factors to overcome hypoxic stress through the formation of new blood vessel or from existing vasculature. In addition to secreted VEGF, several chemokines (G-CSF, GM-CSF, CXCL16, and SDF-1) and exosomes were shown to be important mediators for tumor cells to overcome hypoxic stress (Park *et al.* 2010). In this context, it has been reported that tumor cells under hypoxic stress secrete numerous proteins sequestered in exosomes involved in cell-cell communication, cell growth, and malignant transformation. Other studies have focused on how hypoxia-induced membrane vesicles stimulates angiogenesis in malignant and angiogenic brain tumor glioblastoma multiforme (GBM). Indeed, hypoxic cancer cells release exosomes containing tissue factor (TF) acting on surrounding endothelial cells in a paracrine manner leading to the activation of a protease-activated receptor 2 (PAR2)-ERK signaling pathway (Svensson *et al.* 2011). PAR2 has been recently identified as a regulator of the innate immune response and a mediator of cell proliferation and migration. Also called thromboplastin, TF complexes with the tissue protease factor VIIa and is necessary for the initiation of thrombin formation. Because hypoxic tumors are often characterized by endothelial cells hyperplasia and hypercoagulation, the combined presence of newly generated fibrin and activated platelets has been shown to protect the tumor from NK cells and immune surveillance (Kasthuri *et al.* 2009). Further findings obtained with GBM cells indicated that hypoxic conditions stimulated tumor cells to generate exosomes containing proteins which reflect the hypoxic status of tumor cells. These finding support that the microenvironment deeply impacts the TDE composition. The enrichment in exosomes of specific hypoxia-related RNAs and proteins (cytokines, growth factors, and MMP) could indeed be associated with poor patient prognosis. In addition, hypoxic TDE mediated a strong paracrine stimulation of angiogenesis and activation of cancer cells leading to an acceleration of tumor growth in mouse xenograft model (Kucharzewska *et al.* 2013). TDE systematically contain several members of ADAM family, mostly ADAM10 (Keller *et al.* 2009) able to shed NKG2D ligands from the cell membrane (Zocchi *et al.* 2012). Finally, besides stimulating the production of exosomes with a specific content, hypoxia has also been shown to enhance exosomes release by cancer cells (King *et al.* 2012).

Beside solid tumors, circulating tumor cells, such as leukemic cells escape NK surveillance at a systematic level in blood. It is worthy to note that leukemic cells are constantly recirculating the bone marrow, where the environment is maintained in constant hypoxia (Chow *et al.* 2001). Recent studies have shed light on mechanisms of tumor cell escape from NK-mediated killing which could be used as new therapeutic approaches. These mechanisms include the shedding of soluble (BAG6, and MICA) or exosome-derived inhibitory molecules (TGF-β) in various malignancies such as

acute myeloid leukemia (Szczepanski *et al.* 2011), chronic lymphocytic leukemia (Reiners *et al.* 2013) and Hodgkin's lymphoma (Reiners *et al.* 2013).

5.2 Secreted microRNAs

As described above, under hypoxic conditions, most of cell types undergo important metabolic changes orchestrated by members of the HIF transcription factors family. It is well documented that HIF-1α is a potent inducer of miR-210 (Kulshreshtha *et al.* 2007), which has been described to be released by tumor cells (Kosaka *et al.* 2013, Tadokoro *et al.* 2013). It has been shown that released-miR-210 by leukemic and metastatic cancer cells may be transported by exosomes and entered endothelial cells (Kosaka *et al.* 2013, Tadokoro *et al.* 2013). In the recipient cells miR-210 is able to induce angiogenesis and promote tumor growth. These data underline the role of exosomal miR-210 in the shaping of the TME and the potential action on various cell types present at the tumor site. Although data available are limited, we strongly believed that exogenous miRNAs can impair the anti-tumor function of immune cells (Figure 4). In line with this concept, it has been shown that the TGF-β1-induced miR-1245 down-regulated the NKG2D receptor on NK cells and impaired NKG2D-mediated functions (Espinoza *et al.* 2012). The influence of exogenous miRNAs on NK cells is currently unknown but the comprehension of this new regulatory mechanism may help to improve the outcome of NK-based immunotherapy.

6 Conclusion

Recent developments in cancer immunotherapies have now begun to explore the use of NK cells (Lee and Gasser 2010, Gillgrass and Ashkar 2011). Particularly, strategies designed to improve NK-mediated killing using tumor specific monoclonal antibodies have shown promising results in preclinical and some clinical settings (Alderson and Sondel 2011). This review has summarized the different mechanisms involved in the impairment of NK-mediated tumor killing and highlighted that the majority of these mechanisms are likely evolved within the TME. In this regard, it should be emphasized that the composition and characteristics of the TME are important in determining the anti-tumor immune response. For example, certain cells of the immune system, including NK cells, DCs and effector T cells, are capable of driving potent anti-tumor responses. However, the ability of tumor cells to exploit other cells present in the TME is now widely regarded as a critical factor that switch the immune response from a tumor-destructive profile to a tumor-promoting profile. Such microenvironment may also favor the development of immunosuppressive populations of immune cells, such as MDSCs, TAM, and Treg.

Despite recent advances in cancer immunotherapy, the therapeutic outcome was often disappointing in many protocols in clinic. Given the important immunomodulatory effects of TME, it stands to reason that it may represent a therapeutic target that can be manipulated to improve the anti-tumor immune response. Thus, the first clinical interventions that aim to target the microenvironment to enhance tumor immunity are under active evaluation in the clinic.

Overall, investigations oriented toward the identification of novel therapeutic strategies, aiming to improve the anti-tumor immunotherapy, should pay closer attention to TME to awake or reawake immune cells, and/or to redirect such microenvironment from a pro-tumor to an anti-tumor state. Given its central role in tumor progression and resistance to therapy, hypoxic TME should be considered as a new critical therapeutic target in oncology. We assume that a better characterization of the TME can provide important prognostic and predictive values independently of the tumor phenotype.

Acknowledgments

This work was supported by grants from Public Research Center for Health (REC-LHCE-2009-0201) and "Fondation Cancer" Luxembourg (FC/2012/02) to B.J, from Télévie to E.V, J.P, and S.M, and from the "Fonds National de la Recherche" (FNR), Luxembourg (CORE project C12/BM/3962058) to E.M. J.B is a recipient of an AFR grant (2009-1201) from the (FNR), Luxembourg.

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Figure legends

Figure 1.

Tumor microenvironment activates different mechanisms to impair the NK-mediated anti-tumor immunity.

Under the pressure of the tumor microenvironment (TME), tumor cells adapt to such stress by activating intrinsic resistance mechanism (autophagy) or by regulating their metabolism. Such regulation leads to the secretion of several metabolites which impair the function of NK cells in the tumor site (yellow area). Tumor cells under stress condition may activate the release of tumor derived vesicles containing cytokines, growth factors or microRNA to directly impact the NK function (blue area). Such factors can be secreted directly to the tumor microenvironment to recruit immunosuppressive cells or to educate other stromal cells involved in the impairment of NK cell function (green area).

Figure 2.

Tumor-derived soluble factors regulate directly or indirectly the NKG2D receptors on the surface of NK cells.

A. Tumor cells in hostile tumor microenvironment educate other cells such as cancer-associated fibroblasts (CAF), myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM) and/or regulatory T cells (Treg), mainly through tumor-derived soluble factors (TDSFs). These cells affects the different NK activating receptors impairing NK cell function. **B.** Under hypoxic stress, tumor cells through HIF-1α activate the expression and the release of ADAM-10. Released ADAM-10 cleave MICA ligand on the surface of tumor cells and soluble MICA downregulates the expression of NKG2D on the surface of NK cells leading to tumor escape from NK-mediated killing.

Figure 3.

Hypoxic stress activates autophagy in tumor cells as an intrinsic resistance mechanism to NK mediated killing.

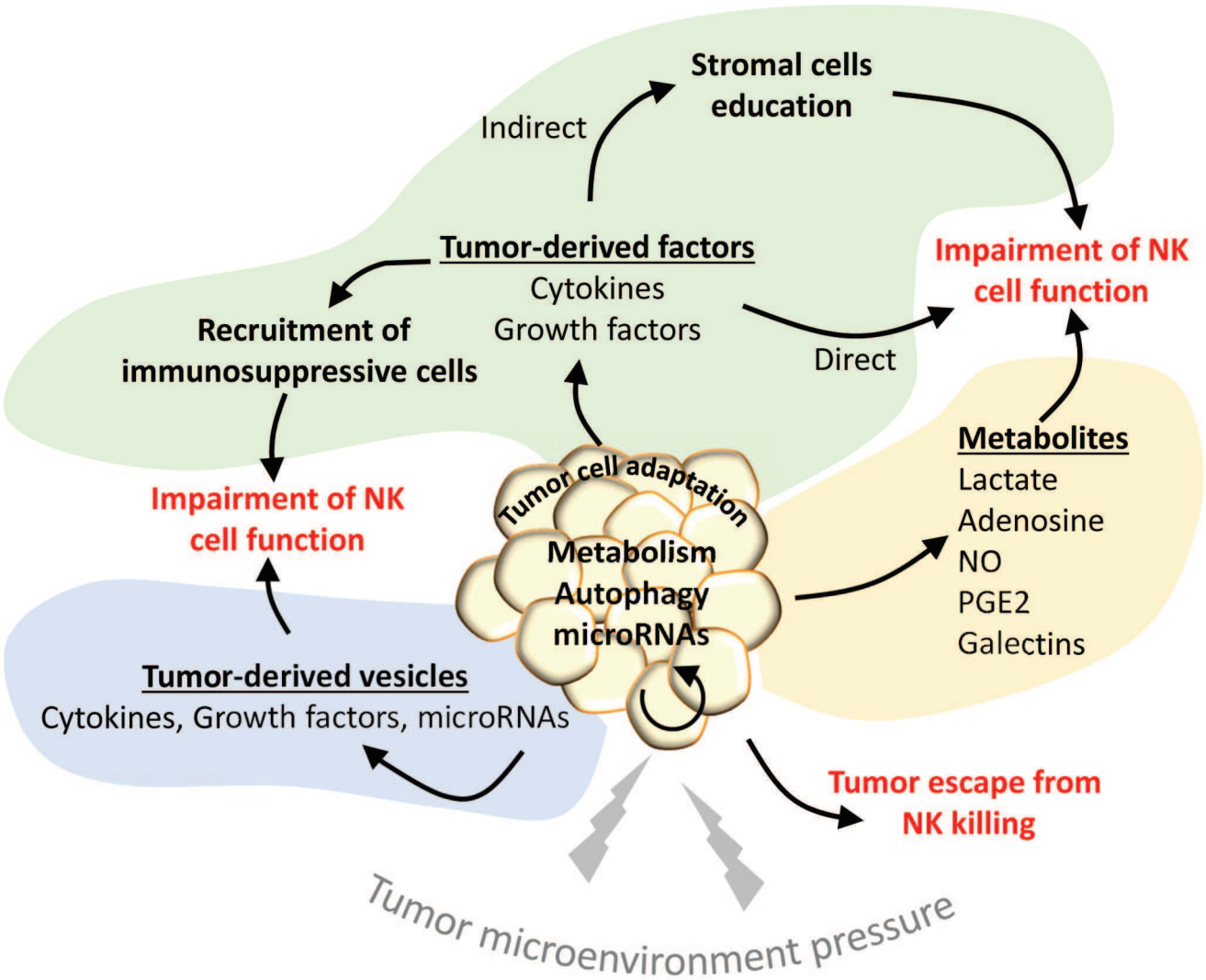
Under hypoxia, tumor cells activate the autophagy degradation process characterized by the formation of autophagosomes. NK-derived cytotoxic granules enter target cells by endocytosis and traffic to large endosomes called gigantosomes. The fusion of gigantosomes and autophagosomes lead to a specific degradation of granzyme B, making tumor cells under hypoxia less sensitive to NK-mediated lysis.

