The Selective Degradation of Synaptic Connexin-43 by Hypoxia-Induced Autophagy Impairs Natural Killer Cell-Mediated Tumor Cell Killing*

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*Running title: Cx43 modulation by hypoxia-induced autophagy in tumor cell

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Keywords: Hypoxia; autophagy; connexin-43; gap junction; natural killer; tumor immunology; melanoma

Background: The regulation of gap junction protein connexin-43 (Cx43), involved in natural killer (NK) cell-mediated tumor killing, is still elusive.

Results: Hypoxia-induced autophagy selectively degrades gap junctional Cx43 from immune synapses and impairs NK-mediated melanoma cell lysis.

Conclusion: Hypoxic microenvironment induces melanoma resistance to NK cells via modulation of Cx43 channels.

Significance: Targeting autophagy prevents gap junctional-Cx43 degradation and potentiates NK-based tumor immunotherapies.

ABSTRACT

Although natural killer cells (NK) play an important role in the control of melanoma, hypoxic stress in the tumor microenvironment may impair NK-mediated tumor cell killing by mechanisms which are not fully understood. In this study, we investigated the effect of hypoxia on the expression and the channel activity of connexin-43 (Cx43) in melanoma cells and its impact on their susceptibility to NK-mediated lysis. Our results demonstrated that hypoxic stress increases Cx43 expression in melanoma cells via hypoxia-inducible factor-1alpha (HIF-1α) transcriptional activity. Hypoxic cells displaying increased Cx43 expression were less susceptible to NK-mediated lysis compared to normoxic cells expressing moderate level of Cx43. Conversely, when overexpressed in normoxic tumor cells, Cx43 improves their susceptibility to NK-mediated killing. We showed that the NK cell immune synapse formed with normoxic melanoma cells is more stable and contains high level of gap-junctional Cx43, whereas that formed with hypoxic cells is less stable and contains significant lower level of gap-junctional Cx43. We provided evidence that the activation of autophagy in hypoxic melanoma cells selectively degrades gap-junctional Cx43 leading to the destabilization of the immune synapse and the impairment of NK-mediated killing. Inhibition of autophagy by genetic or pharmacological approaches as well as expressing of un-degradable form of Cx43, significantly restore its accumulation at the immune synapse and improve NK-mediated lysis of hypoxic melanoma cells. This study provides the first evidence that hypoxic microenvironment negatively affects the immune surveillance of tumors by NK cells through the modulation of Cx43-mediated intercellular communications.
Natural killer cells (NK) are innate lymphocytes endowed with the ability to detect and kill tumor cells. The elimination of target cells by NK largely relies on the formation of an immunological synapse at the intercellular interface (1). Given its important role in tumor regression, NK-based immunotherapies have recently emerged as promising treatments for hematopoietic malignancies and for solid tumors including melanoma (2).

Gap junctions (GJs) are channels formed of connexin (Cx) subunits that mediate direct cell-cell exchange of small molecules. GJ-mediated intercellular communication (GJIC) participates in the regulation of a plethora of cell functions, including differentiation, survival and cell death (3). Non-gap junctional Cx-hemichannels (Cx-HC) also participate in cell behaviors via the release of small mediator molecules to the extracellular medium (4). As Cxs have short half-lives, their turnover regulation and trafficking critically impact the control of GJIC (5). Connexin-43 (Cx43), the main GJ protein found in immune cells (6), has recently been shown to accumulate at the immunological synapse and allows GJIC between effector immune and tumor target cells (7-10). These observations suggest that loss of Cx43 expression during tumor progression may represent a mechanism by which cancer cells evade immune control by the local microenvironment (11).

It is well established that the tumor microenvironment supports tumor growth and limits the effectiveness of solid tumor immunotherapies by promoting neoplastic transformation, cell plasticity, and by inducing tumor cell resistance to host immunity (12,13). Accumulating data suggest that hypoxic stress in the tumor microenvironment promotes the acquisition of tumor cell resistance to NK cells (14,15) and cytotoxic T-lymphocytes attack by several mechanisms (16,17) via the induction of hypoxia-inducible factor-1α (HIF-1α) (18). Recently, we have reported 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) (19).

It has been reported that hypoxia regulates Cx43 expression and channel activity in different cell models (20,21). However, no data are currently available addressing the role of Cx43-channels in melanoma cells in the context of hypoxic microenvironment and their putative involvement in tumor cell susceptibility to NK-mediated lysis. In this study, we provide evidence that ectopic overexpression of Cx43 under normoxia in melanoma tumor cells increases their susceptibility to NK-mediated killing via the stabilization of cytolytic immune synapse. However, under hypoxic stress, although melanoma cells overexpressed Cx43 they were less susceptible to NK-mediated killing. We therefore unraveled that hypoxia-induced autophagy selectively degraded GJ-Cx43 leading to destabilization of the immunological synapse and subsequently the acquisition of resistance to NK-mediated lysis.

**EXPERIMENTAL PROCEDURES**

**Cell cultures, treatments** – The melanoma cells were established from biopsies of primary lesions (M4T, T1, I2) or from metastatic lymph nodes (M4T2, G1) as described previously (22). The M4T melanoma cells used along this study were generated from melanoma biopsy at the Hospital Clinic, Barcelona, Spain (1991-93) and defined by clinicopathological characteristic as primary melanoma. M4T displayed a loss of heterozygosity (LOH) at the microsatellite marker locus D9S265 of the chromosome 9p13-23, a region that contains genes involved in melanoma tumorigenesis (23). M4T cells express wild type BRAF. A753 and SKMel30 cell lines were from the American Type Culture Collection (ATCC) and DSMZ respectively. NK92 and MCF7 cells were obtained from ATCC. Cells were maintained in culture as previously described (7). NK were maintained in RPMI supplemented with 300U/ml recombinant human interleukin-2 (rhIL2).

