Gap Junction Intercellular Communications Regulate NK Cell Activation and Modulate NK Cytotoxic Capacity


J Immunol published online 27 December 2013
http://www.jimmunol.org/content/early/2013/12/26/jimmunol.1301297

Supplementary Material http://www.jimmunol.org/content/suppl/2013/12/26/jimmunol.1301297.DCSupplemental

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Gap junctions (GJs) mediate intercellular communication between adjacent cells. Previously, we showed that connexin 43 (Cx43), the main GJ protein in the immune system, mediates Ag transfer between human dendritic cells (DCs) and is recruited to the immunological synapse during T cell priming. This crosstalk contributed to T cell activation, intracellular Ca\(^{2+}\) responses, and cytokine release. However, the role of GJs in NK cell activation by DCs and NK cell–mediated cytotoxicity against tumor cells remains unknown. In this study, we found polarization of Cx43 at the NK/DC and NK/tumor cell-contact sites, accompanied by cytokine release. Moreover, blocking Cx43 strongly inhibited the NK cell–mediated tumor cell lysis associated with inhibition of granzyme B activity and Ca\(^{2+}\) influx. Our data identify a novel and active role for Cx43-GJIC in human NK cell activation and antitumor effector functions that may be important for the design of new immune therapeutic strategies. The Journal of Immunology, 2014, 192: 000–000.
process and mediated through the formation of a functional IS (18). Moreover, NK-mediated cytotoxicity of target cells largely relies on the formation of functional IS (20). Once an IS is formed, NK cells can induce apoptosis in the target cells by releasing their cytotoxic granules, including granzyme B (GrB) (21). Importantly, Ca2+ influx in target cells is required for the effective internalization of perforin and GrB and for immune-mediated death by apoptosis (21). In the current study, we describe for the first time, to our knowledge, that Cx43, the main GJ of the immune system (21), accumulates at the contact zone of NK cells and DCs or at the interface between NK cells and target cells, facilitating DC-mediated NK cell activation and cytotoxic activity against tumor cells.

Materials and Methods

Cell lines, generation of DCs, and NK cell purification

This study was approved by the Bioethical Committee of Human Research, Faculty of Medicine, University of Chile. Me1 and Me3 are human melanoma cell lines established from metastatic lymph nodes biopsies at the Institute of Biomedical Sciences, University of Chile (22). The myelogenous leukemia cell line K562 was purchased from American Type Culture Collection. The cells were grown at 37°C in an atmosphere with 5% CO2 in RPMI 1640 culture medium (Invitrogen) supplemented with 10% FBS, penicillin (100 U/L), streptomycin (100 mg/ml), and 1 mM L-glutamine (all from Invitrogen).

Leukocytes from healthy donors were isolated by density gradient using Ficoll-Hypaque (AxisShield). Human DCs were obtained as described (10). At day 6, DCs were stimulated overnight with 100 ng/ml melanoma cell lysate named TRIMEL, which was obtained as previously described (23), and with 2 ng/ml recombinant human TNF-α (all from Invitrogen).

Resting (r)NK cells were purified by negative selection from PBMCs using the NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. Purity of the isolated CD3−CD56+ NK cells was >90% according to routinely performed flow cytometry analysis. NK cells were activated overnight (IL-2–activated NK cells [aNK cells]) with 500 U/ml recombinant human IL-2 (ProSpec-Tany TechnoGene).

Inhibition of Cx43 channels and GJs

DCs, NK cells, and tumor cells were pretreated as follows: 4 h with 40 μM Cx43 antisense (Cx43-AS) human oligodeoxynucleotide (ODN) (sequence: 5′-gTAc CGc AaA gAa gATg TgT CgC TC-3′) or 40 μM Cx43 sense (Cx43 s) ODN (24); and 30 min with 300 μM 1848-mimetic peptide (sequence: CNTQQPGCVENVC; extracellular loop 1; >98% purity), 300 μM gap20 control peptide (sequence: EIKKFKYGIEEHC; cytoplasmic loop; >98% purity; both from Chiron Peptides), or 50 μM 18-β-glycyrrhetinic acid (β-GA; Sigma-Aldrich). For incubations lasting >4 h, the ODNs and mimetic peptides were added every 4 h.