Figure 4

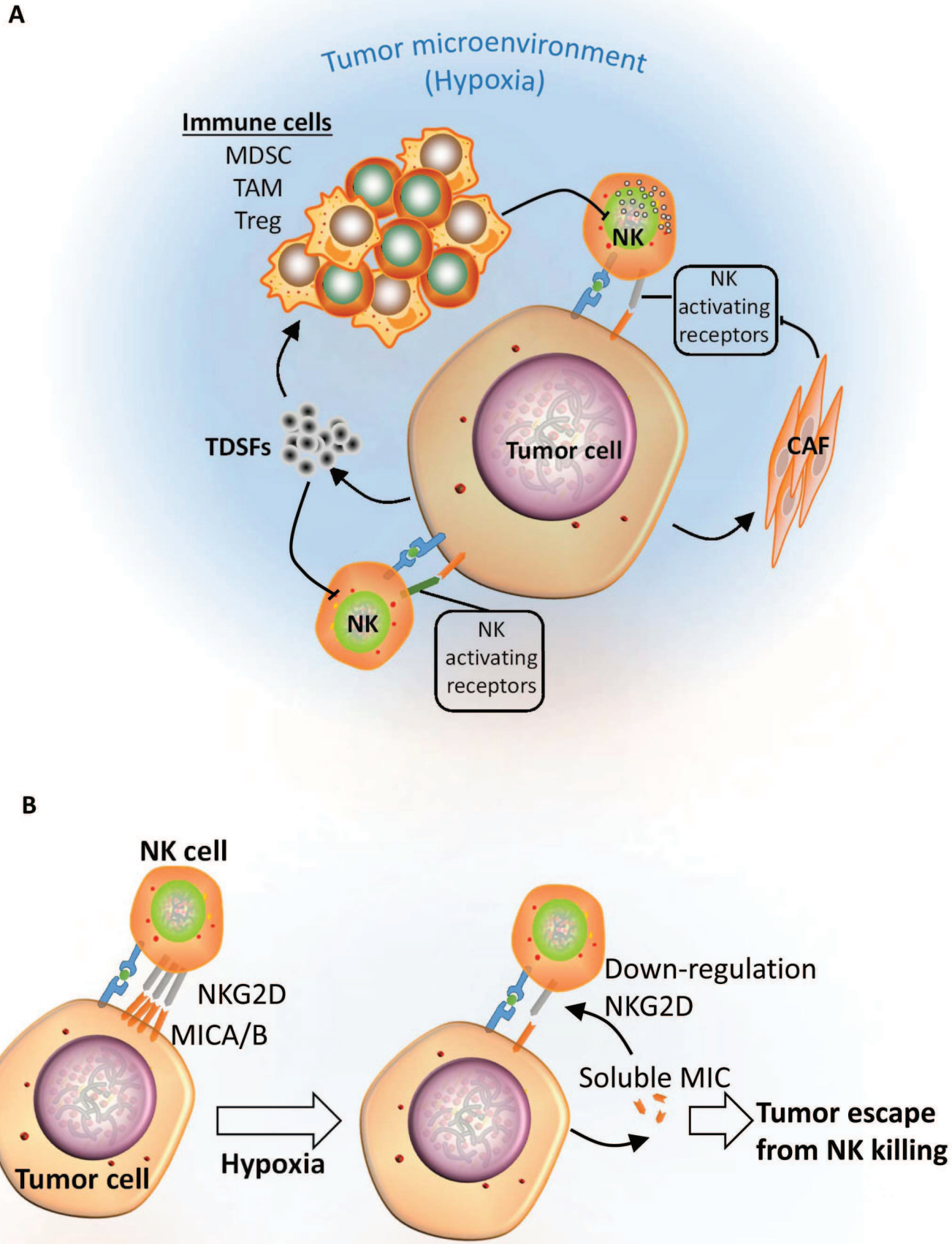
Impairment of NK cell function by tumor derived exosomes

Tumor cells secrete extracellular vesicles called exosomes. Tumor derived exosomes contain numerous factors able to modulate the function of NK cells such as MICA/B, ULBP3, TGF-β, PI-9 and different microRNAs. The most important effects of tumor derived exosomes described so far are the downregulation of NKG2D on the surface of NK cells and the downregulation of perforin, by a yet unknown mechanism.

Baginska et al. Figure 1



Baginska et al., Figure 2



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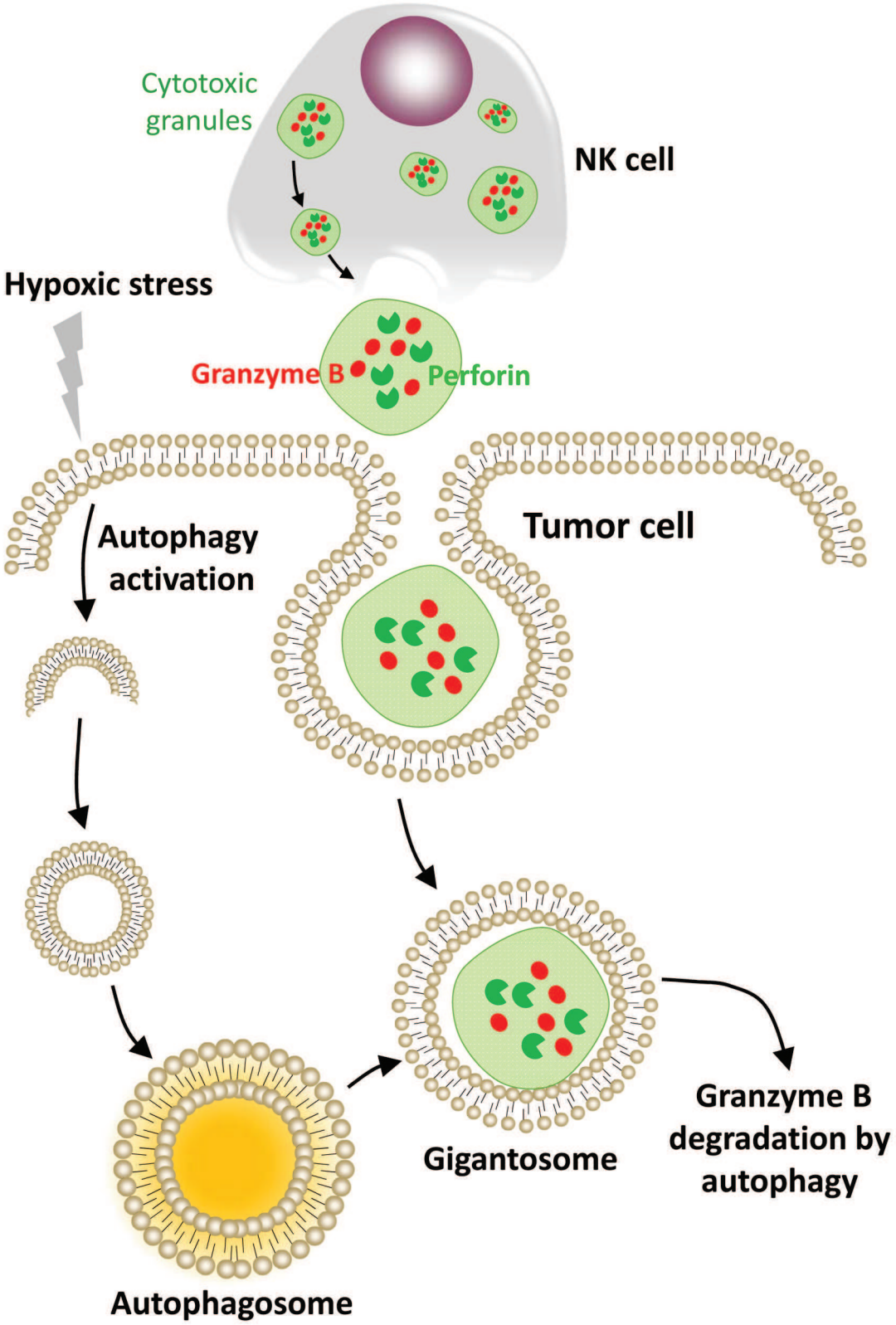

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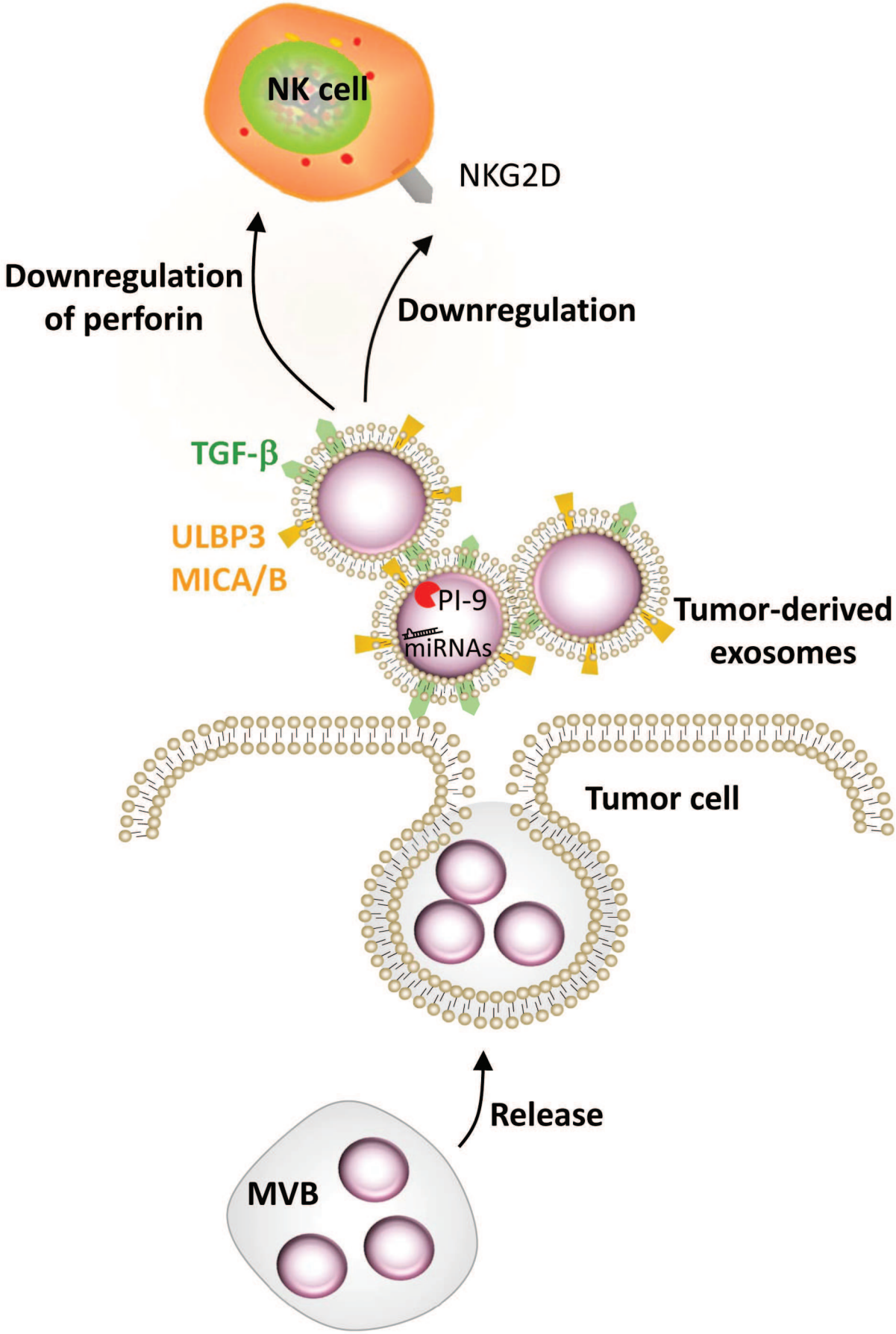

5.2 Article 4

The acquisition of resistance to TNFα in breast cancer cells is associated with constitutive activation of autophagy as revealed by a transcriptome analysis using a custom microarray.

Autophagy 7:7, 1-11; July 2011; © 2011 Landes Bioscience

The acquisition of resistance to TNFα in breast cancer cells is associated with constitutive activation of autophagy as revealed by a transcriptome analysis using a custom microarray

Etienne Moussay,[1] Tony Kaoma,[1,2,†] Joanna Baginska,[1,†] Arnaud Muller,[2] Kris Van Moer,[1] Nathalie Nicot,[2] Petr V. Nazarov,[2] Laurent Vallar,[2] Salem Chouaib,[3] Guy Berchem[1,4] and Bassam Janji[1,*]

[1]Laboratory of Experimental Hemato-Oncology and [2]Microarray Center of the Public Research Center for Health (CRP-Santé); [4]Centre Hospitalier de Luxembourg; Luxembourg City, Luxembourg; [3]Unité INSERM U753; Institut de Cancérologie Gustave Roussy; Villejuif, France

[†]These authors contributed equally to this work.

While the autophagic process is mainly regulated at the post-translational level, a growing body of evidence suggests that autophagy might also be regulated at the transcriptional level. The identification of transcription factors involved in the regulation of autophagy genes has provided compelling evidence for such regulation. In this context, a powerful high throughput analysis tool to simultaneously monitor the expression level of autophagy genes is urgently needed. Here we describe setting up the first comprehensive human autophagy database (HADb, available at www.autophagy.lu) and the development of a companion Human Autophagy-dedicated cDNA Microarray which comprises 234 genes involved in or related to autophagy. The autophagy microarray tool used on breast adenocarcinoma MCF-7 cell line allowed the identification of 47 differentially expressed autophagy genes associated with the acquisition of resistance to the cytotoxic effect of TNFα. The autophagy-core machinery genes *DRAM* (Damage-Regulated Autophagy Modulator), *BNIP3L* (BCL2/adenovirus E1B 19 kDa interacting protein 3-like), *BECN1* (Beclin 1), *GABARAP* (Gamma-AminoButyric Acid Receptor-Associated Protein) and *UVRAG* (UV radiation resistance associated gene) were found upregulated in TNF-resistant cells, suggesting a constitutive activation of the autophagy machinery in these cells. More interestingly, we identified *NPC1* as the most upregulated genes in TNF-resistant compared to TNF-sensitive MCF-7 cells, suggesting a relation between the intracellular transport of cholesterol, the regulation of autophagy and *NPC1* expression in TNF-resistant tumor cells. In conclusion, we describe here new tools that may help investigating autophagy gene regulation in various cellular models and diseases.