Hypoxic treatments were conducted in hypoxia chambers as previously described (16). Autophagy was inhibited by addition of 50μM hydroxychloroquine (HCQ) or 5mM 3-methyladenine (3-MA) (both from Sigma-Aldrich). Cx43-channels were inhibited using Cx43-mimetic peptides (300μM), named gap27 (SRPTEKTIFII) ((10,24) (Synprosis). A scrambled peptide (TFEPIRISITK) was used as a control.
Transfections – Transfections were performed as previously described (16). Cells were stably transduced with the following plasmids: i) hCx43-pUNO1 or control (Invitrogen); ii) pcDNA3.1/Vs/His-Cx43Y286A (25); iii) pcDNA-hAATG5 or control vector (psRNA-LucGL3) (Invitrogen). Knockdowns were performed by transduction with siRNA against HIF-1α (pool of three independent target-specific siRNAs, as previously described (16,17) or p62 (all from Qiagen). Luciferase specific siRNA (Sigma-Proligo) was used as a negative control. siRNAs sequences are available under request.

Confocal microscopy – Melanoma cells were pre-cultured under normoxic or hypoxic conditions and incubated with NK at 3/1 effector/target (E/T) ratio in μ-Slide eight-well chambered coverslips (IBIDI). Time-lapse microscopy was performed as previously described (19). Cells were maintained at 37°C in a CO2 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 40x oil objective lens. Confocal microscopy of fixed melanoma/NK co-cultures was done as previously described (16). Cells were stained with polyclonal antibodies (pAb) anti-Cx43 (Sigma-Aldrich), monoclonal Ab (mAb) anti-phosphotyrosine (p-tyr) (Millipore), or with rhodamine-phalloidin (Invitrogen). Cx43 and p-tyr expression were visualized by using the secondary goat anti-rabbit (AlexaFluor-647-conjugated) and anti-mouse (AlexaFluor-546-conjugated) Abs, respectively (Invitrogen). Cells were analyzed with a Zeiss LSM-510 Meta laser scanning confocal microscope (Carl Zeiss), and images were captured with a 63x oil objective lens. The recruitment of Cx43 to the cell-to-cell contact site (immune synapses) was quantified using the ImageJ NIH software as described (10). Cx43-LC3 co-localization was analyzed using the Imaris software.

Cytotoxicity assay – The cytotoxic activity of NK was measured by conventional 4h 51Cr release assays as described (16).

Immunoblots – Cells were lysed in lysis buffer (50mM Tris, pH 7.4; 150mM NaCl; 1% Triton-X100) supplemented with protease and phosphatase inhibitor cocktails (Roche) for 30min on ice. Western blotting was conducted as previously described (17). Proteins were detected by using Abs to Cx43 (C6219; Sigma-Aldrich), HIF-1α (clone 54; BD Transduction Laboratories), HIF-2α (clone D9E3; Cell Signaling), GzmB (4275; Cell Signaling), p62 (Clone3; BD Transduction Laboratories), ATG5 (2630; Cell Signaling), and actin (clone c-11; Santa Cruz Biotechnology). Alternatively, cells were treated for 6h with 50mg/ml cycloheximide (CHX; Sigma-Aldrich), and Triton-X100 fractionation assays were performed as described (5,6).

Chromatin immunoprecipitation (ChIP) – Normoxic or hypoxic (16h) M4T cells were harvested and processed with the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer’s instructions. For HIF-1α-specific ChIP, chromatin was immunoprecipitated with 5µg mAb (BD Transduction Laboratories). The relative amounts of chromatin immunoprecipitated by the anti-HIF-1α Abs were determined by SYBR green qPCR method (Applied Biosystems) using specific primers for HIF-1α reporter and Renilla luciferase sequences downstream of the SV40 promoter. Luciferase reporter assay – A 2500-bp fragment corresponding to human GJA1 gene promoter containing HRE1-5 sequences was inserted into the Nhel–Xhol sites of pGL3-Basic vector (Promega). Mutations of HRE3 and/or HRE5 were performed by site-directed mutagenesis and verified by sequencing. M4T cells were co-transfected with 0.2µg of pGL4-hRluc/SV40 vector (which contains Renilla luciferase sequences downstream of the SV40 promoter) and 1µg of pGL3 HRE3/5 WT, pGL3 HRE3 Mut, pGL3 HRE5 Mut or pGL3 HRE3/5 Mut vectors. After 48h the cells were grown under normoxia or hypoxia for additional 24h and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter assay (Promega).

Cx43-HC activity – Cx43-HC activity was determined by ethidium bromide (EtBr; 25µM) uptake experiments using flow cytometry as described (27).

Formation and stabilization of cell conjugates analysis – Melanoma and NK92 cells were loaded with the red Dil-CM (Invitrogen) or the blue TFL4 (OncoImmuminin)
cell trackers according to the manufacturer instructions and cocultured for 10 min at 3/1 E/T ratio. The percentages of target cells conjugated with NK cells were immediately analyzed by flow cytometry. To determine the stability, cell conjugates were subjected to increasing dissociation forces by 30 sec of vortexing (low: 2, medium: 5, high: 9; Heidolph TopMix, 94323-Bioblock Scientific) and analyzed as described (28).

Flow cytometry analysis – The PE-conjugated Abs anti-CD69 (Immunotech) and AlexaFluor 488-conjugated anti-CD56 (BD Pharmingen), were used for cell staining. Flow cytometry analysis was performed using a BD Accuri™ C6 flow cytometer. Data were processed using BD Accuri software was used for acquisition, analysis, and calculation of cell counts.