Abs and flow cytometry analysis

Flow cytometry experiments were performed as previously described (10). To detect Cx43, a rabbit polyclonal anti-human Cx43 Ab, directed to the C-terminal domain (C6219; Sigma-Aldrich), plus a secondary donkey anti-rabbit FITC-conjugated Ab (Poly4064; BioLegend) were used. The following mAbs were used for cell staining: anti-CD11c (clone 3.9), anti-CD3 (clone OKT3), anti-CD56 (clone CMSSB), anti-CD69 (clone FN50), anti-CD25 (clone BC96), anti-CD107a (clone HA34; all from eBioscience), anti-macrophage inflammatory protein-1β (MIP-1β, D21-1351; BD Biosciences), and LFA-1 (clone 24B2, 25B12; Abcam). To simultaneously evaluate active LFA-1, CD107a surface expression, and intracellular MIP-1β expression on PBMCs cocultured or not with K562 cells, we performed a multiparameter flow cytometry analysis as previously described (26). Samples were acquired on a FACSCalibur (BD Biosciences) or a CyAn ADP LX 9-color flow cytometer (DakoCytomation) and analyzed using the software Cytlogic (version 1.2.1; CyFlow) or FlowJo (version 8.8.6; Tree Star).

Immune fluorescence staining, confocal microscopy, and quantitative image analysis

Purified rNK or aNK cells were cocultured with autologous iDCs or TRIMEL–stimulated mDCs for 30 min or with K562, Me1, or Me3 tumor cells prestained with the fluorescent dye CM-Dil (15 μg/ml) according to the manufacturer’s indications (Invitrogen) at a ratio of 3:1 for 0, 10, 30, and 60 min. The cell conjugates were gently washed with PBS twice and fixed with 4% parafomaldehyde for 30 min. After gentle washing with PBS, the cells were incubated in ammonium chloride (50 μM) for 10 min. Then the cells were permeabilized for 10 min (0.5% Triton X-100 and 0.5% BSA) and blocked with 0.5% BSA. We allowed the conjugates to adhere to poly-t-lysine–coated slides (Sigma-Aldrich), and the cell mixture was incubated with the PE-conjugated anti-CD11c (clone 3.9; eBioscience), APC-conjugated anti-CD56 (clone CMSSB; eBioscience) mAbs, and anti-Cx43 polyclonal Abs (Cd219; Sigma-Aldrich) overnight at 4°C. Cx43 expression was visualized by using the secondary donkey anti-rabbit FITC-conjugated Ab (Poly4064; BioLegend); additionally, cells were also stained with 5 μg/ml Hoechst 33342 (Invitrogen) and mounted using DAKO fluorescence mounting medium (DakoCytomation). Cells were analyzed by confocal microscopy (LSM 510; 363 numerical aperture 1.4 oil immersion objective; Carl Zeiss).

The recruitment of Cx43 to the cell-contact site was quantified using the ImageJ NIH software and quantified as the ratio of the Cx43 mean fluorescence intensity (MFI) in the cell-contact site versus the Cx43 MFI in the same area but in the opposite side of the cell-contact site. Between 40 and 60 NK cell–DCs or NK cell–target cell conjugates were analyzed on ∼30 fields in at least three experiments. Two independent investigators evaluated the data. The fluorescence emission intensity was displayed on a pseudocolor scale (16 colors) using the Image J software (National Institutes of Health).