Key words: autophagy-related genes and proteins, microarray, database, www.autophagy.lu

Abbreviations: HADb, human autophagy-dedicated database

Submitted: 11/10/10

Revised: 03/14/11

Accepted: 03/14/11

DOI:

*Correspondence to: Bassam Janji; Email: bassam.janji@crp-sante.lu

Introduction

Macroautophagy (hereafter referred to as autophagy) is a highly conserved multistep process for the degradation and recycling of long-lived proteins and cytoplasmic organelles. During this process, portions of cytoplasm, damaged proteins and/or organelles are sequestered in double- or multi-membrane structures called autophagosomes. Upon fusion with lysosomes, sequestered materials are digested by hydrolases. The recycling of these intracellular constituents serves as an alternative energy source during periods of metabolic stress (e.g., starvation) to maintain cell viability.[1] The number of proteins identified as implicated in the orchestration of this highly dynamic process dramatically increased over the past 10 years.

Besides its basic role in the turnover of proteins and organelles, autophagy is activated under environmental stresses (nutrient starvation, pathogen infection), resulting in either cell adaptation and survival, or cell death. In addition, autophagy

is also activated under pathological conditions such as cancer, neurodegenerative, heart, liver and kidney diseases.[2] To date, the role of autophagy in cancers is a topic of intense debate because autophagy could have diametrically opposite consequences for tumor survival or death depending on the cell type and context.[3] In this regard, understanding how the autophagic process is activated and regulated under physiological and pathological conditions represents a fundamental issue in cancer therapy.

It has been suggested that the autophagic process is mainly regulated at a post-translational level. However, the recent identification of transcription factors and microRNAs (miRNAs) involved in the regulation of autophagy genes has shed light on a possible transcriptome regulation of the autophagic genes. In a recent study, Kusama et al. reported an upregulation of *ATG2A* mRNA by doxorubicin and etoposide-induced apoptosis of HeLa cells. In addition, the authors demonstrated that 13 out of 23 human autophagy gene (*ATG)* promoters are regulated by the transcription factor E2F1 in cells, indicating that autophagy might be regulated at the transcriptional level as well.[4] While *Myc* and *STAT3* (Signal transducer and activator of transcription 3) were shown to repress autophagy,[5,6] other transcription factors have been described as potent inducers of autophagy [*p53, E2F, FOXO1* (forkhead box O1) and *HIF-1*α (Hypoxia inducible factor-1)] under stress conditions such as hypoxia, starvation and DNA damages.[7-9] Several other studies highlighted the involvement of transcription factors p53, E2F and HIF-1α in regulating the transcription of *ATG5*, *BNIP3* (BCL2/adenovirus E1B 19 kDa interacting protein 3), *ULK1* (unc-51-like kinase 1), *MAP1LC3B* (Microtubule-Associated Protein 1 Light Chain 3beta) and *DRAM* (Damage-Regulated Autophagy Modulator) genes and subsequently activating autophagy in tumor cells in response to chemotherapy or hypoxia.[8-11] Although these factors are extensively described as oncogenes or tumor suppressors, their implication in autophagy is an emerging topic. Recently, it has been reported that *ATF4* (activating transcription factor 4) facilitates autophagy through direct binding to a cyclic AMP response element binding site in the *LC3B* promoter, resulting in LC3B upregulation.[12] The transcription factor NFκB was also reported in different models as repressor or activator of autophagy.[13,14] Additionally, the identification of miR-30a which targets the key autophagy-promoting gene *beclin 1* provided compelling evidence for a transcriptomic regulation of autophagy genes.[15] Overall, it is now largely admitted that autophagy might be regulated at both the transcriptional level and the post-translational level. Several methods and tools for the analysis of the autophagy flux and the post-translational modifications of autophagy proteins exist and are described in detail,[16] however there is yet no specific tool allowing the exploration of autophagy genes and the analysis of their transcriptional modifications.

In this context, we have implemented a comprehensive and freely available web Human Autophagy Database (HADb) in order to integrate and annotate autophagy genes. Using genomic data from the HADb, we have designed and produced the first human autophagy dedicated microarray. Covering the three types of autophagy, macro-, micro- and chaperone mediated-autophagy, the array targets 234 human genes described to orchestrate the initiation, the execution and the regulation of the autophagic process. The Human Autophagy-dedicated oligonucleotide microarray represents a high throughput tool to simultaneously analyze the expression of the whole repertoire of genes involved in, or related to the autophagic process.

We used the autophagy microarray to profile the transcriptome of well-characterized TNF-sensitive MCF-7 cell line and corresponding TNF-resistant 1001 clone,[17-20] and we compared the resulting gene expression data with those obtained from a commercial whole genome microarray platform. Our results showed that the autophagy microarray exhibits robust performance as a transcriptome analysis platform and is a powerful alternative to commercial high density whole genome microarray for profiling the expression of autophagy genes. The array allowed the identification of a significant number of autophagy genes differentially expressed in MCF-7 compared to 1001 cells. Expression data were validated using molecular biology and biochemical approaches, thus demonstrating the reliability of autophagy microarray. Importantly, in silico data mining on differentially expressed genes generated by the autophagy microarray resulted in the identification of signaling pathways that may contribute to explain the resistance of breast cancer cells to cell death induced by TNFα.

Results and Discussion

Setting up of a human autophagy database (HADb). A comprehensive database called HADb was implemented by integrating genomic data and sequence analysis features of the human genes involved in or related to autophagy. HADb is available, free, at www.autophagy.lu. Autophagy genes were selected using public databases in combination with commercial softwares as described in the "Methods" Section. HADb was established based on a relational database schema dedicated to biology. The database describes the features and sequences of the autophagy genes, transcripts, exons and proteins and provides an exhaustive and up-to-date list of human autophagy genes and corresponding proteins. Detailed information about features and data available for each gene in the HADb are summarized in the **Supplemental Table 1**. HADb integrates, in addition, data related to mechanisms positively or negatively regulating autophagy such as programmed cell death, cell growth and proliferation and response to endoplasmic reticulum stress. The relevance of the autophagy gene list was confirmed by performing functional annotation and enrichment analyses using bioinformatics online tools such as DAVID (Database for Annotation, Visualization and Integrated Discovery) and FatiGO. Although most of the genes described as autophagy-related genes are not annotated as such in ontology databases, autophagy was the most relevant enriched process in the FatiGO significant terms (**Sup. Table 2**, Biological process, level 3) and in the DAVID functional annotation chart (**Sup. Table 3**). Furthermore, the HADb integrates all

autophagy-related genes and proteins described in research reports thereby constituting the first exhaustive database dedicated to autophagy.

A browser was associated with the HADb allowing fast data retrieval and comprehensive display of annotations as well as navigation on the genome. Furthermore, links to other public databases including Ensembl, NCBI, KEGG, HPRD, HGNC InterPro, PDB, Prosite, Gene Expression Atlas and UniProt were introduced to allow additional complementary queries. In addition to general information about genes, transcripts and exons, HADb provides a structural and functional description of the proteins based on Gene Ontology and analysis of protein structure. Thus, users have access to several features useful to characterize proteins such as family, structural domains, protein-protein interactions, biological function, subcellular distribution, post-transcriptional modification, isoforms and ontologies. The database is regularly updated by integrating new findings from the literature.

Development of oligonucleotide array. The HADb was used as a reference database to design the human autophagy dedicated microarray. For each entry in HADb, oligonucleotide probes (60-mer) were designed using CADO4MI software as previously described in reference 21. For the majority of autophagy genes, a single probe was designed to match simultaneously all transcript variants of the gene. For only 12 genes, two probes were necessary to match the different transcript variants. Thus, the final probe set (N = 307) was composed of 246 probes matching 234 autophagy genes, 31 positive controls matching human genes reported as housekeeping genes and 30 negative controls matching viral sequences described as having no similarity with any human transcripts. The list of genes represented on the autophagy microarray is shown in **Supplemental Table 4**.

Evaluation of autophagy-microarray performance using MCF-7/1001 cell-based model. We first evaluated the intrinsic performance of autophagy microarray by performing six repeated self-self hybridization experiments using total RNA extracts from MCF-7 cells. Microarray images exhibited good and reproducible quality parameters with low background signal, signal-to-noise ratio typically higher than 20 and maximum dynamic range extending over three log10. Pearson product-moment correlation coefficients (r) calculated after signal filtering and normalization between the different array experiments were above 0.9 indicating a good level of inter-array reproducibility. We next assessed the biological reliability of autophagy microarrays by comparing the transcriptome of MCF-7 cell line sensitive to the cytotoxic effect of TNFα to that of its TNFα resistant counterpart clone, called 1001. The MCF-7 human cell line has been largely used and described as an appropriate model to study biological mechanisms occurring in breast adenocarcinoma cells. The 1001 clone has been previously characterized and the mechanisms conferring TNF-resistance to cells were extensively studied in reference 20. It has been shown that resistance is associated with a p53 mutation;[19] a defect in the sphingomeylin/ceramide pathway[18] and an upregulation of the actin filament cross-linker L-plastin protein.[22] Since TNFα was described to induce autophagy in MCF-7 cells,[23] we speculated that the expression of autophagy genes could be regulated in TNF-resistant 1001 cells. A list of 47 significantly regulated genes representing 20% of the genes represented on the autophagy array was generated through statistical analysis of expression data by setting the cut-off value for the adjusted p-value to < 0.001 and the fold-change to 2.0 ($\log_2$ FC >1.0 or <-1.0) (**Table 1**). Among this list, 34 genes were identified as significantly upregulated, while 13 were found significantly repressed in TNFα resistant 1001 cells compared to TNFα sensitive cells MCF-7. We compared these data with those obtained using a commercial whole genome transcriptomic platform (operon human oligonucleotide set version 2.0) and obtained an excellent concordance score between the two datasets. As shown in **Figure 1**, the Pearson (r) coefficient calculated using the expression Log_2 ratios of the set of significantly regulated genes was 0.9124 between the autophagy and operon arrays, corresponding to a coefficient of determination R^2 = 0.8324.

Indeed, gene expression profiling using the operon platform yielded a list of significant 1,092 upregulated and 791 downregulated genes in 1001 compared to MCF-7 cells. The most significantly and differentially expressed genes were classified into several gene function categories with the MetaCore™ software (GeneGo). These categories are highly enriched in genes linked to cell adhesion, actin cytoskeleton dynamics and TGFβ-dependent induction of EMT.[22] While the autophagy gene function category appeared at rank #189 (p-value 3.24E-02) in the operon platform, it was the most regulated pathway in the dataset obtained with the autophagy-dedicated array (p-value 2.74E-14) (**Sup. Table 5**).

Altogether, our data indicated that autophagy microarray exhibits solid performance that makes it a useful transcriptome analysis platform. Although it is a low-density array, the autophagy-dedicated microarray has several critical advantages over whole genome arrays. Each oligonucleotide probe on the array was designed carefully and benefits from the additional gene annotation introduced in the HADb database. Furthermore, since the number of probes is limited, several arrays can be printed on the same slide contributing to lower analysis cost. Finally, analysis and interpretation of expression data generated from the autophagy microarrays do not require sophisticated and specifically designed computer algorithms and are less time-consuming. Thus, our thematic microarray may facilitate discovery based on the analysis of the expression profiles of the subset of autophagy genes in a given biological situation.

