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Regulatory Dendritic Cells Restrain NK Cell IFN- γ Production through Mechanisms Involving NKp46, IL-10, and MHC Class I–Specific Inhibitory Receptors

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Cross-talk between mature dendritic cells (mDC) and NK cells through the cell surface receptors NKp30 and DNAM-1 leads to their reciprocal activation. However, the impact of regulatory dendritic cells (regDC) on NK cell function remains unknown. As regDC constrain the immune response in different physiological and pathological conditions, the aim of this work was to investigate the functional outcome of the interaction between regDC and NK cells and the associated underlying mechanisms. RegDC generated from monocyte-derived DC treated either with LPS and dexamethasone, vitamin D3, or vitamin D3 and dexamethasone instructed NK cells to secrete lower amounts of IFN- γ than NK cells exposed to mDC. Although regDC triggered upregulation of the activation markers CD69 and CD25 on NK cells, they did not induce upregulation of CD56 as mDC, and silenced IFN- γ secretion through mechanisms involving insufficient secretion of IL-18, but not IL-12 or IL-15 and/or induction of NK cell apoptosis. Blocking experiments demonstrated that regDC curb IFN- γ secretion by NK cells through a dominant suppressive mechanism involving IL-10, NK cell inhibitory receptors, and, unexpectedly, engagement of the activating receptor NKp46. Our findings unveil a previously unrecognized cross-talk through which regDC shape NK cell function toward an alternative activated phenotype unable to secrete IFN- γ , highlighting the plasticity of NK cells in response to tolerogenic stimuli. In addition, our findings contribute to identify a novel inhibitory role for NKp46 in the control of NK cell function, and have broad implications in the resolution of inflammatory responses and evasion of antitumor responses. *The Journal of Immunology*, 2015, 195: 2141–2148.

Natural killer cells are key players during innate responses against virus-infected cells and tumors, displaying cytotoxic activity and producing substantial amounts of IFN- γ and other proinflammatory cytokines during the development of immune responses against such insults. In addition, NK cells shape the adaptive immune response by interacting with dendritic cells (DC), promoting DC maturation, Th1 polarization,

and CTL responses (1, 2). Such cross-talk between DC and NK cells requires engagement of NKp30 and DNAM-1 and secretion of cytokines, including IL-12, IL-15, IL-18, and IFN- γ (3–7). In addition, NK cells display effector functions upon recognition of target cells through activating receptors such as CD16 (which mediates Ab recognition of target cells); NKG2D; the natural cytotoxicity receptors NKp46, NKp44, and NKp30; DNAM-1; 2B4; members of the killer Ig-like receptor (KIR) family that carry a short cytoplasmic tail (KIR2DS and KIR3DS); and others (8–10).

Myeloid DC exhibit a high degree of plasticity, as they can differentiate into fully mature DC (mDC) or become regulatory DC (regDC) under some circumstances (11–13), a functional characteristic that is accompanied by the expression of particular costimulatory molecules (14, 15). mDC have been shown to stimulate IFN- γ secretion by NK cells in a NKp30- and DNAM-1–dependent manner (3, 4), resulting in a reciprocal activation of both cells. Such cross-talk amplifies the adaptive immune response as NK cells endow DC with the ability to promote Th1- and CTL-mediated effector functions (1–3, 16). In addition, regDC are relevant for tumor progression (17–19) and for the resolution of immune responses (20). Several approaches to generate regDC have been developed to harness the immune response in different pathologic conditions such as autoimmune diseases (21) and in allogeneic organ transplantation (22), highlighting their potential therapeutic value. Known effects of regDC include the induction of T cell anergy due to delivery of defective costimulation to CD4⁺ and CD8⁺ T cells and expansion of regulatory T cells (23). regDC can be generated by a variety of immunoregulatory molecules, including IL-10 (24, 25), TGF- β (26), vasoactive intestinal peptide (27), dexamethasone (DEX) (28, 29), vitamin D3 (VitD3) (28), and galectin-1, a glycan-binding protein

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Abbreviations used in this article: DC, dendritic cell; DEX, dexamethasone; FC, flow cytometry; iDC, immature DC; KIR, killer Ig-like receptor; mDC, mature DC; regDC, regulatory DC; VitD3, vitamin D3.

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that triggers IL-27 production and promotes the generation of IL-10-secreting Tr1 cells (20, 30–32). Moreover, the heterogeneity of regDC-inducing agents reflects the fact that regDC might not represent a specialized DC subset, but instead might encompass a functional state of DC differentiation influenced by particular environmental factors (13–15).