NK cell-derived GzmB detection in target cells – GzmB activity was measured in TFL4 pre-stained melanoma target cells by GranToxiLux kit (OncoImmunin) according to the manufacturer’s instructions after coculture with NK cells for 1 h at a 1/3 T/E ratio, in the presence of a permeable fluorogenic substrate for GzmB. GzmB activity was evaluated in target cells (TFL4+) by flow cytometry. The level of GzmB in target cells was assessed by western blot as previously described (19).

Microarray – Gene expression was profiled using an 8 x 60K Human Whole Genome expression array (Agilent Technologies) according to the manufacturer’s instructions in the Genomics and Bioinformatics platform of Gustave Roussy Cancer Campus. Total RNA from 4 independent clones of M4T-EV and M4T-Cx43 cells were used as samples. Image analyses (quantification, normalization) were performed with Feature Extraction software (Agilent Technologies) and gene expression analysis was performed using Resolver software (Rosetta Inpharmatics). Analysis of genes differentially expressed between M4T-EV and M4T-Cx43 melanoma cells was performed with an absolute fold change > 2 and p-value <10-10.

Statistical analysis – Data were analyzed with GraphPadPrism. Statistical analyses were performed using a two-tailed Student t test or where appropriate by ANOVA. Differences were considered statistically significant when the p value was < 0.05.

RESULTS

Hypoxia increases the expression of Cx43 in melanoma cells via HIF-1α-dependent transcriptional activation – We analyzed the effect of hypoxia on the expression of Cx43 in human melanoma cells. Five human melanoma cells tested (M4T, T1, G1, I2 and M4T2) showed a clear hypoxia-time-dependent increase in Cx43 protein levels (Fig. 1A). The increase in the expression of Cx43 is correlated with the induction of HIF-1α as no difference in the expression of HIF-2α was observed in M4T cells cultured under normoxia or hypoxia (Fig. 1B). We next investigate the molecular mechanism by which hypoxia upregulates the expression of Cx43 using M4T cell line as a model. We showed that a pool of three independent siRNAs targeting HIF-1α resulted in a complete inhibition of Cx43 protein expression in hypoxic M4T cells (Fig. 1C). In silico analysis of the Cx43 promoter region revealed the presence of six putative HRE (Fig. 1D, left). ChIP data indicate that under hypoxia, HIF-1α directly interacts with HRE-3 and -5 (Fig. 1D, right). The functional interaction between HIF-1α and HRE-3 and -5 was further assessed by luciferase reporter assay using vectors encoding either wild type (WT) or mutated (Mut) HREs at indicated positions (Fig. 1E, left). Our results showed that the activity of the Cx43 promoter containing WT HREs increased upon hypoxic stress and it was not affected by the mutation of HRE-3. However, it was significantly decreased by the mutation of HRE-5. Since no additional decrease in the luciferase activity was observed by the double mutations of HRE-3 and -5, our results indicate that only HRE-5 is the functionally active cis element in the Cx43 promoter (Fig.1E, right).

Ectopic overexpression of Cx43 enhances NK-mediated tumor cell killing by stabilizing the immune synapse – It has been reported that the inhibition of Cx43-channels strongly decreased NK-mediated tumor cell lysis (10). In keeping with this, we investigated whether the overexpression of Cx43 in M4T melanoma cell model influences their susceptibility to NK-mediated lysis. Our
results confirm that Cx43 overexpressed in M4T melanoma cells (Fig. 2A, left) forms functional Cx43-HCs (Fig. 2A, right) and that M4T melanoma cells overexpressing Cx43 (M4T-Cx43) were significantly more susceptible to NK-mediated lysis as compared to M4T cells transfected with control empty vector (M4T-EV) (Fig. 2B). The increased susceptibility of M4T-Cx43 to NK cell killing was no longer observed when the cells were treated with gap27 mimetic peptide (specific Cx43 inhibitor) (Fig. 2B) suggesting that, in M4T cell model, NK-mediated killing is enhanced by the Cx43 expression at the surface of target cells. Similar results were obtained by overexpressing Cx43 in other melanoma cell lines (T1, M4T2) and in the breast cancer cell line MCF7 (Fig. 2C upper and lower panels). In addition, the increased susceptibility of M4T-Cx43 cells to NK-mediated lysis was associated with a higher accumulation of Cx43 at the immunological synapse between NK/target as demonstrated by p-tyr staining (Fig. 2D), and this was not associated with an increase in their ability to form conjugate with NK as no significant difference in the conjugate formation was observed between NK and either control or Cx43 overexpressing M4T cells (Fig. 2E). However, Cx43 overexpressing cells form more stable NK/melanoma conjugates compared to control cells (Fig. 2F). Furthermore, the expression of the NK activation marker CD69 was increased at the surface of NK cells when co-cultured with Cx43 overexpressing melanoma cells (Fig. 2G). This result indicates that NK cells become more active when co-cultured with target cells displaying higher expression levels of Cx43. In line with this data, we showed that Cx43 positive melanoma cells (Cx43+) displayed higher level of NK-derived active GzmB as compared to Cx43 negative melanoma cells (Cx43-). The level of active GzmB in target cells seems to be strikingly related to the expression of Cx43, as the Cx43-mimetic peptide gap27 dramatically decreases GzmB activity only in Cx43+ target cells (Fig. 2H). Moreover, it is unlikely that the overexpression of Cx43 increases the tumor cell susceptibility to NK-mediated killing by regulating the global gene expression profile (Fig. 2I), or by regulating NK activating (MIC-A/B, ULBP1, ULBP2, ULBP3) and inhibitory (MHC class I) ligand expression on the surface of target cells (data not shown).

Altogether these results show that overexpression of Cx43 in melanoma cells resulted in an increase in their susceptibility to NK-mediated lysis most likely due to a mechanism involving the stabilization of cytolytic immunological synapse.