Gene expression profiles obtained with autophagy-microarrays revealed alteration of NPC1 expression and lipid distribution in TNF-resistant 1001 cells. Gene profiling experiments using autophagy microarray provided strong evidence that the acquisition of TNF resistance in MCF-7 cells is associated with a significant alteration of autophagy gene expression pattern suggesting a concomitant activation of autophagy in the cells (**Table 1**). This hypothesis was supported by our results showing the formation of numerous autophagosomes in 1001 compared to MCF-7 cells (**Fig. 2A**). In

Table 1. Differentially expressed autophagy genes between TNF-sensitive MCF-7 and TNF-resistant 1001 cells

	Symbol	Name	Log_2FC	adj. p value
1	NPC1	Niemann-Pick disease type C1	3.67	5,2E-08
2	NAMPT	Nicotinamide phosphoribosyltransferase	3.59	3,2E-07
3	CASP4	Caspase 4 apoptosis-related cysteine peptidase, transcript variant alpha	3.24	3,2E-07
4	ITGA3	Integrin alpha3 (CD49C), transcript variant a	3.20	3,5E-08
5	TMEM166	Transmembrane protein 166 (FAM176A)	3.05	1,7E-05
6	PEA15	Phosphoprotein enriched in astrocytes 15	2.37	1,8E-07
7	DRAM	Damage-regulated autophagy modulator	2.32	4,0E-07
8	MYC	Myc proto-oncogene protein	2.30	6,2E-08
9	BCL2L1	BCL2-like 1 nuclear gene encoding mitochondrial protein, transcript variant 1	2.17	1,2E-07
10	SH3GLB1	SH3-domain GRB2-like endophilin B1 (BiF)	2.13	5,3E-07
11	PIK3C3	Phosphoinositide-3-kinase class 3	2.13	9,0E-06
12	RAB32	RAB32 member RAS oncogene family	2.12	3,8E-07
13	RB1CC1	RB1-inducible coiled-coil protein 1	2.10	3,4E-07
14	NBR1	Neighbor of BRCA1 gene 1	2.06	1,8E-07
15	JUN	Jun oncogene	1.99	4,5E-07
16	CAPN2	Calpain 2 (m/II) large subunit	1.96	1,6E-06
17	EGFR	Epidermal growth factor receptor	1.89	2,6E-06
18	ATF4	Activating transcription factor 4	1.87	2,8E-07
19	KIAA0831	KIAA0831 (Atg14L)	1.83	1,2E-06
20	HSPA5	Heat shock 70 kDa protein 5 (GRP78)	1.73	1,7E-07
21	ERO1L	ERO1-like (*S. cerevisiae*)	1.65	2,8E-07
22	BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	1.62	4,3E-07
23	TP53INP2	Tumor protein p53 inducible nuclear protein 2	1.54	3,8E-06
24	BECN1	Beclin 1 autophagy related	1.51	8,6E-08
25	ITGB1	Integrin beta 1 (CD29), transcript variant 1A	1.46	7,6E-07
26	EDEM1	ER degradation enhancer mannosidase alpha-like 1	1.45	3,4E-07
27	FKBP1A	FK506 binding protein 1A 12 kDa	1.45	9,0E-07
28	GABARAP	GABA(A) receptor-associated protein	1.39	3,4E-06
29	HIF1A	Hypoxia-inducible factor 1alpha subunit, transcript variant 1	1.34	6,5E-04
30	CANX	Calnexin, transcript variant 1	1.28	1,7E-05
31	CFLAR	CASP8 and FADD-like apoptosis regulator (c-FLIP)	1.25	7,2E-07
32	TP53	Tumor protein p53, transcript variant 1	1.11	1,6E-05
33	UVRAG	UV radiation resistance associated gene	1.10	1,6E-04
34	VEGFA	Vascular endothelial growth factor A	1.04	1,7E-05
35	XBP1	X-box binding protein 1, transcript variant 1	-3.69	6,2E-08
36	G6PD	Glucose-6-phosphate dehydrogenase	-3.55	5,2E-08
37	CTSD	Cathepsin D	-2.89	3,6E-08
38	RPS6KB1	Ribosomal protein S6 kinase 70 kDa, polypeptide 1	-2.78	9,0E-06
39	P4HB	Procollagen-proline 2-oxoglutarate 4-dioxygenase, beta	-1.87	1,1E-07
40	AKT1	v-akt murine thymoma viral oncogene homolog 1, transcript variant 1	-1.69	1,2E-07
41	VAMP7	Vesicle-associated membrane protein 7	-1.30	8,8E-05
42	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27)	-1.29	2,4E-06
43	TMEM49	Transmembrane protein 49	-1.27	4,7E-06
44	c12orf44	Chromosome 12 open reading frame 44	-1.20	1,2E-05
45	HSPB8	Heat shock 22 kDa protein 8	-1.19	3,0E-05

Gene profiling experiments were performed using human autophagy dedicated microarray as described in the methods section. A log2 fold change of 1 corresponds to a two-fold change in the expression level of a transcript. Positive Log_2FC correspond to upregulated genes and negative Log_2FC correspond to downregulated genes in 1001 cells when compared to MCF-7. An adjusted p value is assigned to each gene expression.

Table 1. Differentially expressed autophagy genes between TNF-sensitive MCF-7 and TNF-resistant 1001 cells

46	NRG3	Neuregulin 3	-1,16	1,3E-03
47	ATG4D	ATG4 autophagy related 4 homolog D	-1,06	6,6E-06

Gene profiling experiments were performed using human autophagy dedicated microarray as described in the methods section. A log2 fold change of 1 corresponds to a two-fold change in the expression level of a transcript. Positive Log_2FC correspond to upregulated genes and negative Log_2FC correspond to downregulated genes in 1001 cells when compared to MCF-7. An adjusted p value is assigned to each gene expression.

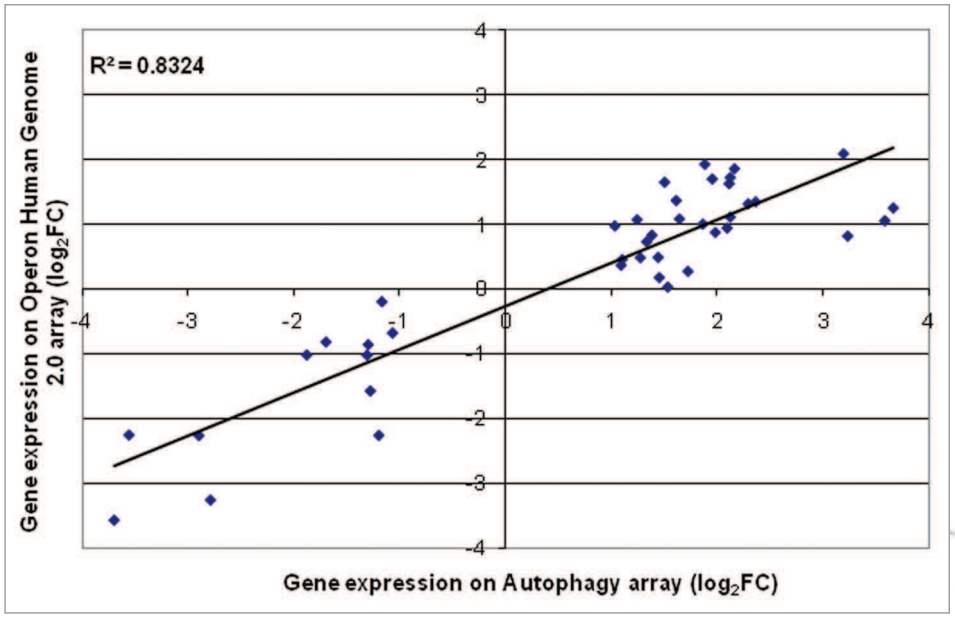

Figure 1. Correlation of gene expression between autophagy array and shole genome transcriptomic operon platform. RNA isolated from MCF-7 and 1001 cells were reverse transcribed into cDNA, labeled with Cy3 and Cy5, and hybridized on both human autophagy microarray or operon whole genome transcriptome analysis platform. Only genes statistically regulated between MCF-7 and 1001 cells ($p < 0.001$ and FDR <5%) presenting a log_2 fold change (log_2FC) greater than 1 or -1 were plotted. The Pearson product-moment correlation coefficient (PMCC or R) was calculated from log_2 fold changes obtained from both array platforms and the coefficient of determination was calculated as the square of the PMCC (R^2).

addition, the expression of key autophagy genes was analyzed at mRNA and protein level. **Figure 2B** show an upregulated expression of Beclin 1, LC3 and p62 and a downregulated expression of Bcl2 mRNA in 1001 compared to MCF-7. Western blot analysis confirms the overexpression of *beclin 1* and LC3 and the downregulation of Bcl-2 proteins (**Fig. 2C**). Although its mRNA was found upregulated, p62 protein level was dramatically decreased in 1001 cells compared to MCF-7. This result suggests that p62 mRNA is upregulated to compensate for the continuous degradation of this protein by the autophagy flux. In order to discriminate between the increase in the autophagic flux and the blocking in autophagic clearance, we assessed the protein level of LC3 and p62 in 1001 untreated or treated with chloroquine (CQ). CQ is a potent inhibitor of autophagy, which blocks the fusion of autophagosomes and lysosomes. Results in **Figure 2D** clearly show an accumulation of p62 and LC3-II in 1001 cells treated with CQ suggesting a blockade in the autophagic flux responsible for the clearance of these proteins. Although an accumulation of LC3-II was also detected in CQ-treated MCF-7 cells, no difference in the expression level of p62 was observed in untreated and CQ-treated MCF-7 cells. This result suggests that the autophagic flux is increased in 1001 cells compared to the basal level of autophagy observed in MCF-7 cells.

It has been reported that p53 is involved in the control of the autophagy program by transcriptionally activating the autophagy gene DRAM.[11] While the causal mechanisms underlying the activation of autophagy in TNF-resistant cells remains to be determined, it is unlikely that p53 is involved in the activation of autophagy in 1001 cells since these cells express a mutated form of p53. Similarly, the involvement of the transcription factor E2F1 in the regulation of autophagy genes in 1001 cells could be excluded since no difference in the expression of this transcription factor was observed in MCF-7 and 1001 cells (data not shown).

Interestingly, one of the most significant overexpressed genes in 1001 compared to MCF-7 cells was found to be *NPC1*. Indeed, Niemann-Pick disease, type C1 is an autosomal recessive disease most commonly caused by a mutation of NPC1, resulting in the accumulation of cholesterol in late endosomes or lysosomes. It has been reported that the transport of the cholesterol from the late endosomes/Lysosomes to the sterol-regulatory pool at the endoplasmic reticulum was associated with the downregulation of the NPC1 gene.[24] In line with this study, we next analyzed the subcellular distribution of cholesterol in MCF-7 and in 1001 cells overexpressing *NPC1*. **Figure 2E** show a

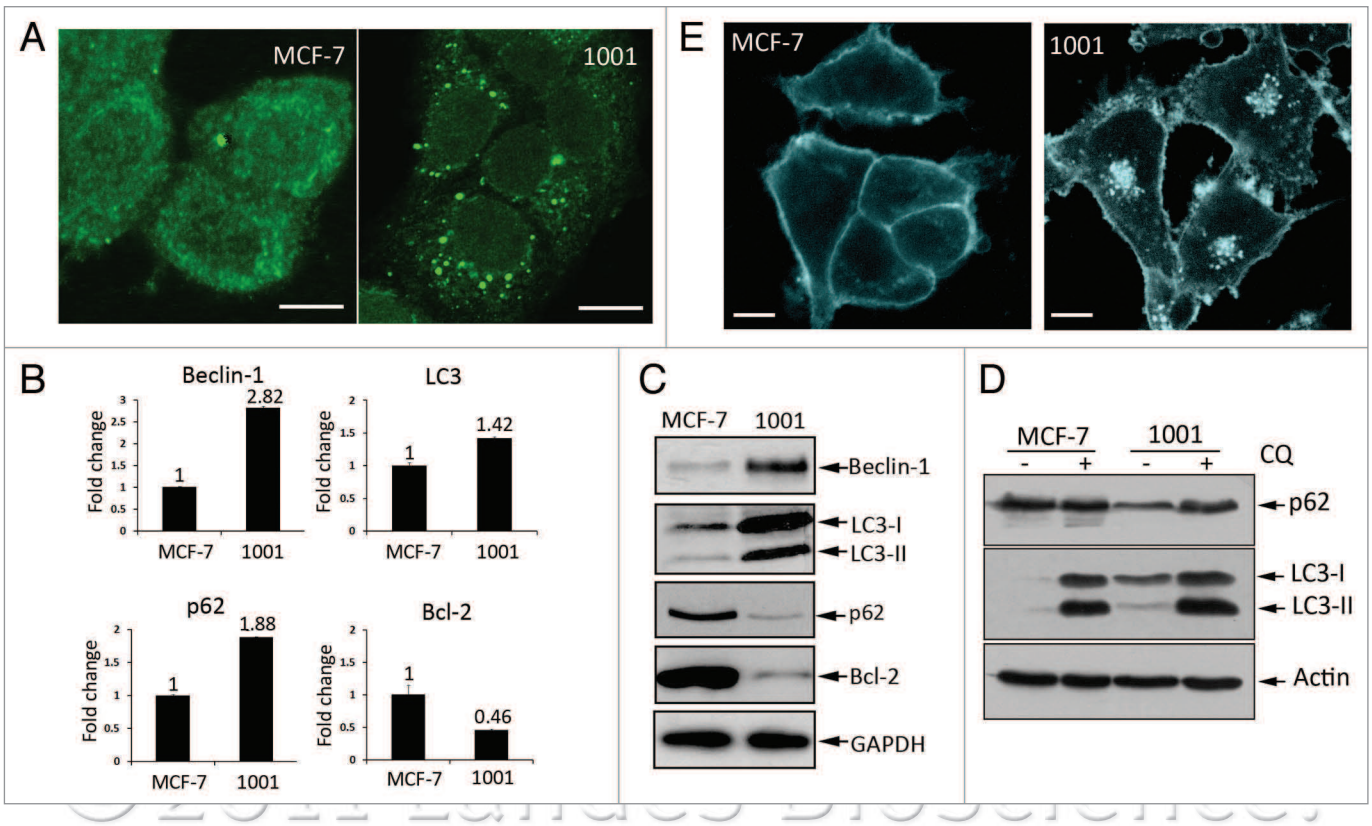

Figure 2. (A) Immunofluorescence analysis of autophagosomes formation in TNF-resistant 1001 cells. MCF-7 and 1001 cells were labeled with anti-LC3 primary antibody and Alexa-Fluor 488-conjugated secondary antibody. No autophagosomes were observed in MCF-7 (diffuse green staining) and several autophagosomes (green dot-like structures) were observed in 1001 cells. (B) mRNA expression level of autophagy genes Beclin 1, LC3, p62 and Bcl-2 was assessed by real time PCR using specific primers. The expression level of mRNA of each gene in 1001 cells is represented as a fold change relative to that in MCF-7. Results are given as the mean ± standard deviation (SD) of three independent experiments. (C) Expression of autophagy markers Beclin 1 and LC3, p62 and Bcl-2 in MCF-7 and 1001 cells. Immunoblot analysis was performed on total protein extracts (50 μg) using anti-beclin 1, -LC3, -p62 or -Bcl-2 specific antibody. Anti-GAPDH antibody was used as a loading control. (D) Expression of autophagy marker p62 and LC3 in MCF-7 and 1001 cells untreated (-) or treated with the autophagy inhibitor chloroquine (CQ, Sigma, C6628). Cells were treated with 60 μM of CQ for 72 h. Immunoblot analysis was performed as described in (C) using anti-p62 or -LC3 specific antibody. Anti-actin antibody was used as a loading control. (E) Subcellular distribution of cholesterol in MCF-7 and 1001 cells. Cells were labeled with filipin (50 μg/ml in PBS), as described in the methods section, to monitor the cellular distribution of cholesterol. The images are representative of three independent experiments. Bar = 10 μm.