Although the impact of regDC on the T cell compartment has been well established, their possible effects on NK cell physiology remain unknown. In this study, we present evidence that indicates that regDC restrain IFN- γ secretion by NK cells through mechanisms involving insufficient production of IL-18, secretion of IL-10, engagement of MHC class I-specific inhibitory receptors, and, unexpectedly, the activating receptor Nkp46. Our results contribute to unravel the mechanisms that regulate the interactions between human regDC and NK cells at the crossroad of innate and adaptive immunity and unveil a previously unanticipated function for Nkp46 in the regulation of NK cell activation.

Materials and Methods

Abs and reagents

Human rIL-12 and rIL-15 were from PeproTech; human rIL-18 was from MBL International; human rGM-CSF and LPS (*Escherichia coli* 0111:B4 strain) were from Sigma-Aldrich; and human rIL-4 was from BD Pharmingen. The following mAb against human molecules were used: unlabeled anti-NKG2D (clone 1D11; eBioscience); PE anti-Nkp46 (9E2; BioLegend, San Diego, CA); PE anti-NKG2A (R&D Systems), AlexaFluor488 anti-NKG2C (R&D Systems); FITC anti-CD69 (FN50; BD); PE anti-CD25 (BC96; eBioscience); PE anti-T-bet (4B10; eBioscience); PE anti-Helios (22F6; BioLegend); PE anti-IL-18R α (H44; BioLegend); PE anti-IL-18R β (R&D Systems); anti-Nkp30 (clone AZ20); anti-Nkp46 (clone BAB281) (provided by A. Moretta, Laboratory of Molecular Immunology, Department of Experimental Medicine, University of Genoa, Genoa, Italy); fluorochrome-labeled and unlabeled isotype-matched control mAb (IC; eBioscience or BioLegend); PE anti-CD3 (clone UCHT-1; eBioscience); PE/Cy5 anti-CD56 (clone N901; Beckman Coulter); FITC anti-CD14 (clone HCD14; BioLegend); FITC anti-HLA-DR (clone L243; BioLegend); PE anti-CD86 (clone IT2.2; BioLegend); FITC anti-CD83 (clone HB15e; BioLegend); PE anti-CD1a (clone HI149; eBioscience); anti-MHC class I (clone W6/32, purified from hybridoma culture supernatants); anti-IL-10 (clone JES3-9D7; BD); and anti-TGF- β (clone 9016; R&D Systems). DEX and VitD3 were from Sigma-Aldrich, and Zombie Green was from BioLegend. The anti-Gal-1 (F8G7) mAb was generated, as described (33).

DC and NK cells

Monocytes and NK cells were isolated from blood from healthy volunteers (provided either by the Blood Bank of the Province of Buenos Aires, La Plata, Argentina, or by the Service of Transfusion Medicine, of the Hospital Churrucavisca, Buenos Aires, Argentina). Monocytes were isolated by immunomagnetic selection of CD14⁺ cells using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). NK cells were isolated using the RosetteSep NK cell enrichment mixture (StemCell) and Ficol-Paque Plus (Amersham Biosciences) centrifugation. Purity of isolated cells was always >90%, as assessed by flow cytometry (FC) as CD14⁺ or CD3⁻CD56⁺ cells. Monocytes were cultured for 6 d with GM-CSF and IL-4 to obtain immature DC (iDC). To obtain mDC, iDC were cultured for 5 or 24 h with 0.1 μ g/ml LPS (*E. coli* 0111:B4 strain; Sigma-Aldrich). To obtain regDC, iDC were cultured for 5 or 24 h with 0.1 μ g/ml LPS and 5 nM DEX (regDC^{DEX}), 10 nM VitD3 (regDC^{VitD3}), or 5 and 10 nM VitD3 (regDC^{DEX+VitD3}). DC were cultured with NK cells at a 1:1 ratio for 18 h, in the absence or in the presence of blocking/neutralizing mAb in RPMI 1640 (Life Technologies) supplemented with 10% inactivated FBS (Life Technologies), sodium pyruvate, glutamine, and gentamicin (Sigma-Aldrich). In some experiments, NK cells were cultured for 24 h with 10 ng/ml IL-12, 1 ng/ml IL-15, and 10 ng/ml IL-18 in the absence or in the presence of DEX. Cell cultures were used for analysis of apoptosis, cytokine production, and cell surface expression of specific receptors. Studies have been approved by the institutional review committee of the Institute of Biology and Experimental Medicine. To obtain conditioned media, DC were washed and cultured for up to 24 h with fresh medium, and cell culture supernatants were stored at -70°C until used.