**Hypoxia-dependent expression of Cx43 is associated to a decrease in melanoma cell susceptibility to NK-mediated killing** – Our results described above predict that hypoxic M4T cells, which express high levels of Cx43, must be more susceptible to NK-mediated killing as compared to normoxic M4T cells. Surprisingly, hypoxic melanoma cells were significantly less susceptible to NK-mediated lysis as compared to normoxic cells (Fig. 3A). This paradox role of Cx43 overexpressed, either ectopically or under hypoxic stress, in NK-mediated killing susceptibility raises an important issue of whether Cx43 overexpressed on hypoxic cells forms functional Cx43-HCs. We therefore evaluated the activity of Cx43-HCs in normoxic and hypoxic M4T cells by EtBr uptake. As depicted in Fig. 3B, an increase in the EtBr uptake was observed in hypoxic cells, and incubation of these cells with the Cx43-inhibitor mimetic peptide (gap27) significantly decreased the EtBr uptake. These results provide evidence that hypoxia-dependent induction of Cx43 expression contributes to the formation of functional Cx43-HCs in hypoxic melanoma cells.

Our results showed that despite the increased level of Cx43 protein expression and the formation of functional Cx43-HC, the NK-mediated killing of hypoxic cells was impaired as compared to that of normoxic M4T cells. This impairment was not related to a decreased expression of NK activating (MIC-A/B, ULBP1, ULBP2, ULBP3) and inhibitory (MHC class I) ligands on the surface of M4T-Cx43 cells (data not shown).

We next assessed the expression of CD69 as NK cell activation marker. We observed a moderate but statistically significant increase in the expression of CD69 at the surface of NK cells co-cultured during 4 hours with normoxic tumor cells when compared to its basal expression level of NK cells cultured alone (Fig. 3C). Surprisingly, the increase in the expression of CD69 at the
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surface of NK cells co-cultured with hypoxic tumor cells remained very low, or even not significant, after 4 hours of co-culture. This result indicates that NK cells become slightly more active when co-cultured with normoxic target cells as compared to their activity when they are co-cultured with hypoxic target cells. Nevertheless, the induction of CD69 in NK cells remains low. We therefore speculate that this could be related to the time point that we have used to measure the expression of CD69 rather than to an impairment in the activity of NK cells. Indeed, recent report by Tran et al. (29) provided evidence that a high level of lymphocytes depict CD69 expression after 6 hours of stimulation, peaked at 24 hours and down regulated after 48 hours.

**Hypoxia decreases the accumulation of Cx43 at the immune synapse formed between melanoma and NK cells** – We next investigated whether hypoxic stress affects the localization of Cx43 at the cytolytic immunological synapse between NK and M4T cells where it is supposed to stabilize the synapse and improves NK-mediated lysis. While Cx43 is primarily detected at the immune synapse formed between normoxic M4T and NK cells, its localization at the synapse formed between hypoxic M4T target and NK cells was dramatically decreased (Fig. 3D). The reduction in Cx43 immune synapse localization correlated with the formation of i) less stable immune synapses characterized by a decrease in both actin cytoskeleton polarization (Fig. 3D) and p-tyr staining (Fig. 3E), ii) fewer conjugates with NK cells (Fig. 3F) and iii) lower level and less activity of NK-derived GzmB (Fig. 3G upper and lower panels, respectively). The hypoxia-mediated decrease in the immunological synapsis localization of Cx43 was also observed in other melanoma cell lines such as A735 and SKMel30 cells (Fig. 3H).

Taken together, these results strongly argue that the defect in the localization of Cx43 at the immunological synapse between hypoxic target and NK cells could be responsible for the impairment of melanoma cell susceptibility to NK-mediated killing.

**Hypoxia-induced autophagy promotes the degradation of gap junctional-Cx43 at the NK/melanoma immune synapse** – Recent evidence has demonstrated that autophagy contributes to the turnover of the gap junctional-Cx43 (GJ-Cx43) (5,30). We therefore analyzed whether the activation of autophagy in hypoxic melanoma cells is involved in a selective decrease in GJ-Cx43 expression at the immune synapse. Fig. 4A showed that hypoxic M4T cells displayed an increased number of autophagosomes and decreased expression of p62 (Fig. 4B), indicating that the autophagy flux is activated in M4T cells under hypoxia. This was supported by our data showing that the number of LC3 positive puncta per cell was increased following treatment of hypoxic cells with the autophagy inhibitor HCQ (Fig. 4C).

We next evaluated the impact of inhibiting autophagy on the localization of the melanoma Cx43 at the immune synapse formed between NK and normoxic or hypoxic target cells. Fig. 4D showed a significant decrease in the localization of Cx43 at the immune synapse of hypoxic M4T/NK cells, which was rescued by both HCQ treatment (Fig. 4D) or by targeting ATG5 (Fig. 4E). Indeed, M4T stably expressing siRNA against ATG5 showed 80% of ATG5 protein inhibition, and this was correlated with an increase in the expression of p62, indicating that the autophagy flux was efficiently blocked (Fig. 4E right panel). Taken together, our results strongly argue that hypoxia-induced autophagy could be responsible for a selective degradation of GJ-Cx43 localized at the immune synapse. Consistent with this, we showed a co-localization of Cx43 and LC3 at the proximity of the immune synapse of hypoxic M4T/NK cells, which was abrogated following HCQ or 3MA treatment (Fig. 4F, G and H).