Calcein-acetoxymerthoxy and GJ detection

GJ IC was measured using calcein transfer assay as previously described (27). DCs, isolated NK cells, K562, or melanoma cells were loaded with calcein-acetoxymerthoxy (AM) (1 μM; Sigma-Aldrich) or the plasma membrane red marker CM-Dil (15 μg/ml; Invitrogen) for 30 min at 37°C according to the manufacturer’s instructions. The membrane-permeable calcein-AM is hydrolyzed by intracellular nonspecific esterase, and the resulting green fluorescent hydrophilic calcein is then trapped inside the cells. GJs are permeable to calcein but not to CM-Dil. Dil-stained cells were cocultured with calcein-stained cells for different times and at different ratios in the presence or absence of the Cx43 inhibitors. After cocultures, the cells were collected and analyzed by flow cytometry.

IFN-γ ELISPOT assay

Isolated rNK cells were cocultured with autologous TRIMEL-stimulated mDCs for 12 h at a ratio 10:1 in the presence or absence of 300 μM Cx43-mimetic peptides 1848 and gap20. IFN-γ release was tested by ELISPOT assay according to the manufacturer’s instructions (Mabtech).

^{51}Cr release assay

The cytotoxic activity of isolated aNK cells was measured by conventional 4-h [{^{51}Cr}] (PerkinElmer) release assays using triplicate cultures in round-bottom 96-well plates in the presence or absence of the different Cx43 inhibitors. E:T cell ratios were as indicated, on 5000 target cells/well. Specific lysis was calculated according to the formula: percent specific lysis = [(experimental release − spontaneous release)/maximum release−spontaneous release] × 100.

Measurement of GrB activity

Isolated aNK and K562 cells were preincubated or not with 300 μM Cx43-mimetic peptide 1848 or control peptide gap20. GrB activity was measured by GranToxiLux kit (OncoImmobilin) according to the manufacturer’s protocol. Labeled K562 cells were cocultured with NK cells for 1 h at a 1:3 ratio, in the presence of a permeable fluorogenic substrate for GrB. GrB activity was evaluated in K562 cells (TFL4^CD56^-) by flow cytometry.

Measurement of intracellular calcium

K562 or Me3 cells were loaded with 5 μM Fluor-4 AM (Invitrogen) according to the manufacturer’s protocol. Intracellular Ca^{2+} levels were measured by flow cytometry in target cells cocultured with aNK cells at a 1:3 ratio at different time points as previously described (28). Cells were incubated with the Cx43 antisense 1848 or control peptide gap20 before and during the cocultures. Fluor-4 AM fluorescence intensity (F1/F0) intensity was plotted as a function of time.

Results

NK cells and DCs communicate through functional Cx43-mediated GJs

Previously, we demonstrated that Cx43 accumulates in the IS during DC-mediated priming of CD4^+ T cells, promoting lymphocyte
FIGURE 1. NK cells and DCs communicate through functional Cx43-mediated GJs. (A) The distribution of Cx43 was analyzed using a polyclonal anti-Cx43 Ab plus a secondary FITC-conjugated mAb by confocal microscopy 30 min after coincubation of autologous iDC-rNK cells or mDC-rNK cells. The cell-contact site is indicated with a red arrow, and the opposite site is indicated with a white arrow. Images are representative of three independent experiments. The right panel shows the Cx43 accumulation at the cell-contact site, which was measured as the ratio of the Cx43 MFI at the cell-contact site versus the opposite site and was evaluated in the different cocultures. Values are reported as mean ± SD of three independent experiments. Scale bar, 5 μm. (B) Isolated aNK cells were preloaded with calcein-AM and cocultured for 50 min with autologous iDCs or TRIMEL-stimulated mDCs preloaded with Dil. The calcein transfer from the NK cells to the DCs was assessed by flow cytometry. The numbers in the dot plots represent percentage of Dil+Calcein+ cells. The bar graph shows Dil+Calcein+ cells as a percentage of the maximum (mDCs); n = 3 independent experiments. (C) Isolated aNK cells were preloaded with calcein and cocultured for 50 min with autologous TRIMEL-stimulated mDCs preloaded with Dil. Cells were pretreated for 4 h with the Cx43-AS or the Cx43 s. Percentages of Dil+Calcein+ cells are indicated. The bar graph shows Dil+Calcein+ cells (as a percentage of the maximum [control (Ctrl)]); n = 3 independent experiments. **p < 0.01, ***p < 0.001.