ELISA for cytokines

Secretion of IFN- γ by human NK cells was analyzed by ELISA, as described (34). Detection of IL-12 and IL-10 was performed using the

ELISA MAX Standard kits from BioLegend, and detection of IL-18 was performed using the anti-human IL-18 matched pair of mAb clone 125-2H and biotinylated clone 159-12B (R&D Systems) and HRP-labeled streptavidin (BioLegend).

Flow cytometry

Expression of cell surface receptors on DC and NK cells was analyzed by FC using fluorochrome-labeled mAbs, as previously described (35). Expression of Helios and T-bet was analyzed by intracellular FC using Cytofix/Cytoperm (BD). Cells were analyzed in a FACSCanto II flow cytometer (BD). Results were expressed as geometric mean fluorescence intensity or as percentage of positive cells. Data were analyzed using FlowJo X software (Tree Star).

Viability and apoptosis

Apoptosis of NK cells was assessed using annexin V conjugated to FITC (BD) or allophycocyanin (eBioscience), using the apoptosis detection kit (BD) following manufacturer's instructions. Viability of DC was assessed using Zombie Green following manufacturer's instructions.

Statistical analysis

Two-way ANOVA with Bonferroni's correction was used for comparison of expression of cell surface markers on DC and NK cells. One-way ANOVA with Dunnett's or Bonferroni's post hoc test or paired *t* tests were used for comparison between experimental groups, as indicated in the legend for each figure. Data were analyzed using GraphPad Prism 6.0 software.

Results

regDC restrain NK cell-derived IFN- γ production through secretion of suboptimal amounts of IL-18

We first generated different regDC from iDC differentiated from monocytes and matured with LPS in the presence of DEX, VitD3, or DEX and VitD3, and performed their phenotypical and functional characterization (Fig. 1). Although these regDC were similar to mDC in terms of expression of CD1a (data not shown) and MHC class II, they expressed lower amounts of the costimulatory molecules CD83 and CD86 (Fig. 1A, 1B). Importantly, all regDC, regardless of the stimulus used, exhibited a poor capacity to produce IL-12 when compared with mDC (Fig. 1C). Moreover, regDC generated with DEX or with DEX and VitD3, but not regDC generated with VitD3 alone, secreted less IL-10 than mDC, but these differences did not reach statistical significance (Fig. 1D). To investigate the effect of regDC on NK cells, we performed cocultures of iDC, mDC, and different regDC with freshly isolated NK cells and assessed the production of IFN- γ as surrogate parameter indicative of NK cell effector function (Fig. 2). As expected, mDC, but not iDC, induced robust IFN- γ secretion by NK cells. In contrast, regDC generated with DEX, VitD3, or DEX and VitD3 elicited secretion of very low amounts of this cytokine (Fig. 2A). As DEX-induced regDC showed lower CD86 expression, elicited a more robust effect on NK cell-derived IFN- γ than other regDC, and displayed similar amounts of CCR7 than mDC (indicating that they can migrate to secondary lymph nodes similar to mDC), subsequent experiments were performed with DEX-induced regDC. Moreover, because these previous experiments were performed with allogeneic combinations of cells, we repeated the experiment with syngeneic combinations (using NK cells that were cryopreserved at the time that monocytes were isolated). As with allogeneic combinations of cells, DEX-induced regDC failed to stimulate IFN- γ secretion by syngeneic NK cells (Fig. 2B), thus substantiating the results obtained with allogeneic cell combinations.

To explore the mechanisms underlying regDC-NK cell crosstalk, we performed new experiments using extensively washed regDC (Fig. 2C), as well as mDC- and regDC-derived conditioned media to evaluate their effect on IFN- γ secretion by NK cells (Fig. 2D). In both cases, we observed that NK cells secreted high