To further assess the effect of hypoxia-induced autophagy on the selective degradation of GJ-Cx43 localized at the immune synapse, we analyzed the accumulation of GJ-Cx43 isolated from normoxic or hypoxic M4T/NK cell cocultures untreated or pretreated with cycloheximide. Previous reports have been shown that GJ-Cx43 is enriched in TritonX-100 insoluble fraction, whereas non-GJ-Cx43 is enriched in TritonX-100 soluble fraction (26). Our results (Fig. 4I left panel) showed that even under hypoxic conditions, where TritonX-100 soluble Cx43 was upregulated, the TritonX-100 insoluble GJ-Cx43 was dramatically down-regulated most likely by an autophagy-
dependent degradation manner. Similarly, in cycloheximide-treated hypoxic cells, a selective decrease in the accumulated GJ-Cx43 was observed, which was rescued by HCQ treatment or by targeting ATG5 (Fig. 4I right panel). However, no significant difference in the non-GJ-Cx43 (intracellular Cx43 and Cx43-HCs) was observed under the same conditions. Overall, these results indicate that hypoxia accelerates the removal of Cx43 at the NK/target immune synapse by a selective degradation of GJ-Cx43 through an autophagy-dependent mechanism.

**Inhibition of autophagy-mediated GJ-Cx43 degradation in hypoxic melanoma cells restores their susceptibility to NK-mediated lysis** – We next assessed how autophagy selectively degrades GJ-Cx43. Indeed, autophagy has long been viewed as a random cytoplasmic degradation system. However, emerging new evidences suggest that autophagy could be a selective degradation process (31). The discovery and characterization of the autophagic adapter protein cargos have provided mechanistic insight into this process. The most common of these cargo recognition molecules is p62 (32). p62 is able to act as cargo receptor for degradation of ubiquitinated substrates. A direct interaction between the autophagic adapter p62 and the autophagosomal marker protein LC3, mediated by a so-called LIR (LC3-interacting region) motif is required for efficient selective autophagy. To investigate whether GJ-Cx43 is selectively degraded by autophagy through p62-dependent manner, we targeted p62 by siRNA and analyzed the expression of GJ-Cx43 at the immunological synapse of hypoxic melanoma cells. Our results clearly showed that targeting the cargo protein p62 (Fig. 5A left panel) inhibited the degradation of the synaptic GJ-Cx43 in hypoxic M4T melanoma cells co-cultured with NK cells (Fig. 5A right panel). These results indicate that p62 is required for the selective degradation of GJ-Cx43 by autophagy.

To further support our data showing that hypoxia-induced autophagy is responsible for a selective degradation of synaptic GJ-Cx43, we used an endocytic-impaired form of Cx43 (Cx43Y286A) mutated in the tyrosine sorting signal. Cx43Y286A was previously described to be undegradable by the autophagy machinery (5,25). We first showed that Cx43Y286A was localized at the immune synapse formed between NK and either normoxic or hypoxic M4T cells, where it is supposed to stabilize the synapse and improve NK-mediated lysis. Our results (Fig. 5B left panel) clearly showed that Cx43Y286A variant was still present at the immune synapse of hypoxic cells indicating that Cx43Y286A resists to autophagy mediated degradation. We next assessed the NK-mediated lysis of hypoxic cells expressing Cx43Y286A. Our results provided clear evidence that, similar to targeting ATG5, the expression of Cx43Y286A in hypoxic M4T cells significantly restored their susceptibility to NK-mediated lysis. Cx43Y286A was functionally involved in the restoration of NK-mediated lysis of hypoxic target cells as such restoration was no longer observed when the Cx43 mimetic peptide gap27 was used (Fig. 5B right panel). Taken together, our results strongly argue that blocking autophagy in hypoxic M4T cells restores NK-mediated killing mainly by preventing the degradation of GJ-Cx43.

**DISCUSSION**

Impaired expression and function of Cxs have been observed in different tumor tissues and cell lines, including melanoma (11,33). Recent data strongly suggest that Cx43 may act as a tumor suppressor gene and predicts the clinical outcomes in cancer patients treated with chemotherapy (34). Indeed, several reports have demonstrated that Cx43 expression increased the sensitivity of tumor cells to apoptosis through GJ-channel- and HC-dependent and independent manners (35). Thus, GJ-channels allow the passage of apoptotic signals such as Ca\(^{2+}\) or IP\(_3\) (36).

Several reports support the prominent role for Cx43-mediated intercellular communications in different immunological processes, including tumor immunity (6,37,38). In line with this, we showed that ectopic overexpression of Cx43 in normoxic melanoma cells stabilizes the immune synapse formation and improves NK-mediated killing through the formation of GJ-Cx43 between NK and target cells. Under hypoxic stress, Cx43 is overexpressed but its localization at the immune synapse is dramatically impaired by autophagy. Such impairment leads to the destabilization of the immune synapse and to tumor escape from NK-mediated killing. Given
that normoxic melanoma cells formed stable conjugates and that hypoxic cells, displaying a defect in the GJ-Cx43, formed less conjugates with NK than normoxic M4T-Cx43 cells, it is tempting to speculate that Cx43 localized at the immunological synapse may contribute to cell adhesion between NK and their targets, as previously described in endothelial cell/B lymphocyte interactions (39).

In response to hypoxia, the transcription factor HIF-1α is rapidly stabilized and translocated to the nucleus, where it binds to HREs motif (5’-RCGTTG-3’) to induce the transcription of many critical genes to sustain cell survival under hypoxic stress (18). Here, we identified that HIF-1α induces Cx43 expression in M4T melanoma cells through the binding to the promoter of the Cx43 gene (GJA1). In line with this result we showed that hypoxia induces the expression of Cx43 in M4T and several melanoma cells including T1, G1, I2 and M4T2. Similar to M4T, there is a hypoxia time-dependent increase in the expression of Cx43 in G1 and I2 cells. However in T1 cells the expression of Cx43 was gradually increased after 24 and 48 hours and reached plateau at 72 hours of hypoxia. In the case of M4T2 cells, we observed an increase in Cx43 after 24 hours of hypoxia and then its level decline slightly after 48-72 hours, although it remains higher than in normoxia. As suggested from our results, it seems that the levels of Cx43 in melanoma cells exposed to chronic hypoxia depends on the balance between its expression by HIF-1α and its degradation through autophagy. Moreover, Cx43 has an intrinsic short half-life (1-3 hours), which can vary in different cell lines.