Cx43 channels are required for DC-mediated NK cell activation

Human NK cells are activated by mature monocyte-derived DCs (16, 30). To investigate whether Cx43-mediated intercellular communication is involved in mDC-mediated activation of autologous rNK cells, CD69 and CD25 surface expression was evaluated in NK cells cocultured with mDCs in the presence of the Cx43-mimetic peptide 1848, which blocks docking between adjacent Cx43 Hchs (14). Induction of CD69 and CD25 on NK cells was strongly inhibited after treatment with the 1848 Cx43-mimetic peptide (Fig. 2A, 2B). In contrast, incubation with the activation through the formation of functional GJs and increased intracellular Ca2+ signaling in T cells (14). Given that the reciprocal activation of DCs and NK cells is a cell contact–dependent process and shares some mechanisms with the DC-mediated T cell priming such as the formation of a functional IS (18), we aimed to examine whether Cx43-GJ channels may be involved in DC–NK intercellular communication. In line with previously published data (6), positive Cx43 expression was observed in NK cells, and the expression levels were similar to rNKs and aNKs, respectively (Supplemental Fig. 1A). Cx43 levels were increased in mDCs compared with nonstimulated iDCs (Supplemental Fig. 1B), as previously reported (10, 11). The Cx43 cellular distribution was then investigated by confocal microscopy in conjugates of iDCs or mDCs with autologous rNK cells. Cx43 was found to preferentially accumulate in the interface between mDCs and the rNK cells, but was homogeneously distributed when rNK cells were incubated with iDCs (Fig. 1A).

GJ-mediated bidirectional communication between cells of the immune system has been previously described (7, 14). The establishment of bidirectional GJIC between DCs and NK cells was monitored using a calcein transfer assay. iDCs or mDCs were loaded with the GJ nondiffusible dye CM-Dil (Fig. 1B, 1C) or the GJ diffusible dye calcein-AM (Supplemental Fig. 1C) and cocultured for 50 min with autologous rNK cells loaded with calcein-AM or CM-Dil, respectively. There was an ∼2-fold increase in calcein-AM dye transfer from mDCs to rNK cells compared with the transfer from iDCs (Fig. 1B). This result is consistent with the augmented Cx43 expression levels in mDCs (Supplemental Fig. 1B). Inhibition of Cx43 by a Cx43-AS ODN that targets Cx43 expression in NK cells and DCs (Supplemental Fig. 2) blocked the calcein-AM transfer from rNK cells to mDCs, confirming that Cx43 is required for functional GJIC between these immune cells (Fig. 1C). Similar results were obtained using β-GA, which inhibits GJ formation by dephosphorylation of Cxs (29) (Supplemental Fig. 1C). These results demonstrate a role for Cx43 in mediating bidirectional intercellular communication between NK cells and mDCs.

FIGURE 2. Cx43 GJIC is required for DC-mediated NK cell activation. (A and B) Autologous mDCs and rNK cells were cocultured for 20 h with NK cells (in a 3:1 ratio of NK/DCs), in the presence or absence of the Cx43-mimetic peptide 1848 or the control peptide gap20. CD69 (A) and CD25 (B) surface expression was determined in the CD3+ CD56+ NK cell population by flow cytometry and plotted as fold induction, relative to the level of NK cells alone (A) or as percentage of the maximum expression (B). Data correspond to the mean ± SD from three independent experiments. (C) Autologous TRIMEL-stimulated mDCs and rNK cells were cocultured for 12 h at a 10:1 ratio of NK cells/DCs in the presence or absence of the Cx43-mimetic peptide 1848 or the control peptide gap20. The secretion of IFN-γ was assessed by ELISPOT. Results correspond to the mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