dramatic differences in cholesterol distribution between MCF-7 and 1001 cells. Filipin staining provides clear evidence that accumulation of cholesterol in 1001 is associated with the overexpression of NPC1. Based on the report of Garver et al. we suggest that the overexpression of NPC1 in 1001 cells is a secondary event in response to an altered cholesterol distribution. While further studies need to be conducted to determine the causal mechanisms underlying the increase of autophagy in NPC1-overexpressing 1001 cells, several reports demonstrated an association between autophagy and cholesterol-accumulation in NPC1 disease.[25] In addition, a recent study provided evidence that NPC-dependent defects in lipid trafficking increase the expression of Beclin 1 and subsequently activate basal autophagy.[26]

It is noteworthy that the basal high level of *NPC1* mRNA was increased by 2.25 fold ($\log_2$ FC 1.5) when 1001 cells were treated with TNFα (**Table 2**). This result is consistent with previous reports indicating that in the absence of TNFα, the progression of NPC liver disease is slower and confirm the role of TNFα as a lipid metabolism regulator.[27,28]

Microarray data mining highlights the activation of autophagy in TNF-resistance cells either directly or indirectly via the ER stress and suggests a potential involvement of VEGF. To gain more insight into pathways involved in the acquisition of TNF-resistance, we analyzed the significant differentially expressed genes identified in our study (**Table 1**) using Ingenuity Pathway Analysis software. Based on the molecular interactions referenced in the Ingenuity® Knowledge Base and the transcription factor-oriented analysis, we inferred the interaction networks within our expression dataset, generating a potential model of autophagy interactome. Thus, all statistically differentially expressed genes listed in **Table 1** ($p < 0.001$) were imported to IPA®, transcription factors were selected and their targets were determined. p53 was removed from the network because this gene is mutated at p53 DNA-interaction site (point mutation P280K) in 1001 cells as previously described in reference 19. Downstream nodes were then connected to obtain the final model (**Fig. 3**). This

Table 2. Effect of TNF-α treatment on autophagy genes expression in 1001 cells

	Symbol	Name	Log_2FC	adj. p value
1	NAMPT	Nicotinamide phosphoribosyltransferase	3.50	2,32E-06
2	DRAM	Damage-regulated autophagy modulator	2.89	1,20E-06
3	ULK1	UNC-51-like kinase 1	2.19	1,20E-06
4	SESN2	Sestrin-2	2.16	2,35E-04
5	CFLAR	CASP8 and FADD-like apoptosis regulator (c-FLIP)	2.15	1,38E-06
6	SQSTM1	Sequestosome 1 (p62)	2.04	1,32E-05
7	BNIP3L	BCL2/adenovirus E1B 19 kDa interacting protein 3-like	1.53	4,84E-04
8	HSPA5	Heat shock 70 kDa protein 5 (GRP78)	1.53	2,35E-04
9	SPHK1	Sphingosine kinase 1, transcript variant 1	1.50	6,94E-07
10	NPC1	Niemann-Pick disease type C1	1.50	6,51E-06
11	DDIT3	DNA-damage-inducible, transcript 3	1.40	8,12E-04
12	PRKCD	Protein kinase C delta, transcript variant 1	1.22	3,14E-04
13	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21), transcript variant 1	1.17	5,32E-04
14	TP53	Tumor protein p53, transcript variant 1	-1.05	8,12E-04
15	HSPCA	Pos. control heat shock 90 kDa protein 1alpha	-1.38	8,72E-06
16	PARP1	Poly [ADP-ribose] polymerase 1	-1.92	8,23E-06
17	PRKDC	Protein kinase DNA-activated catalytic polypeptide, transcript variant 1	-2.22	6,94E-07
18	BIRC5	Baculoviral IAP repeat-containing 5 (survivin), transcript variant 1	-2.83	2,98E-05

Autophagy gene profiling experiments were performed using human autophagy dedicated microarray on untreated and TNF-treated 1001 cells, as described in the methods section and the legend of Table 1. The overlapped genes between Table 1 and 2 are highlighted in gray.

model is composed by two interconnected networks, one related to endoplasmic reticulum (ER) stress function, the other centered on the transcription factor MYC. It suggests that the autophagic cell survival pathway may be activated by ER stress and/or may depend on MYC which regulates the expression of proteins involved in the autophagy core machinery such as BECN1, BNIP3L, BCL2L1, UVRAG and PIK3C3 (phosphoinositide-3-kinase, class3). Activation of autophagy triggered by ER stress involves HIF1α-dependent overexpression of factors including ERO1L (Endoplasmic oxidoreductin-1-like protein), HSPA5 (heat shock 70 kDa protein 5) also called BIP, VEGFA (Vascular Endothelial Growth Factor A), JUN and ATF4 (activating transcription factor 4). Since HIF1-α seems to directly regulate the expression of MYC and appears as the key node in the network, it is reasonable to assume that 1001 cells are constantly under hypoxia-like condition, which ultimately and constitutively activates the autophagy in 1001 cells. Furthermore, our interactome model highlights the central position of VEGF in integrating signals from HIF1α, ERO1L, ATF4 and Jun and regulating the expression of NAMPT (Nicotinamide phosphoribosyltransferase) and NPC1. Interestingly, a link between HIF1α-dependent induction of VEGF and NPC1 gene was disclosed in the model. Whether the upregulation of VEGF occurs directly by HIF or indirectly via ATF4, ERO1L or JUN is not apparent from our data and should be studied in more detail. Nevertheless, ATF4 could be involved in the acquisition of 1001 cells resistance to TNFα since it has been documented that ATF4 is a critically important transcription factor playing a major role in cell adaptation to ER Stress by integrating a stress response and activating autophagy.[29,30] In addition ATF4 plays a major role in protecting MCF-7 cells from Bortezomib-induced cell death and other anti-cancer drugs.[12,30]

Given the central role of *HIF1*α and *MYC* in the network described in **Figure 3A**, the upregulated expression of Myc and HIF1α revealed by microarray data was validated by real-time PCR. **Figure 3B** shows 5.21 and 3.72 fold increases in the expression of Myc and HIF1-α respectively in 1001 compared to MCF-7 cells. In addition, the overexpression of some HIF1-α downstream genes, such as Ero1-Lα, BIP and VEGF, in 1001 cells was also confirmed at protein level by western blot (**Fig. 3C**).

TNF-treatment repressed the expression of the autophagy inhibitor gene survivin and the apoptosis inducer gene PARP1 in TNF-resistant cells. Results from this study provide strong evidence for a change in the cell transcriptome background between the TNF-sensitive MCF-7 and their TNF-resistance counterpart 1001 cells which ultimately regulate several signaling pathways including autophagy. To determine whether a different subset of genes was regulated by TNFα in 1001 resistant cells, we profiled gene expression in 1001 cells untreated or treated with TNFα using autophagy dedicated microarrays. Analysis of significant expression data yielded 13 upregulated and five downregulated genes in TNF-treated compared to untreated 1001 cells (**Table 2**). Among the 13 upregulated genes, six (46%) were also overexpressed in 1001 cells when compared to MCF-7 cells (**Table 1**). More particularly, NPC1, which was identified as one of the most consistently relevant overexpressed genes in 1001 compared to MCF-7 cells, was even more upregulated in 1001 cells upon

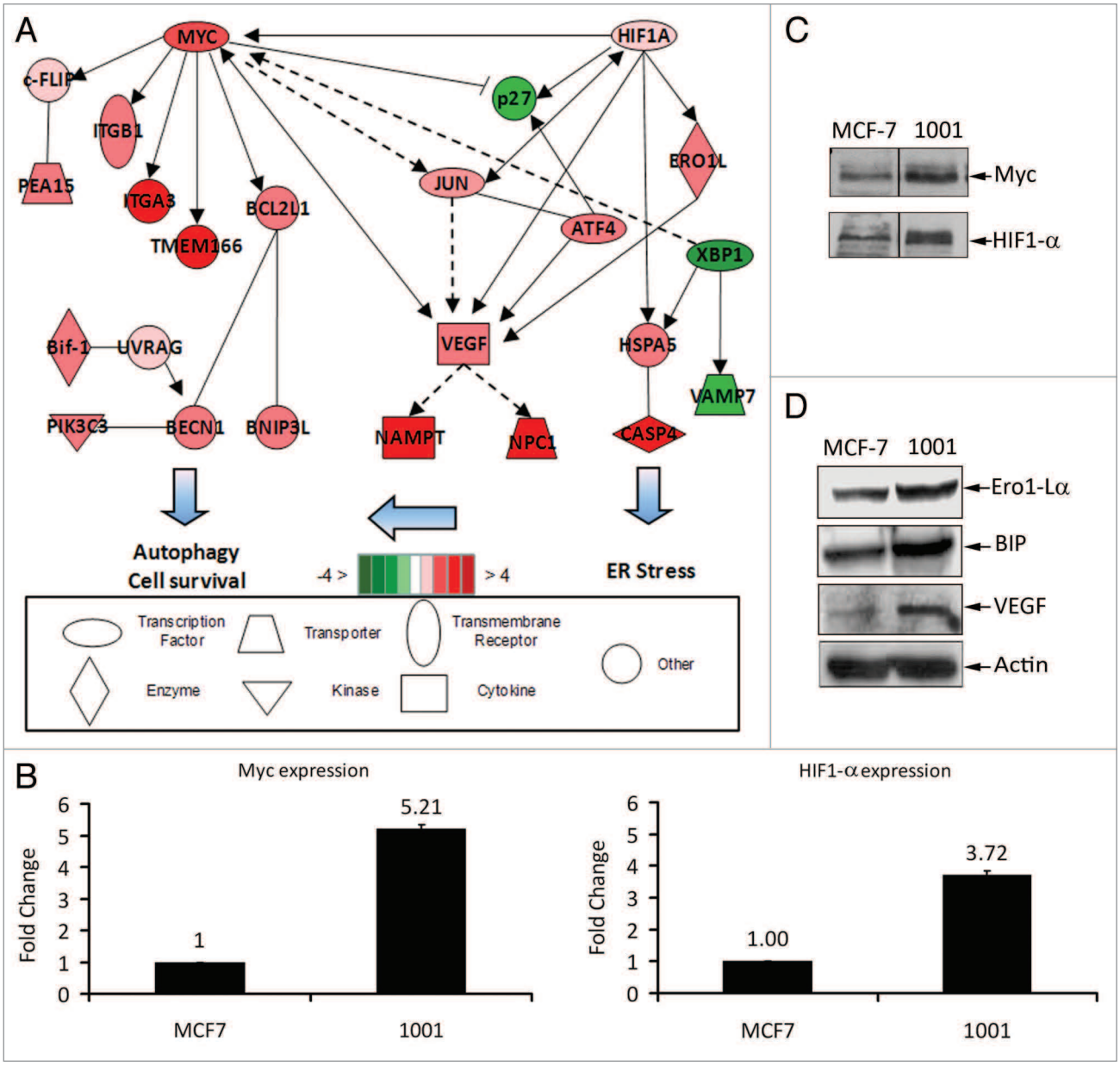

Figure 3. (A) Data mining of autophagy microarray results performed by Ingenuity software highlights the involvement of MYC and HIF1-α downstream pathways in the activation of autophagy in TNF-resistant cells. Solid lines indicate a direct interaction and dotted lines indicate an indirect interaction; arrows indicate that protein A acts directly (solid line) or indirectly (dotted line) on protein B. Green represents downregulation while red depicts upregulation in 1001 compared to MCF-7 cells. The intensity of color represents the average of log2 fold change from three independent experiments. Symbols affected to each gene reflect cell functions. (B) MYC and HIF1-α mRNA level was assessed by real-time PCR using specific primers. mRNA expression level was determined as described in Figure 2B. (C) Expression of Myc and HIF1-α protein in MCF-7 and 1001 cells. Immunoblot analysis was performed as described in Figure 2C using anti-Myc or -HIF1α specific antibody. (D) Expression of Ero1-Lα, BIP and VEGF protein in MCF-7 and 1001 cells. Immunoblot analysis was performed as described in Figure 2C. Anti-actin antibody was used as a loading control.