Although the relationship between hypoxic stress and Cxs is not well documented in tumor cells, it has been proposed that the expression of Cx46 may protect breast cancer cells from hypoxia-induced cell death (40). Indeed, the role of Cx43 in hypoxia has been well described in other cell types; for instance Cx43 is a key mediator of cell protection during ischemia/hypoxia in cardiomyocytes (41).

It has been proposed that human melanoma cells are addicted to functional autophagy for their survival and invasion (42). In this regard, we have previously reported that the activation of autophagy under hypoxic conditions resulted in the degradation of NK-derived GzmB in tumor cells, thus compromising the ability of NK to eliminate their targets in vitro and in vivo (19). In this study we demonstrated that autophagy impairs NK-mediated killing of hypoxic melanoma cells through an additional mechanism involving immune synapse destabilization by selective degradation of GJ-Cx43 localized at the NK/melanoma interface.

Recently, several lines of evidence highlighted the role of autophagy in the selective degradation of the pool of Cx43 in plasma membrane GJ plaques (5,30). Briefly, using Cx43-GFP-expressing Hela cells, it has been reported that the GJ-Cx43 is internalized in structures called annular GJs, which colocalized with autophagosomes. The inhibition of autophagy resulted in an increase in total levels of exogenously expressed Cx43 and a significant increase in cytoplasmic annular GJs. Using mouse liver tissue and NIH3T3 cells, Bejarano et al (5) confirmed the degradation of GJ-Cx43 by autophagy and highlighted the role for ubiquitination in the selective targeting of the GJ-Cx43 to starvation-induced autophagosomes. Furthermore, they demonstrated that ubiquitin ligase Nedd4-mediated ubiquitination and the subsequent Eps15-mediated endocytosis were required for Cx43 autophagic degradation. Moreover, it has been shown that a mutation in the PY motif (Y286) of Cx43 blocked its autophagic degradation. Indeed, Cx43 carrying such a mutation fails to interact with Nedd4 (25) and therefore prevents the subsequent interaction with Eps15 (5). In the current study we strongly argue that a similar mechanism of autophagy-mediated degradation occurs during NK/target interactions in hypoxic conditions. In support of this, we showed that hypoxia did not affect the accumulation of the Cx43-Y286A mutants at the NK/M4T cytolytic immune synapses.

Together the results reported here provide new insight into the role of Cx43 in regulating melanoma susceptibility to NK-mediated lysis and could therefore lead to new therapeutic approaches that involve manipulation of Cx43 expression. Our data also suggest that strategies targeting autophagy in combination with compounds that selectively enhance Cx43 expression in tumor cells (43,44) would significantly enhance the therapeutic efficacy of NK-based melanoma immunotherapies.
Acknowledgments: We thank Dr. Henrique Girão (University of Coimbra, Portugal) for providing the Cx43Y286A pcDNA3.1/V5/His vector.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: AT and BJ: designed the study, performed and analyzed the experiments shown in all figures. BJ and SC: wrote the paper. KVM and MZN: performed and analyzed the experiments. SC: conceived and coordinated the study. All authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES
*This project was supported by funding from the European Union Seventh framework Program (FP7/2007-2013) under grant agreement No.246556 (European project RBUCE-UP) and by grants from INSERM; Université Paris-Sud; Luxembourg Institute for Health (L.I.H) LHCE-2013-1105 and Fondation Cancer Luxembourg (FC/2012/02).

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3The abbreviations used are: ChIp, Chromatin immuno-precipitation; CHX, cycloheximide; Cx43, connexin-43; Cx-HC, connexin hemichannel; EtBr, ethidium bromide; EV, empty vector; GJ, gap junction; GJ-Cx43, gap junctional-Cx43; GJIC, GJ-mediated intercellular communication; GzmB, granzyme B; HCQ, hydroxychloroquine; HRE, hypoxia responsive elements; HIF, hypoxia-inducible factor; MHC, major histocompatibility complex; MIC, MHC class I-related chain; NK, natural killer cell; p-tyr, phosphotyrosine; 3-MA, 3-methyladenine; ULBP, UL16-binding protein.
FIGURE LEGENDS

FIGURE 1. Hypoxic stress increases the expression of Cx43 by transcriptional activation through HIF-1α. A, B. Cells were incubated under normoxia (N) or hypoxia (H) at indicated time (in panel B: 24 hours). The expression of Cx43, HIF-1α, HIF-2α (in panel B only), and β-actin in indicated melanoma cell lines (M4T, T1, G1, I2, and M4T2) were determined by western blot and Cx43/Actin ratio were quantified by Image J software. C. M4T Cells were incubated under normoxia (N) or hypoxia (H) in the absence (-) or presence (+) of a pool of three independent HIF-1α-specific siRNAs. The expression of HIF-1α, Cx43 and actin was assessed by western blot and HIF/Actin and Cx43/Actin ratio were quantified by Image J software. D. (Left) Schematic representation of the Cx43 (GJA1) promoter. The positions of six putative HREs are shown. (Right) ChIP was performed in M4T cells incubated under normoxia (N) or hypoxia (H) using anti-HIF-1α antibodies. The HIF-1α ChIP/Input DNA ratios for the HRE-3 and -5 are shown. E. (Left) Sequence of HRE-5 and HRE-3. The mutation positions in HRE-5 and HRE-3 are indicated by arrowheads. (Right) M4T cells were transfected with pGL3 vectors encoding wild type (WT) or mutated (Mut) HRE-3, HRE-5 or both (3-5) and grown under normoxia (N) or hypoxia (H). After 24h, firefly and Renilla luciferase activities were measured. The luciferase activity is reported as fold change relative to N. (***, p<0.001).