Human NK cells are activated by mature monocyte-derived DCs (16, 30). To investigate whether Cx43-mediated intercellular communication is involved in mDC-mediated activation of autologous rNK cells, CD69 and CD25 surface expression was evaluated in NK cells cocultured with mDCs in the presence of the Cx43-mimetic peptide 1848, which blocks docking between adjacent Cx43 Hchs (14). Induction of CD69 and CD25 on NK cells was strongly inhibited after treatment with the 1848 Cx43-mimetic peptide (Fig. 2A, 2B). In contrast, incubation with the
control gap20 peptide did not affect CD69 or CD25 expression levels (Fig. 2A, 2B). Similarly, treatment of mDCs and NK cells with the Cx43-mimetic peptide 1848 severely impaired IFN-γ secretion by NK cells (Fig. 2C). Although Cx43-GJIC between NK cells and DCs was bidirectional (Fig. 1, Supplemental Fig. 1), Cx43-mediated interaction between NK and DCs did not affect surface expression of different markers on DCs, including MHC class I and II, the maturation molecules CD83 and CD86, and NK-associated MICA and MICB molecules known to act as key ligands for NKG2D and promote NK cell–mediated recognition and cytolysis (31). Moreover, Cx43 blocking or silencing affected neither the expression of these markers (Supplemental Fig. 3A, 3B) nor the NK-induced TNF-α or IL-12 release by iDCs or activated DCs (Supplemental Fig. 3C, 3D). Finally, Cx43-mimetic peptides neither affect NK cell viability nor DC maturation state (Supplemental Fig. 2).

Cx43 accumulates at site of contact between NK cells and tumor cells, which contributes to NK cell–mediated cytotoxic activity against tumor cells

FIGURE 3. Cx43 accumulates at the site of cell contact during the killing of NK cell–sensitive tumor cell lines. (A) Representative images of Cx43 distribution (green) 30 min after coculture of aNK and K562 cells prestained with Dil (red). A series of images (1–7) obtained by serial optical sections of the same cells at 1-μm thickness along the z-axis are shown. Blue: Hoechst nuclear staining. The cell-contact site is indicated with a red arrowhead, whereas the opposite site is indicated with a white arrowhead. (B) Cx43 accumulation at the cell-contact site, depicted in pseudocolor, in aNK cell and K562 conjugates. Bottom panel shows Hoechst nuclear staining. Scale bars in (A) and (B), 5 μm. (C) Cx43 accumulation at the cell-contact site was measured as the ratio of the Cx43 MFI at the cell-contact site versus at the opposite site and was evaluated at different times of coculture between aNK cells with K562 cells. Values are reported as mean ± SD of three independent experiments. (D) A pseudocolor image shows Cx43 accumulation at the cell-contact site formed between aNK and Mel3 cells 60 min after coculture. The black and white picture depicts nuclear Hoechst staining. Scale bar, 5 μm. (E) Cx43 accumulation at the cell-contact site formed between aNK cells and Mel3 cells was measured as described in (C). Values are reported as the mean ± SD of three independent experiments. (F) aNK cells and K562 cells communicate through functional Cx43 channels. Calcein transfer assays were carried out after 10 min of coculture of isolated aNK cells (labeled with calcein) and K562 cells (labeled with Dil) pretreated or not with the Cx43-mimetic peptide 1848 or gap20. The percentage of Dil+Calcein+ cells was measured as described in (F) and corresponds to the results from three independent experiments. These data are comparable with results obtained with Mel3 as target cells. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. Cx43-mediated intercellular communication contributes to NK cell cytotoxic activity against tumor cells. (A) IL-2–activated aNK cells were cocultured with K562 cells at different E:T ratios and preincubated with Cx43 and GJ inhibitors or their respective controls. Cytotoxicity was assessed by conventional [51Cr] release assays. The results are plotted as a percentage of specific lysis and are representative of at least three independent experiments and comparable with those obtained in the melanoma cell lines (Mel1 and Mel3) (Fig. 3D, 3E and data not shown). Moreover, NK cells and target cells (K562 and Mel3 cells) formed bidirectional coupling through Cx43 channels, which was effectively reduced by blocking Cx43-GJIC (Fig. 3F, Supplemental Fig. 4A).