TNFα treatment. Our data also suggested that TNFα could regulate autophagy-driving factors such as DRAM, ULK1, p62, BNIP3L and the sphingosine kinase 1 (SPHK1). Surprisingly, no common downregulated genes were identified between the gene lists shown in **Tables 1 and 2**. This suggested that TNFα treatment of 1001 cells impacts on the expression of a subset of genes that is distinct from those involved in TNF resistance. Of interest, we identified survivin encoding gene *Birc5* as one of the most downregulated genes in TNF-treated cells (**Table 2**). Given the role of survivin in autophagy inhibition, by interacting with LC3,[31] it is likely that TNFα accelerates basal autophagy in 1001 cells by downregulating the expression of survivin. Moreover, the apoptosis inducer PARP was found repressed by TNFα in 1001 cells. This suggested that TNFα could inhibit apoptosis and favor autophagy in these cells.

Conclusion

There is strong evidence that transcriptome regulation of autophagy genes plays a pivotal role in the execution phase of autophagy and in the interplay between the autophagy and the apoptotic machineries.[32,33] By allowing the simultaneous monitoring of the expression level of autophagy genes, the human autophagy-dedicated microarray represents a powerful tool to study autophagy interactome in biological samples. Using appropriate bioinformatics tools on well-established cell-based model, we demonstrated the reliability of the autophagy microarray to generate and disclose novel unexpected insight into the mechanisms leading to the acquisition of resistance to TNFα in tumor cells.

Whole genome DNA microarrays are extremely valuable investigation tools generating huge amounts of informative data at once. However, these data can be valorized only through a set of specifically designed computer algorithms including sophisticated statistical and complex bioinformatics software which are not available in the majority of research labs. In addition, analyzing thousands of genes remains a difficult task that required a massive amount of work, and most of the Supplemental or additional data generated by whole genome microarray are irrelevant. In this context, low-density thematic DNA microarrays have the competitive advantage of being easy and fast to use for the analysis of the expression of a subset of genes related to a particular cellular phenomenon or a precise signaling pathway. Our oligonucleotide microarray represents an affordable alternative to pangenomic arrays and is available for all researchers interested in screening the autophagy interactome.

Methods

Implementation of the human autophagy database (HADb) and cDNA microarray. The database and oligonucleotide microarray were built by integrating all genes described as directly or indirectly involved in the autophagic process including genes regulating survival signals, cellular growth, stress or apoptosis. A first list of manually selected genes was established based on the literature in PubMed. Additional entries were added from biological public databases (NCBI RefSeq and GenBank, Gene Ontology, and UniProt) and the commercial pathway database Ingenuity® Pathway Analysis (IPA 7, database Aug 2009). The additional entries were manually curated to verify their implication in autophagy. Two gene subsets were then created: one comprising genes involved in the core autophagic machinery and the other including those involved in autophagy regulatory mechanisms. The current list of genes is composed of 234 entities whose relevance was investigated using bioinformatics tools available online. Thus, a gene enrichment and functional annotation chart was created using DAVID software.[34,35] A functional enrichment analysis by means of a two-tailed Fisher's exact test was performed using FatiGO software.[36] We used both methods to compare our set of autophagy-related genes, present in the database and on the autophagy-microarray with the whole human genome set as background. All features associated with autophagy-related genes were collected from diverse public databases and gathered in a database hardcore (GMOD/Chado).[37] This relational database schema was coupled to a web interface allowing users to browse the entries, query the database, and have a clear presentation of the multiple data related to genes, transcripts, exons, and proteins involved in autophagy. Additional links to international reference databases were also included.

Autophagy array probe design and fabrication. The probe design and array fabrication was performed as previously described in reference 21. Briefly, oligonucleotide probes (60-mer) matching human autophagy genes, human housekeeping genes (positive controls) and viral sequences (negative controls) were designed using the program CADO4MI (Computer-Assisted Design of Oligonucleotides for Microarrays) software.[21,38] Specificity of the probes was assessed by identifying the complementary target sequences referenced in RefSeq and UniGene. Probes with unique target in both databases were automatically selected by CADO4MI while the others were curated manually. When necessary, two probes were designed to hybridize different variants of the gene transcripts. Oligonucleotides were then synthesized with a 5'-end C6-amine modification by Eurofins MWG Operon (Ebersberg, Germany; customized order). Microarrays were produced by contact printing using a Microgrid II microarrayer (Genomic Solutions, Huntingdon, United Kingdom) by spotting oligonucleotides in triplicates onto epoxy-coated glass slides (SME2, Arrayit, Sunnyvale, CA, USA) at a concentration of 25 μM in microspotting plus solution (T-MSP4X, Arrayit). The layout of the slides was designed to allow the printing of two independent arrays per slide.

Gene expression profiling experiments. A human breast adenocarcinoma MCF-7 cell line was obtained from ATCC (HTB-22). The TNF-resistant clone 1001 derived from MCF-7 was previously described in reference 18. Cells were grown to 70–80% confluence in Dulbecco's modified Eagles's medium containing 10% fetal bovine serum (Lonza, DE14-801F), 100 units/ml potassium penicillin and 100 μg/ml streptomycin sulfate (Lonza, 17-602E) and UltraGlutamine (DMEM, Lonza, BE12-604F/U1). For TNFα treatment, 1001 cells were incubated with 75 ng/ml of recombinant human TNFα (ImmunoTools 11343017) during 72 h. Cells were washed twice with PBS (Lonza, BE17-513F), and total RNA was extracted using the TRIzol reagent (Invitrogen 15596-018) according to the manufacturer's instructions. RNA concentration and integrity were analyzed by the Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and Agilent 2100 Bioanalyzer RNA 6000 nano assay (Agilent Biotechnologies 5067-1511). RNA was stored frozen at -80°C in DEPC-treated water (Invitrogen 750024) until use. Only high quality RNA with a ribosomal RNA ratio greater than 1.8 and no evidence of degradation was used. RNAs from three independent cultures were pooled before staining. Two-color microarray experiments were performed as previously described with AlexaFluor® 555 or 647 NHS-ester dyes (Invitrogen, A32755).[21] Microarrays were scanned at 10-μm resolution with two laser channels

(532 and 635 nm) using a GenePix® 4000B scanner and GenePix® Pro 6.0 (Molecular Devices Corporation, CA) with photomultiplier tube (PMT) gains adjusted to obtain less than 1% saturated spots. Gene expression experiments were performed in triplicate.

Microarray image and data analysis, quality control and statistical evaluation of gene expression. Microarray images were quantified using the GenePix Pro 6.1 software (Molecular Devices, Sunnyvale, CA). Replicate spots were summarized and filtered before the statistical analysis; only spots with intensities significantly higher than the background level were considered. Information was imported from GenePix Result (GPR) files, processed and further analyzed by R/Bioconductor tools. Several normalization methods were applied to remove experimental bias in the dataset. Briefly, spatial effects were removed using two-dimensional approximation of zero log-ratio level by radial basis functions. Dye-effect was removed by a Lowess normalization with the smoothing parameter f = 0.67.[39] Between-array normalization was next performed by median-based centring and scaling of log-ratio distributions. The quality of microarrays was controlled by distributions of log-ratio values of the good-quality spots, their total number of per array, average correlation with other arrays and spatial homogeneity. To find genes with statistically significant regulation in each class (MCF-7 vs. 1001 and 1001 + TNFα vs. 1001) and between these two classes, the empirical Bayes method,[40] implemented in R/Bioconductor's limma package (R-code available at http://www.bioinformatics.lu), was used. Raw data have been submitted to the EMBL-EBI ArrayExpress repository under the reference E-MEXP-3074 and can be visualized at the website at http://www.ebi.ac.uk/arrayexpress/. Genes with adjusted p-value lower than 0.05 were considered as significantly regulated. Microarray Data mining was performed using the databases from Ingenuity Pathway Analysis system (Ingenuity® Systems, IPA® 8) and MetaCore™ (MetaCore™ version 6.5 build 27009, GeneGo Inc., MI).

Validation of autophagy-microarray data with commercial whole genome microarray data. RNA from MCF-7 and 1001 cell lines were hybridized on both cDNA Autophagy microarrays (CRP-Santé, Luxembourg) and operon human version 2.0 oligonucleotide library (University Medical Center Utrecht, The Netherlands). Two-color microarray experiments were performed as described above. Base 2 Logarithmic fold changes ($\log_2$FC) obtained from both array platforms were plotted and the coefficient of determination (R^2) was calculated as the square of the sample Pearson product-moment correlation coefficient (r).

Reverse transcription (RT) and real-time PCR (RT-PCR). Total RNA was extracted as described above and the cDNA was synthesized using SuperScript III First-Strand Synthesis System (Invitrogen 18080-051). Real-time PCR was performed using MYC and HIF1α specific primers purchased from Eurogentec. Myc primers were 5'-CCC GGA GTT GGA AAA CAA TG-3' (Forward) and 3'-CCT CTT CAG AAA TGA GCT TTT GC-3' (Reverse). HIF1-α primers were 5'-GAA AGC GCA AGT CCT CAA AG-3' (Forward) and 5'-TGG GTA GGA GAT GGA GAT GC-3' (Reverse). Beclin 1 primers were 5'-GGA TGG TGT CTC TCG CAG AT-3' (Forward) and 3'-TTG GCA CTT TCT GTG GAC AT-3' (Reverse). LC3 primers were 5'-AGC AGC ATC CAA CCA AAA TC-3' (Forward) and 3'-CTG TGT CCG TTC ACC AAC AG-3' (Reverse). BCL2 primers were 5'-ATC GCC CTG TGG ATG ACT GA-3' (Forward) and 3'-GGG CCG TAC AGT TCC ACA AA-3' (Reverse). P62 primers were 5'-CAG CTT CTG CTG CAG CCC CG-3' (Forward) and 3'-TCC TCA TCG CGG TAG TGC GC-3' (Reverse). Five nanograms of cDNA were used in a 25 µl PCR reaction mixture containing 12.5 µl SYBR® Green PCR MasterMix and 300 nM of each primer. PCR amplifications were performed on ABI 7300 Real-Time PCR System (40 cycles at 95°C for 15 sec and 60°C for 60 sec). Values were normalized to 28S rRNA and were processed by using the $2^{-\Delta\Delta Ct}$ calculation method.

Cholesterol staining. MCF-7 and 1001 cells were grown on cover slips in complete medium until reaching 70–80% of confluence. After two washes with PBS, cells were fixed in 4% paraformaldehyde (Thermo Scientific 28908) for 20 min, washed twice with PBS and stained with filipin (Sigma, F9765) for 75 min at room temperature. Cells were washed and cover slips were mounted with anti-fading glycerol-based medium (Agar Scientific Ltd., R1320) and visualized immediately with Zeiss laser scanning confocal microscopy (LSM-510-Meta).

Immunoblotting analysis. Protein extracts from cells were prepared as previously described and the protein concentration was determined using the Bradford assay kit from BioRad.[41] Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane which was incubated with rabbit polyclonal anti-Beclin1 (Cell Signaling 3738), anti-LC3 (Cell Signaling 2775), anti-Ero1-Lα (Cell signaling 3264), anti-BIP (Cell signaling 3177) or anti-VEGF (Epitomics 1909-1), mouse monoclonal anti-p62 (BD 610833), anti-Bcl2 (Dako, M0887), anti-actin (Sigma, A5441), anti-HIF-1α (BD Transduction Laboratories 610959), anti-MYC (Invitrogen 46-0603) or anti-GAPDH (Abcam, ab8245). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch 111-035-144) and goat anti-mouse IgG (Jackson ImmunoResearch 115-035-146). Protein bands were revealed by enhanced chemiluminescence ECL (GE Healthcare, RPN2135V1).

Indirect immunofluorescence. Cells were fixed with 4% paraformaldehyde, detergent permeabilized with 0.4% Triton X-100 and stained with primary rabbit monoclonal anti-LC3B antibody (Cell Signaling, 3868) and secondary Alexa Fluor 488-coupled rabbit anti IgG antibody. Cells were analyzed with a Zeiss laser scanning confocal microscope (LSM-510-Meta).

Acknowledgements

This work was supported by grants from the Luxembourg Ministry of Culture, Higher Education and Research (REC-LHCE-2009 0201) to Bassam Janji, the Télévie (N° 7.4552.05) and the National Research Fund (FNR) of Luxembourg to Etienne Moussay. Joanna Baginska is a recipient of an AFR grant from the FNR. The authors thank Sandrine Pierson

for her outstanding technical assistance and Olivia Roland for the editing of the manuscript.