FIGURE 2. Ectopic overexpression of Cx43 in melanoma cells improves their susceptibility to NK-mediated lysis. A. (Left) M4T melanoma cells were transfected with empty vector (EV) or vector encoding Cx43. The overexpression of Cx43 was determined by western blot. (Right) The uptake of EtBr by M4T-EV or -Cx43 cells was determined in the presence of control (-) or Cx43 inhibitor peptide gap27 (+). The percentage of EtBr+ cells was reported. B. The percentage of NK-mediated lysis of M4T-EV or -Cx43 cells. Cells were co-cultured with NK92 cells at 10/1 (10) or 20/1 (20) effector/target (E/T) ratio in the presence of control (Ctrl) or Cx43 inhibitor peptide gap27. C. (Upper) Melanoma cell lines T1, M4T2 and the breast cancer cell line MCF7 were stably transfected with EV or vector encoding Cx43. Cx43 and β-actin protein levels were determined by western blot. (Lower) NK-mediated cytotoxicity was determined at a 10/1 E/T ratio. D. M4T-EV or -Cx43 cells were cocultured with NK92 cells. NK/target (T) conjugates were analyzed by phase contrast (PC) microscopy. Cx43 and p-tyr accumulation at the immune synapse is indicated by white arrows. Scale bar, 5 μm. E, F. M4T-EV or -Cx43 cells were loaded with Dil-CM and cocultured for 10 min with TFL4-loaded NK92 cells at a 3/1 E/T ratio. The percentages of target cells conjugated with NK cells were assessed by flow cytometry (E). F. Cell conjugates were subjected to increasing dissociation forces (vortex set-ting: 1: low, 2: medium, 3: high velocity) for 30 sec. The percentages of target cells conjugated with NK cells were reported as percentage relative to the maximum (no vortex). G. NK92 cells were cocultured for 4h with M4T-EV or -Cx43 cells. The expression of CD69 on the surface of NK cells was determined by flow cytometry. H. M4T cells were transfected with the Tomato fused to Cx43 (Tom-Cx43). Cells were labeled with TFL4 and cocultured with NK92 cells for 1h at a 3/1 E/T ratio in the presence of a permeable fluorogenic substrate for GzmB (GranToxiLux). GzmB activity was evaluated on TFL4*Tom-Cx43 or TFL4*Tom-Cx43* M4T cells by flow cytometry. The bars graph shows the percentage of GranToxiLux positive target cells cocultured with NK92 cells in the presence of control (-) or gap27 peptides (+). I. Volcano plots of gene expression (log2 fold change, FC) and adjusted p values for M4T cells stably transfected with Cx43 versus the control EV. The data were obtained by analyzing the gene expression profile of four independent clones per condition. (* p<0.05; **p<0.01; ***p<0.001).

FIGURE 3. Hypoxia decreases both the accumulation of Cx43 at the immune synapse and the NK-mediated lysis of melanoma cells. A. M4T cells were incubated 24h under normoxia (N) or hypoxia (H). The percentage of NK92-mediated cytotoxicity was determined at 10 to 1 effector/target (E/T) ratio. B. Hemi-channel activity was evaluated in M4T cells cultured under normoxia (N) or hypoxia (H) during 24h in the presence of control (-) or Cx43 inhibitor peptide gap27 (+). The activity was reported as a percentage of maximum. C. NK92 cells were cocultured for 4h with N or H M4T cells at a 3/1 E/T ratio. CD69 surface expression was determined by flow
Cx43 modulation by hypoxia-induced autophagy in tumor cell

cytometry. D. (Left) M4T-Cx43 cells were incubated under normoxia (N) or hypoxia (H) conditions for 24h and cocultured with NK92. Conjugates between NK and target cells were analyzed by phase contrast (PC) microscopy. F-actin (rhodamine-phalloidin) and Cx43 were stained and analyzed by fluorescence microscopy. The accumulation of Cx43 at the immune synapse (IS) is indicated by arrow. Scale bar: 5 µm. (Right) Quantification of F-actin and Cx43 accumulation at the IS.

E. The expression of HIF-1α (NK) was used as a control for GzmB detection. (Lower) Quantification of the number of GFP-Cx43 positive autophagosomes (LC3+). puncta per cell was quantified in M4T-Cx43 cells cultured under normoxia (N) or hypoxia (H) for 24h under normoxia (N) or hypoxia (H) for 24h in the absence or presence of 50µM HCQ. D. M4T-GFP-Cx43 cells were cultured under normoxia (N) or hypoxia (H) for 24h in the absence or presence of 50µM HCQ. Cells were cocultured with NK92 and the localization of Cx43 was evaluated. Immune synapses between target (T) and NK are indicated (arrows); scale bar, 10 µm.

E. (Left) M4T cells expressing Tom-Cx43 (Red) were transfected with GFP-control plasmid or GFP-siRNA plasmid targeting ATG5 (siATG5). Cells were pre-cultured 24h under hypoxia (H) and subsequently cocultured with NK92 cells and the localization of Cx43 at the IS is indicated by white arrows. (Upper) The expression of HIF-1α and LC3-I and –II (as autophagy marker) was determined by western blot. (Lower) A735 and SKMel30 melanoma cells were expressing GFP-Cx43 were subsequently cocultured with NK92 cells and the localization of Cx43 at the IS is indicated by white arrows. was analyzed by confocal microscopy. Enlarged (X 200) regions (boxes) are shown on the right. (*p<0.05; **p<0.01; ***p<0.001).