Both Cx43 Hchs as well Cx43-GJs play an important role in the intercellular communication of death signals (32). However, an
important and unexplored aspect of these Cx43 channels is whether they play a role in NK cell–mediated cytotoxicity. Using conventional $^{51}$Cr release assays, we observed that NK cell cytotoxic activity against tumor cells was significantly reduced following treatment with Cx43-AS, the Cx43-mimetic peptide 1848, or the GJ inhibitor β-GA (Fig. 4A, 4B). Overall, inhibition of GJs and specific inhibition of Cx43 reduced NK cell–mediated tumor lysis by ~50% (Fig. 4B).

In contrast to Hch-based signaling, communication via GJ channels requires expression of Cx proteins in both donor and recipient cells. To further analyze how Cx43 regulates NK cell cytotoxic activity, Cx43 expression was silenced using Cx43-AS only in NK cells, only in K562 cells, or in both. Knockdown of Cx43 in NK cells resulted in a significant reduction of NK cytotoxic activity (Fig. 4C). However, cytotoxic activity was dramatically impaired when Cx43 expression was targeted in K562 cells only, or in both NK and K562 cells (Fig. 4C), probably due to the fact that the Cx43 knockdown was more effective in K562 cells than in NK cells (Supplemental Fig. 2B). Taken together, these findings reveal that Cx43 accumulation at the site of interaction between NK cells and tumor cells appears to regulate NK cell–mediated cytotoxic activity against tumor cells.

Cx43-mediated intercellular communication contributes to an efficient GrB activity and calcium influx in target cells during the NK cell attack

GrB is a proapoptotic serine protease that plays a crucial role in NK cell–mediated cytotoxicity (21, 33, 34). Following the recognition of target cells and activation, NK cells release mature GrB specifically in the IS, from where it enters target cells in cooperation with perforin, rapidly inducing their death. In order to assess the contribution of GJs to this process, GrB activity was examined in control (treated with gap20 peptide) or Cx43-deficient (treated with the Cx43 mimetic peptide) K562 cells. Significantly lower intracellular GrB activity was detected in K562 cells cocultured with NK cells following inhibition of Cx43 (Fig. 5A). This finding suggests that Cx43 channels may somehow contribute to the efficient GrB activity in the target cells during NK cell–mediated cytotoxicity. The engagement of activating receptors at the lytic IS site during target cell recognition induces the so-called inside-out signals that prompt an extracellular conformational change in LFA-1. This integrin switch from a closed to an open, extended conformation facilitates ligand binding and target cell adhesion (35) and in turn can support LFA-1–dependent signals for cytoxic granule polarization, maturation, and NK-mediated cytotoxicity (36). Inhibition of Cx43-mediated intercellular communication during tumor recognition by NK cells did not affect LFA-1 active conformational change in NK cells (Fig. 5B). CD107a (LAMP-1)-deficient cells have reduced levels of perforin in lytic granules and disturbed motility of the lytic granules, which leads to the inability to deliver apoptosis-inducing GrB to target cells and to the inhibition of NK cell cytotoxicity (36, 37). We evaluated CD107a expression on the surface of NK cells; however, no difference in CD107a levels were observed upon blockade of Cx43-GJs in NK cells and K562 cells (Fig. 5C). This suggests that the reduced cytotoxicity observed following GJ blockage was not due to reduced degranulation by NK cells. Furthermore, inhibition of Cx43-mediated intercellular communication during tumor recognition by NK cells did not affect CD69 or MIP-1β expression levels in NK cells (Supplemental Fig. 4B, 4C).