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/autophagy/article/15454

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Supplementary table 1

Feature	Category	Information	Main Source	Other Sources
Gene	General	Official symbol	HGNC	
		Synomym		
		Official name		RefSeq, Uniprot
		Other names		RefSeq, Uniprot
		Gene type		
		Organism		
		Chromosome location	RefSeq	
		Map		
		Length		
	External links	EC_number	HGNC	
		Ensembl		
		Entrez Gene		
		HGNC		
		HPRD	RefSeq	
	Comments	Summary	RefSeq	
	Sequence		RefSeq	
Transcript	General	Name	RefSeq	
		Type of transcript		
		Organism		
		Length		
		Map		
		Chromosome location		
	Exon	exon number	RefSeq	
		length		
		Transcript location		
		Sequence		
		is coding	Internal ressources	
		Chromosome location	RefSeq	
	Variation	source	RefSeq	
		Class (mutation type)		
		Events		
		Mutation effect		
		Transcript location		
		dbSNP identifier		
	CDS	Length		
		Transcript location	RefSeq	
		Reference to protein		
		Chromosome location	RefSeq	
	Sequence	primary sequence	RefSeq	
		Sequence		
Protein	Primary source		Uniprot	
	General	Official symbol	Uniprot	
		Synomym		
		Official name		
		Other names		
		Length		
		Protein existence		
	External link	Enzym id	HGNC	
		RefSeq	RefSeq	
		KEGG	Uniprot	
		InterProt		
		Prosite		
		PDB		
	General annotations	Interaction	IntAct	
		Tissues specificity	HPRD	
	Interaction		Uniprot	
	Pathway		Internal ressources	
	Ontologies	Gene Ontology	Uniprot	
	Feature annotations	amino-acid modification	Uniprot	
		post traductional modification	HPRD	Uniprot
		Sites	Uniprot	
		Regions		
		Molecule process		
		Secondary structures		
	Isoform	position	Uniprot	
		Length		
		Description		
		Sequence	Internal ressources	
	Sequence		Uniprot	

Supplementary table 2

Term	#1 vs #2	p value	Adjusted p value
autophagy (GO:0006914)	98.5%1.4%	2.51E-28	1.90E-26
death (GO:0016265)	84.6% 15.3%	5.42E-27	2.06E-25
cellular developmental process (GO:0048869)	70.7% 29.2%	1.73E-13	4.38E-12
response to stress (GO:0006950)	75.6% 24.3%	3.58E-11	6.80E-10
response to biotic stimulus (GO:0009607)	83.1% 16.8%	1.31E-10	2.00E-09
protein localization (GO:0008104)	77.6% 22.3%	2.14E-10	2.71E-09
regulation of biological process (GO:0050789)	62.8% 37.1%	2.52E-10	2.73E-09
regulation of a molecular function (GO:0065009)	81.1% 18.8%	4.20E-10	3.99E-09
cellular component organization and biogenesis	66.7% 33.3%	2.83E-09	2.39E-08
cell communication (GO:0007154)	62.9% 37.0%	7.27E-09	5.52E-08
cell cycle (GO:0007049)	72.9% 27.0%	4.33E-07	2.84E-06
response to chemical stimulus (GO:0042221)	77.4% 22.5%	4.49E-07	2.84E-06
regulation of biological quality (GO:0065008)	75.9% 24.0%	5.88E-07	3.43E-06
macromolecule metabolic process (GO:0043170)	57.1% 42.9%	2.02E-05	0.00011
establishment of localization (GO:0051234)	61.9% 38.0%	0.00014	0.00075
response to abiotic stimulus (GO:0009628)	82.2% 17.7%	0.00021	0.00101
defense response (GO:0006952)	70.2% 29.7%	0.00077	0.00347
response to external stimulus (GO:0009605)	69.8% 30.1%	0.00088	0.00375
primary metabolic process (GO:0044238)	54.5% 45.4%	0.00261	0.01044
anatomical structure development (GO:0048856)	59.7% 40.2%	0.00937	0.03563
localization of cell (GO:0051674)	69.4% 30.5%	0.01203	0.04356

Supplementary table 3

Category	Term	Count	P-Value	Fold Enrichment	FDR
GOTERM_BP_FAT	**autophagy**	34	4.10E-56	58.2	7.20E-53
GOTERM_BP_FAT	regulation of apoptosis	68	4.30E-32	5.5	7.50E-29
GOTERM_BP_FAT	regulation of programmed cell death	68	7.90E-32	5.4	1.40E-28
GOTERM_BP_FAT	regulation of cell death	68	9.80E-32	5.4	1.70E-28
GOTERM_CC_FAT	**autophagic vacuole**	15	1.70E-25	68.4	2.30E-22

Supplementary table 4

	ID	Gene symbole	Description
1	APH0001	APR2	chromosome 17 open reading frame 88 (C17orf88)(Apoptosis-related protein 2)
2	APH0002	ARSB	arylsulfatase B
3	APH0003	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
4	APH0004	GAA	glucosidase, alpha; acid (Pompe disease, glycogen storage disease type II)
5	APH0005	ITGB4	integrin, beta 4
6	APH0006	NPC1	Niemann-Pick disease, type C1
7	APH0007	SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
8	APH0008	PTEN	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
9	APH0009	RB1	retinoblastoma 1 (including osteosarcoma)
10	APH0010	TSC1	tuberous sclerosis 1
11	APH0011	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
12	APH0012	STK11	serine/threonine kinase 11
13	APH0013	ARSA	arylsulfatase A
14	APH0014	TP53	tumor protein p53
15	APH0015	TSC2	tuberous sclerosis 2
16	APH0016	BCL2	B-cell CLL/lymphoma 2
17	APH0017	FKBP1A	FK506 binding protein 1A, 12kDa
18	APH0018	P4HB	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide
19	APH0019	MAP1LC3C	microtubule-associated protein 1 light chain 3 gamma
20	APH0020	CXCR4	chemokine (C-X-C motif) receptor 4
21	APH0021	NRG3	neuregulin 3
22	APH0022	RAB24	RAB24, member RAS oncogene family
23	APH0023	CLN3	ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease)
24	APH0024	ATG9A	ATG9 autophagy related 9 homolog A (S. cerevisiae)
25	APH0025	ITGA6	integrin, alpha 6
26	APH0026	ITPR1	inositol 1,4,5-triphosphate receptor, type 1
27	APH0027	BIRC5	baculoviral IAP repeat-containing 5 (survivin)
28	APH0028	BAK1	BCL2-antagonist/killer 1
29	APH0029	CASP4	caspase 4, apoptosis-related cysteine peptidase
30	APH0030	CASP8	caspase 8, apoptosis-related cysteine peptidase
31	APH0031	ERN1	endoplasmic reticulum to nucleus signaling 1
32	APH0032	GRID2	glutamate receptor, ionotropic, delta 2
33	APH0033	HIF1	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
34	APH0034	ARNT	aryl hydrocarbon receptor nuclear translocator
35	APH0035	ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)
36	APH0036	CANX	calnexin
37	APH0037	CAPN2	calpain 2, (m/II) large subunit
38	APH0038	CAPNS1	calpain, small subunit 1
39	APH0039	CTSB	cathepsin B
40	APH0040	CTSD	cathepsin D
41	APH0041	CTSL1	cathepsin L1
42	APH0042	EEF2	eukaryotic translation elongation factor 2

Supplementary table 4

	ID	Gene symbole	Description
43	APH0043	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
44	APH0044	ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
45	APH0045	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
46	APH0046	LAMP2	lysosomal-associated membrane protein 2
47	APH0047	PIK3C3	phosphoinositide-3-kinase, class 3
48	APH0048	MAPK1	mitogen-activated protein kinase 1
49	APH0049	MAPK3	mitogen-activated protein kinase 3
50	APH0050	MAPK8	mitogen-activated protein kinase 8
51	APH0051	MAPK10	mitogen-activated protein kinase 10
52	APH0052	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
53	APH0053	PTPN2	protein tyrosine phosphatase, non-receptor type 2
54	APH0054	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
55	APH0055	RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1
56	APH0056	UVRAG	UV radiation resistance associated gene
57	APH0057	ULK1	unc-51-like kinase 1 (C. elegans)
58	APH0058	PEX3	peroxisomal biogenesis factor 3
59	APH0059	BECN1	beclin 1, autophagy related
60	APH0060	PEA-15	phosphoprotein enriched in astrocytes 15
61	APH0061	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10
62	APH0062	FADD	Fas (TNFRSF6)-associated via death domain
63	APH0063	SQSTM1	sequestosome 1
64	APH0064	ST13	suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein)
65	APH0065	ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
66	APH0066	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
67	APH0067	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
68	APH0068	DDIT3	DNA-damage-inducible transcript 3
69	APH0069	EIF2S1	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa
70	APH0070	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1
71	APH0071	RAB5A	RAB5A, member RAS oncogene family
72	APH0072	BAG3	BCL2-associated athanogene 3
73	APH0073	BAG1	BCL2-associated athanogene
74	APH0074	BAX	BCL2-associated X protein
75	APH0075	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
76	APH0076	CASP3	caspase 3, apoptosis-related cysteine peptidase
77	APH0077	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
78	APH0078	PEX14	peroxisomal biogenesis factor 14
79	APH0079	RAB7A	RAB7A, member RAS oncogene family
80	APH0080	DIRAS3	DIRAS family, GTP-binding RAS-like 3
81	APH0081	ATG12	ATG12 autophagy related 12 homolog (S. cerevisiae)
82	APH0082	HGS	hepatocyte growth factor-regulated tyrosine kinase substrate
83	APH0083	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3
84	APH0084	ATG5	ATG5 autophagy related 5 homolog (S. cerevisiae)

Supplementary table 4

	ID	Gene symbole	Description
85	APH0085	NRG2	neuregulin 2
86	APH0086	DAPK1	death-associated protein kinase 1
87	APH0087	FRAP1	FK506 binding protein 12-rapamycin associated protein 1
88	APH0088	HDAC1	histone deacetylase 1
89	APH0089	XBP1	X-box binding protein 1
90	APH0090	AKT1	v-akt murine thymoma viral oncogene homolog 1
91	APH0091	CAPN1	calpain 1, (mu/I) large subunit
92	APH0092	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
93	APH0093	LAMP1	lysosomal-associated membrane protein 1
94	APH0094	RHEB	Ras homolog enriched in brain
95	APH0095	RGS19	regulator of G-protein signaling 19
96	APH0096	HDAC6	histone deacetylase 6
97	APH0097	DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1
98	APH0098	PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit
99	APH0099	PRKCD	protein kinase C, delta
100	APH0100	PRKCQ	protein kinase C, theta
101	APH0101	ATG7	ATG7 autophagy related 7 homolog (S. cerevisiae)
102	APH0102	GNAI3	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3
103	APH0103	CAMKK2	calcium/calmodulin-dependent protein kinase kinase 2, beta
104	APH0104	HSPA8	heat shock 70kDa protein 8
105	APH0105	IL24	interleukin 24
106	APH0106	PRKDC	protein kinase, DNA-activated, catalytic polypeptide
107	APH0107	WDR45	WD repeat domain 45
108	APH0108	GABARAP	GABA(A) receptor-associated protein
109	APH0109	GABARAPL2	GABA(A) receptor-associated protein-like 2
110	APH0110	ATF6	activating transcription factor 6
111	APH0111	HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1
112	APH0112	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9
113	APH0113	EEF2K	eukaryotic elongation factor-2 kinase
114	APH0114	ATG4B	ATG4 autophagy related 4 homolog B (S. cerevisiae)
115	APH0115	NRG1	neuregulin 1
116	APH0116	CHMP2B	chromatin modifying protein 2B
117	APH0117	DAPK2	death-associated protein kinase 2
118	APH0118	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A
119	APH0119	HSPB8	heat shock 22kDa protein 8
120	APH0120	PELP1	proline, glutamate and leucine rich protein 1
121	APH0121	ERO1L	ERO1-like (S. cerevisiae)
122	APH0122	EDEM1	ER degradation enhancer, mannosidase alpha-like 1
123	APH0123	WDFY3	WD repeat and FYVE domain containing 3
124	APH0124	ATG2A	ATG2 autophagy related 2 homolog A (S. cerevisiae)
125	APH0125	WIPI2	WD repeat domain, phosphoinositide interacting 2
126	APH0126	MBTPS2	membrane-bound transcription factor peptidase, site 2