FIGURE 4. Hypoxia-induced autophagy impairs Cx43 expression at the immune synapse between hypoxic melanoma cells and NK cells. A. The formation of autophagosomes was analyzed by confocal microscopy analysis in Tomato-LC3 expressing M4T-Cx43 cells cultured for 24h under normoxia (N) or hypoxia (H). Scale bar, 10 µm. B. The expression of HIF-1α, p62 and β-actin in normoxic (N) or hypoxic (H) M4T-Cx43 cells were determined by western blot. C. The number of LC3+ puncta per cell was quantified in M4T-Cx43 cells cultured under normoxia (N) or hypoxia (H) for 24h in the absence (C) or presence of 50µM HCQ. D. M4T-GFP-Cx43 cells were cultured under normoxia (N) or hypoxia (H) for 24h in the absence or presence of 50µM HCQ. Cells were cocultured with NK92 and the localization of Cx43 was evaluated. Immune synapses between target (T) and NK are indicated (arrows); scale bar, 10 µm.

E. (Left) M4T cells expressing Tom-Cx43 (Red) were transfected with GFP-control plasmid or GFP-siRNA plasmid targeting ATG5 (siATG5). Cells were pre-cultured 24h under hypoxia (H) and cocultured for 15min with NK92 cells. Cx43 localization at the immune synapse is indicated by an arrowhead. Scale bar, 10 µm. (Lower) ATG5 and p62 expression was assessed by western blot in normoxic (N) or hypoxic (H) M4T-Tom-Cx43 cells stably transfected with psiRNA-hATG5 (psiATG5) or control (Ctrl) empty vectors. The ATG5/actin and p62/actin ratios were calculated by densitometric quantification of the blot. F, G and H. M4T-GFP-Cx43 cells were transfected with Tom-LC3 (Red) and cultured under normoxia (N) or hypoxia (H) in the absence or presence of HCQ (F-H) or 3MA (G, H). Melanoma cells were then cocultured with NK92 and the colocalization of Cx43 with LC3+ autophagosomes was analyzed by confocal microscopy. Enlarged images of the immune synapses (IS; box) are shown below each representative image. Cx43-containing autophagosomes are indicated (arrows). PC: phase contrast images; scale bar: 10 µm. G. The percentage of Cx43 colocalized with LC3 was evaluated over the time. H. Quantification of the number of GFP-Cx43 positive autophagosomes (LC3+) at the IS proximity (2 µm). I. Hypoxia-induced autophagy selectively degrades GJ-Cx43 in hypoxic melanoma cells. (Left) M4T-Cx43 cells were incubated under normoxic (N) or hypoxic (H) conditions for 24h, and subsequently cocultured with NK92 cells for 30 min. Triton insoluble (TX100-ins) or soluble (TX100-sol) fractions were analyzed by immunoblot for Cx43 detection. The TX100-sol fraction (middle panel) resolves predominately a faster-migrating isoform (P0) that represent non-junctional Cx43 (which is largely unphosphorylated). The TX100-ins fraction (upper panel) resolves at least three distinct Cx43 isoforms: the unphosphorylated P0 and two (P1 and P2) phosphorylated isoforms. (Right upper): M4T-Tom-Cx43 cells transfected with control (C) or

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siRNA-plasmids targeting ATG5 (siATG5) were incubated under normoxia (N) or hypoxia for 24h in the absence or presence of HCQ. Cells were treated with protein synthesis inhibitor cycloheximide for additional 6h, and subsequently cocultured with NK92 cells for 30 min. TX100-sol or TX100-ins fractions were processed for immunoblot against Cx43 and actin. (Right lower) Densitometric quantification of the hypoxia-induced Cx43 degradation in TX100-sol or TX100-ins fractions. Values are reported as % of Cx43 levels relative to normoxic conditions. (**p<0.01).

FIGURE 5. Prevention of autophagy-mediated degradation of Cx43 in hypoxic melanoma cells restores their susceptibility to NK-mediated lysis. A. Role of cargo protein p62 in selective degradation of GJ-Cx43 in hypoxic M4T melanoma cells. M4T cells expressing Tomato-Cx43 protein were transfected with control (C) or p62 siRNA (p62) and cultured under hypoxia. The expression of HIF-1α and p62 was assessed by western blot (left panel). Cells were cocultured with NK92 cells at 5/1 effector/target ratio and the accumulation of synaptic Cx43 (indicated by arrowheads) was monitored by confocal microscopy. T: Target cell; Bar: 10 μm. B. (Left) M4T cells were stably transfected with Cx43\textsuperscript{Y286A}. Cells were incubated under normoxia or hypoxia for 24h and cocultured with NK92 cells for 15min. Cx43 accumulation at the immune synapse (IS; arrows) was analyzed by confocal microscopy and quantified (Left lower panel). PC: phase contrast images; scale bar, 5 μm. (Right) M4T melanoma cells expressing wild type form of Cx43 (columns 1-3) were transfected with control (C) or plasmid encoding ATG5 siRNA (siATG5), as well as with Cx43\textsuperscript{Y286A} (columns 4 and 5). Cells were incubated under normoxia (N) or hypoxia for 24h. NK92-mediated cytotoxicity was determined in the presence of control (-) or inhibitory Cx43-mimetic peptide gap27 (+). NK-mediated lysis values are showed as percentage of the maximum (***, p<0.001).
Figure 5

A

Hypoxia

\[ \text{C} \quad \text{p62} \]

- HIF-1α
- p62
- Actin

Control

Hypoxia

siRNA p62

Cx43

NK

B

Normoxia

Hypoxia

PC

Cx43Y286A

NK-mediated lysis (% of the maximum)

0 25 50 75 100 125

0 0.5 1 1.5 2

Cx43Y286A at the IS (Ratio)

siATG5

gap27

Hypoxia

*** *** ***

*** ***
The Selective Degradation of Synaptic Connexin-43 by Hypoxia-Induced Autophagy Impairs Natural Killer Cell-Mediated Tumor Cell Killing
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J. Biol. Chem. published online July 28, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M115.651547

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