During NK cell–mediated cytotoxicity, a transient Ca$^{2+}$ influx is induced in the target cells, which is necessary for the apoptosis

**FIGURE 5.** Cx43-mediated GJC contributes to efficient GrB activity and calcium influx in target cells during the NK cell–mediated cytotoxicity of tumor cells. (A) aNK and K562 cells were pretreated or not with the Cx43-mimetic peptide 1848 or the control peptide gap20. K562 cells were labeled with TFL4 and cocultured with the aNK cells for 1 h at an E:T ratio of 3:1 in the presence of a permeable fluorogenic substrate for GrB. GrB activity was evaluated in K562 cells (TFL4+CD56$^+$) by flow cytometry. Histograms depict one of three representative experiments. The graph represents the average percentage of GS$^+$ K562 cells ± SD of three independent experiments. (B) PBMCs preincubated or not with the Cx43-mimetic peptide 1848 or the control peptide gap20 were cocultured or not for 2 h with K562 cells at a 1:1 ratio. Active conformation LFA-1 expression was determined by multiparameter flow cytometry on CD3$^+$ CD56$^+$ NK cells. The bars represent the average values of the MFI ± SD of three independent experiments. (C) PBMCs preincubated or not with the Cx43-mimetic peptide 1848 or the control peptide gap20 were cocultured or not with K562 cells at a 1:1 ratio. CD107a surface expression was determined on CD3$^+$ CD56$^+$ NK cells. Bars represent the average MFI ± SD of three independent experiments. (D) Intracellular Ca$^{2+}$ levels were measured by flow cytometry in Fluor-AM–preloaded target cells incubated for different time points with aNK cells at an E:T ratio of 3:1. Cells were treated with the Cx43-mimetic peptide 1848 or the control peptide gap20. Fluor-AM fluorescence ratio ($F_{T}/F_{0}$) intensity was plotted as a function of time. The data are represented as the results from three different experiments using K562 as target cells. $^p < 0.05$, $^{**p} < 0.001$. Ctrl, Control.
triggered by the endocytosed GrB (21, 37, 38). It is known that Cx43 regulates Ca\textsuperscript{2+} influx in various cell types (39), and we have previously reported that Cx43 regulates Ca\textsuperscript{2+} signaling at the IS in T cells (14). Calcium signaling impacts on the granule trafficking pathways through the regulation of Ca\textsuperscript{2+}-responsive proteins to facilitate the rapid movement and fusion of secretory granules with the plasma membrane and is essential for degranulation events in NK cells (40). Additionally, Cx43 channels can mediate the intercellular transfer of death signals (32), and previous evidence suggests that Ca\textsuperscript{2+} could be a major cell death messenger passing through Cx43 channels (41). In order to evaluate whether GJ contribution to GrB cytotoxicity could be facilitated by Cx43-mediated regulation of Ca\textsuperscript{2+} signaling, intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the 1848 mimetic peptide significantly impaired intracellular Ca\textsuperscript{2+} signaling, in intracellular Ca\textsuperscript{2+} levels were evaluated in Fluo4-AM preloaded target cells cocultured with control and Cx43-deficient NK cells. Inhibition of Cx43 by the...
monophosphate is a key component of regulatory T cell-mediated suppression. J. Exp. Med. 204: 1303–1310.
Supplementary Data

**Supplemental Fig. 1: NK cells and DCs communicate through functional GJs.**

**A)** Cx43 expression was analyzed by flow cytometry in resting or IL-2-activated CD3^+CD56^+ NK cells (rNK or aNK, respectively). Grey histogram corresponds to isotype-matched control.

**B)** Cx43 expression was assessed in iDCs and TRIMEL-stimulated mDCs. Percentages of CD11c^+Cx43^+ cells are indicated. One of three representative experiments is shown.