Supplementary table 4

	ID	Gene symbole	Description
127	APH0127	SH3GLB1	SH3-domain GRB2-like endophilin B1
128	APH0128	APR3	chromosome 2 open reading frame 28
129	APH0129	GRID1	glutamate receptor, ionotropic, delta 1
130	APH0130	AMBRA1	autophagy/beclin-1 regulator 1
131	APH0131	WIPI1	WD repeat domain, phosphoinositide interacting 1
132	APH0132	ATG2B	ATG2 autophagy related 2 homolog B (S. cerevisiae)
133	APH0133	DRAM	damage-regulated autophagy modulator
134	APH0134	WDR45L	WDR45-like
135	APH0135	GOPC	golgi associated PDZ and coiled-coil motif containing
136	APH0136	RAPTOR	raptor
137	APH0137	NLRC4	NLR family, CARD domain containing 4
138	APH0138	SPHK1	sphingosine kinase 1
139	APH0139	GBL	G protein beta subunit-like
140	APH0140	ATG3	ATG3 autophagy related 3 homolog (S. cerevisiae)
141	APH0141	MAP1LC3B	microtubule-associated protein 1 light chain 3 beta
142	APH0142	CAPN10	calpain 10
143	APH0143	ATG16L1	ATG16 autophagy related 16-like 1 (S. cerevisiae)
144	APH0144	TMEM49	transmembrane protein 49
145	APH0145	GABARAPL1	GABA(A) receptor-associated protein like 1
146	APH0146	ATG10	ATG10 autophagy related 10 homolog (S. cerevisiae)
147	APH0147	TMEM166	transmembrane protein 166
148	APH0148	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha
149	APH0149	ATG4C	ATG4 autophagy related 4 homolog C (S. cerevisiae)
150	APH0150	ATG4D	ATG4 autophagy related 4 homolog D (S. cerevisiae)
151	APH0151	CASP1	caspase 1, apoptosis-related cysteine peptidase
152	APH0152	ATG16L2	ATG16 autophagy related 16-like 2 (S. cerevisiae)
153	APH0153	ATG4A	ATG4 autophagy related 4 homolog A (S. cerevisiae)
154	APH0154	FKBP1B	FK506 binding protein 1B, 12.6 kDa
155	APH0155	NRG4	neuregulin 4
156	APH0156	BCL2L1	BCL2-like 1
157	APH0157	MAPK9	mitogen-activated protein kinase 9
158	APH0158	NKX2-3	NK2 transcription factor related, locus 3 (Drosophila)
159	APH0159	RICTOR	rapamycin-insensitive companion of mTOR
160	APH0160	ATG9B	ATG9 autophagy related 9 homolog B (S. cerevisiae)
161	APH0161	CHMP4B	chromatin modifying protein 4B
162	APH0162	EIF4G1	eukaryotic translation initiation factor 4 gamma, 1
163	APH0163	FOXO3	forkhead box O3
164	APH0164	PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha
165	APH0165	IRGM	PREDICTED: misc_RNA
166	APH0166	LAMP2	lysosomal-associated membrane protein 2
167	APH0167	MAPK1	mitogen-activated protein kinase 1
168	APH0168	WDFY3	WD repeat and FYVE domain containing 3

Supplementary table 4

	ID	Gene symbole	Description
169	APH0170	CAPN10	calpain 10
170	APH0171	NRG1	neuregulin 1
171	APH0172	ARSB	arylsulfatase B
172	APH0173	TSC1	tuberous sclerosis 1
173	APH0174	BCL2	B-cell CLL/lymphoma 2
174	APH0175	CAMKK2	calcium/calmodulin-dependent protein kinase kinase 2, beta
175	APH0200	APOL1	Apolipoprotein L1
176	APH0201	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1
177	APH0202	C12orf44	chromosome 12 open reading frame 44
178	APH0203	CCL2	C-C motif chemokine ligand 2
179	APH0204	CCR2	C-C chemokine receptor type 2
180	APH0205	FAS	Tumor necrosis factor receptor superfamily member 6
181	APH0206	IFNG	Interferon, gamma
182	APH0207	KIAA0226	KIAA0226
183	APH0208	KIAA0652	UPF0630 protein KIAA0652
184	APH0209	KIAA0831	KIAA0831
185	APH0210	KLHL24	Kelch-like protein 24
186	APH0211	MYC	Myc proto-oncogene protein
187	APH0212	NBR1	Neighbor of BRCA1 gene 1
188	APH0213	PARP1	Poly [ADP-ribose] polymerase 1
189	APH0214	PIK3R4	Phosphoinositide-3-kinase regulatory subunit 4
190	APH0215	PINK1	Serine/threonine-protein kinase PINK1, mitochondrial
191	APH0216	RAB33B	Ras-related protein Rab-33B
192	APH0217	RB1CC1	RB1-inducible coiled-coil protein 1
193	APH0218	SESN2	Sestrin-2
194	APH0219	SIRT1	silent mating type information regulation 2 homolog 1
195	APH0220	SIRT2	silent mating type information regulation 2 homolog 2
196	APH0221	SPNS1	spinster homolog 1 (Drosophila)
197	APH0222	TM9SF1	Transmembrane 9 superfamily member 1
198	APH0223	TMEM74	transmembrane protein 74
199	APH0224	TP53INP2	Tumor protein p53 inducible nuclear protein 2
200	APH0225	TP63	Tumor protein p63
201	APH0226	TP73	Tumor protein p73
202	APH0227	TUSC1	Tumor suppressor candidate gene 1 protein
203	APH0228	ULK2	Serine/threonine-protein kinase ULK2
204	APH0229	ULK3	Serine/threonine-protein kinase ULK3
205	APH0230	BID	BH3-interacting domain death agonist
206	APH0231	CCND3	G1/S-specific cyclin-D3
207	APH0232	PARK2	E3 ubiquitin-protein ligase parkin
208	APH0233	PTK6	Tyrosine-protein kinase 6
209	APH0234	DLC1	deleted in liver cancer 1
210	APH0235	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)

Supplementary table 4

	ID	Gene symbole	Description
211	APH0236	FAM48A	family with sequence similarity 48, member A
212	APH0237	FOS	FBJ murine osteosarcoma viral oncogene homolog
213	APH0238	FOXO1	forkhead box O1
214	APH0239	JUN	jun oncogene
215	APH0240	KIF5B	kinesin family member 5B
216	APH0241	MAPK8IP1	mitogen-activated protein kinase 8 interacting protein 1
217	APH0242	MTMR14	myotubularin related protein 14
218	APH0243	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
219	APH0244	RAB11A	RAB11A, member RAS oncogene family
220	APH0245	RAB1A	RAB1A, member RAS oncogene family
221	APH0246	RAB32	RAB32, member RAS oncogene family
222	APH0247	RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian)
223	APH0248	SAR1A	SAR1 homolog A (S. cerevisiae)
224	APH0249	USP10	ubiquitin specific peptidase 10
225	APH0250	VAMP3	vesicle-associated membrane protein 3 (cellubrevin)
226	APH0251	VAMP7	vesicle-associated membrane protein 7
227	APH0252	VEGFA	vascular endothelial growth factor A
228	APH0253	ZFYVE1	zinc finger, FYVE domain containing 1
229	APH0254	GNB2L1	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1
230	APH0255	BIRC6	baculoviral IAP repeat-containing 6
231	APH0256	CALCOCO2	calcium binding and coiled-coil domain 2
232	APH0257	CD46	CD46 molecule, complement regulatory protein
233	APH0258	CFLAR	CASP8 and FADD-like apoptosis regulator
234	APH0259	CX3CL1	chemokine (C-X3-C motif) ligand 1
235	APH0260	IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
236	APH0261	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
237	APH0262	MAP2K7	mitogen-activated protein kinase kinase 7
238	APH0263	NAF1	nuclear assembly factor 1 homolog (S. cerevisiae)
239	APH0264	NAMPT	nicotinamide phosphoribosyltransferase
240	APH0265	NCKAP1	NCK-associated protein 1
241	APH0266	NFE2L2	nuclear factor (erythroid-derived 2)-like 2
242	APH0267	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
243	APH0268	TBK1	TANK-binding kinase 1
244	APH0269	DLC1	deleted in liver cancer 1
245	APH0270	EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
246	APH0271	TP63	Tumor protein p63
247	NC_10181072		Lactate dehydrogenase-elevating virus
248	NC_10834856		Gallid herpesvirus 3
249	NC_10937870		Human parainfluenza virus 3
250	NC_10937870b		Human parainfluenza virus 3
251	NC_11072117		Echovirus 5
252	NC_11119715		Enterobacteria phage T4

Supplementary table 4

	ID	Gene symbole	Description
253	NC_11119715b		Enterobacteria phage T4
254	NC_11138120		Lelystad virus
255	NC_11138120b		Lelystad virus
256	NC_12175745		Human coronavirus 229E
257	NC_12175745b		Human coronavirus 229E
258	NC_13095680		Lactococcus phage
259	NC_13095743		Lactococcus phage
260	NC_15829254		Human adenovirus type 3
261	NC_16445223		Human adenovirus type 3
262	NC_16445223b		Human adenovirus type 3
263	NC_16445223c		Human adenovirus type 3
264	NC_17426217		Enterobacteria phage M13
265	NC_17426217b		Enterobacteria phage M13
266	NC_17736952		Pseudomonas phage phi8
267	NC_17736965		Pseudomonas phage phi8
268	NC_17975154		Vibrio phage VSKK
269	NC_9632407		Human adenovirus type 17
270	NC_9632407b		Human adenovirus type 17
271	NC_KS60_2019		Kaposi's sarcoma-associated herpesvirus
272	NC_KS60_2151		Kaposi's sarcoma-associated herpesvirus
273	NC_KS60_2156		Kaposi's sarcoma-associated herpesvirus
274	NC_KS60_2610		Kaposi's sarcoma-associated herpesvirus
275	NC_KS60_3924		Kaposi's sarcoma-associated herpesvirus
276	NC_KS60_4644		Kaposi's sarcoma-associated herpesvirus
277	PC_NM000034	ALDOA	Aldolase A, fructose-bisphosphate
278	PC_NM000402	G6PD	Glucose-6-phosphate dehydrogenase
279	PC_NM000942	PPIB	Peptidylprolyl isomerase B (cyclophilin B)
280	PC_NM000969	RPL5	Ribosomal protein L5
281	PC_NM000975	RPL11	Ribosomal protein L11
282	PC_NM000981	RPL19	Ribosomal protein L19
283	PC_NM001005	RPS3	Ribosomal protein S3
284	PC_NM001013	RPS9	Ribosomal protein S9
285	PC_NM001032	RPS29	Ribosomal protein S29
286	PC_NM001087	AAMP	Angio-associated, migratory cell protein
287	PC_NM001101	ACTB	Actin, beta
288	PC_NM001456	FLNA	Filamin A, alpha (actin binding protein 280)
289	PC_NM001916	CYC1	Cytochrome c-1
290	PC_NM001967	EIF4A2	Eukaryotic translation initiation factor 4A, isoform 2
291	PC_NM002046	GAPD	Glyceraldehyde-3-phosphate dehydrogenase
292	PC_NM003194	TBP	TATA box binding protein
293	PC_NM003200	TCF3	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)
294	PC_NM003528	HIST2H2BE	Homo sapiens histone cluster 2, H2be

Supplementary table 4

	ID	Gene symbole	Description
295	PC_NM004048	B2M	Beta-2-microglobulin
296	PC_NM005345	HSPA1A	Heat shock 70kDa protein 1A
297	PC_NM005348	HSPCA	Heat shock 90kDa protein 1, alpha
298	PC_NM005566	LDHA	Lactate dehydrogenase A
299	PC_NM006009	TUBA3	Tubulin, alpha 3
300	PC_NM006013	RPL10	Ribosomal protein L10
301	PC_NM006082	K-ALPHA-1	Tubulin, alpha, ubiquitous
302	PC_NM007363	NONO	Non-POU domain containing, octamer-binding
303	PC_NM016553	NUP62	nucleoporin 62kDa
304	PC_NM018955	UBB	Ubiquitin B
305	PC_NM030773	TUBB1	Beta tubulin 1, class VI
306	PC_X53416_3	ABP-280	Filamin 3'
307	PC_X53416_5	ABP-280	Filamin 5'

Supplementary Table 5

MetaCore™ Pathway Maps (GeneGo)

	AutophagyV2	
#	**Maps**	**pValue**
1	**Autophagy_Autophagy**	**2.47E-14**
2	Signal transduction_AKT signaling	6.54E-08
3	Transcription_Role of Akt in hypoxia induced HIF1 activation	2.03E-07
4	Apoptosis and survival_Endoplasmic reticulum stress response pathway	2.37E-07
5	Immune response_CD40 signaling	7.43E-07
6	Immune response_IL-15 signaling	7.43E-07
7	Apoptosis and survival_BAD phosphorylation	2.03E-06
8	Development_Thrombopoietin-regulated cell processes	2.88E-06
9	Development_PEDF signaling	4.42E-06
10	PGE2 pathways in cancer	7.89E-06

	Operon	
#	**Maps**	**pValue**
1	Cell adhesion_Chemokines and adhesion	8.45E-10
2	Development_WNT signaling pathway. Part 2	3.36E-08
3	Development_TGF-beta-dependent induction of EMT via SMADs	1.08E-07
4	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	1.85E-07
5	Cytoskeleton remodeling_Cytoskeleton remodeling	5.03E-07
6	Development_Regulation of epithelial-to-mesenchymal transition (EMT)	8.40E-07
7	Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK.	8.64E-07
8	Development_TGF-beta-dependent induction of EMT via MAPK	6.70E-06
9	Cell adhesion_Endothelial cell contacts by junctional mechanisms	1.13E-05
⋮	⋮	⋮
189	**Autophagy_Autophagy**	3.24E-02

All pValues < 1,2E-5 and FDR < 0.001 for brevity

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