**C)** Isolated IL-2 activated NK cells were pre-loaded with Dil and co-cultured for 50 min with autologous mDCs pre-loaded with calcein at a ratio 3:1 (NK cell:DC). Cells were pre-treated 30 min with the GJ blocker β-GA (50 μM). Bars graph shows Dil^+Calcein^+ cells (as a percentage of the maximum number of cells - Ctrl); n=3 independent experiments; (**) p<0.01.
Supplemental Fig. 2: Treatments with a Cx43-AS ODN greatly decreases Cx43 expression in DCs, NK cells and K562 cells, and with Cx43-mimetic peptides do not affect the viability or the maturation level of NK cells or mDCs, respectively. A, B) Cells were treated for 4 h with 40 μM of Cx43-AS or 40 μM control Cx43-s. Cx43 protein levels were measured by flow cytometry within the CD11c⁺ population in mDCs (A), aNK cells (B, left), or K562 cells (B, right). C) Isolated aNK cells were incubated for 8 h with 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. The percentage of dead cells was determined by propidium iodide (PI) staining and analyzed by flow cytometry. D) iDCs and TRIMEL stimulated-mDCs were incubated for 16 h with 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. The surface expression levels of MHC I and MHC II were determined by flow cytometry. Dot-plots are representative of 3 independent experiments; the numbers correspond to the average mean fluorescence intensity in CD11c⁺MHC II⁺ cells.
Supplemental Fig. 3: Cx43 is not required for NK cell-mediated DC activation. A)
iDCs or mDCs were co-cultured with rNK or aNK cells for 16 h at a 3:1; (DCs:NK cells)
Cells were pre-incubated or not for 4 h with 40 μM of Cx43-AS or Cx43-sense control. The MHC I, MHC II, CD83 and CD86 expression levels in the CD11c+ cells were determined by flow cytometry and showed as fold increase relative to the iDC level (n = 3 independent experiments). 

B) iDCs or mDCs were co-cultured with aNK cells for 16 h at a 3:1; (DCs:NK cells) ratio. Cells were treated or not with 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. The percentages of CD11c+MICA+ and CD11c+MICB+ cells were determined by flow cytometry. Data are representative for two independent experiments. 

C, D) iDCs or mDCs were co-cultured with aNK cells for 16 h at a 3:1; (DCs:NK cells) ratio. Cells were treated or not with 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. The percentages of CD11c+TNF-α+ (A) and CD11c+IL-12+ cells were determined by flow cytometry; n = 6 (C), n = 3 (D) independent experiments; (* p<0.05; ** p<0.01).
Supplemental Fig. 4: Inhibition of Cx43-mediated intercellular communication during tumor recognition by NK cells did not affect CD69 or MIP-1β expression on NK cells.

A) NK cells and Mel3 melanoma cells communicate through functional GJs. Isolated aNK cells were pre-loaded with Dil and co-cultured for 10 min with Mel3 cells pre-loaded with calcein at a (3:1) ratio (NK cells:Mel3 cells). Cells were pre-treated with 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. Percentages of Dil⁺Calcein⁺ cells are indicated. B) Isolated rNK or aNK cells were co-cultured or not for 4 h with K562 cells at a 3:1 effector:target cell ratio, in the presence of 300 μM of the Cx43-mimetic peptide 1848 or the control peptide gap20. CD69 surface expression was determined by flow cytometry in CD3⁻CD56⁺ NK cells. The histogram represents the result for a single aNK cells staining; bars represent the average values of the mean fluorescence intensity ± SD of three independent experiments. C) PBMCs pre-incubated or not with the Cx43-mimetic peptide 1848 or the control peptide gap20 were co-cultured or not for 2h with K562 cells at a 1:1 ratio. Intracellular MIP-1β expression was determined by multiparameter flow
cytometry on NK cells. The bars represent the average values of the mean fluorescence intensity ± SD of three independent experiments.