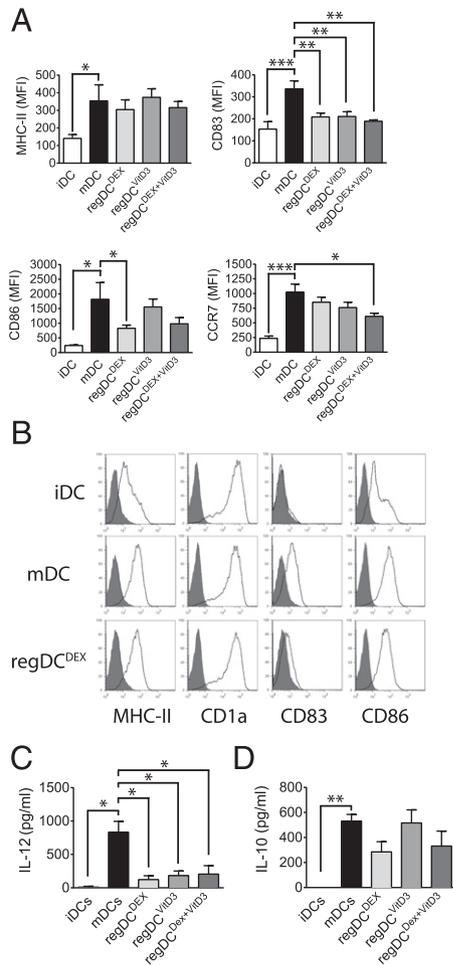


FIGURE 1. Characterization of regulatory DC. iDC, mDC, and regDC generated in the presence of DEX (regDC^{DEX}), VitD3 (regDC^{VitD3}), or a combination of DEX and VitD3 (regDC^{DEX+VitD3}) were used to assess expression of CD1a (data not shown); MHC class II molecules (MHC-II), CD83, CD86, and CCR7 by FC [(A); representative histograms for these markers on regDC^{DEX} are shown in (B)]; secretion of IL-12 (C); and secretion of IL-10 (D). Concentration of cytokines in cell culture supernatants was assessed by ELISA. Mean ± SEM of DC from at least four different donors is shown in (A), (C), and (D). **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Two-way ANOVA with Bonferroni's post hoc test (A) and one-way ANOVA with Dunnett's post hoc test (C and D) were used.

amounts of IFN- γ upon coculture with extensively washed mDC (Fig. 2C) or upon culture with conditioned media from mDC (Fig. 2D), but not when they were cocultured with extensively washed regDC (Fig. 2C) or when they were exposed to conditioned media from regDC (Fig. 2D). As regDC were generated with DEX and glucocorticoids such as methylprednisolone exert inhibitory effects on NK cells (36), we analyzed the direct effect of DEX in our experimental setting (Fig. 2E). We observed that DEX only partially inhibited IFN- γ secretion by NK cells stimulated with IL-12 and IL-18. These results suggest the occurrence of additional mechanisms through which conditioned media from regDC restrain IFN- γ production by NK cells. Moreover, the unresponsiveness of NK cells cultured with regDC was not due to regDC-induced NK cell apoptosis (Fig. 2F) or to NK cell-induced DC death (Fig. 2G). Remarkably, coculture of NK cells with either mDC or regDC induced a significant increase in the percentages of CD25⁺ NK cells (Fig. 2H, 2K) and CD69⁺ NK cells (Fig. 2I, 2K), suggesting that regDC promote NK cell activation. However, only mDC, but not regDC, induced a statistically significant upregu-

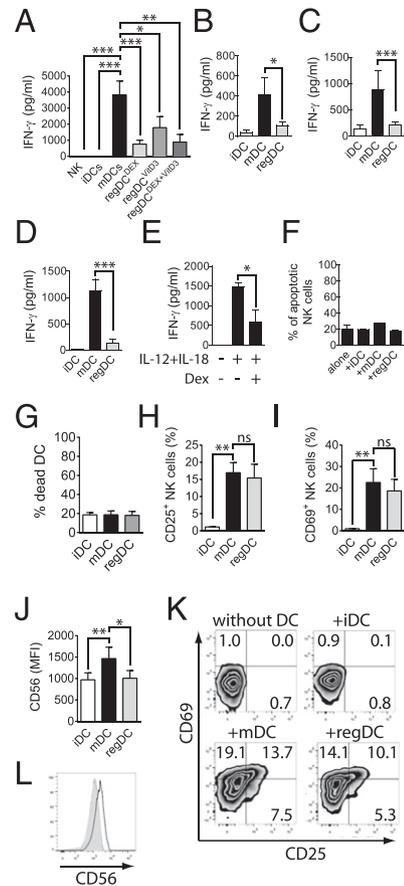


FIGURE 2. regDC restrain IFN- γ production by NK cells through soluble factors and cell surface receptors. Allogeneic iDC, mDC, or regDC generated in the presence of DEX (regDC^{DEX}), VitD3 (regDC^{VitD3}), or a combination of DEX and VitD3 (regDC^{DEX+VitD3}) were cocultured with NK cells for 18 h, and IFN- γ was assessed in cell culture supernatants by ELISA (A). Also, syngeneic (B) or extensively washed (C) iDC, mDC, or regDC generated with DEX were cocultured with NK cells for 18 h, and IFN- γ was assessed in cell culture supernatants by ELISA. Additionally, NK cells were cultured with conditioned media from iDC, mDC, or regDC generated in the presence of DEX (D), or with IL-12 and IL-18 in the absence or in the presence of DEX (E), and IFN- γ was assessed in cell culture supernatants by ELISA. NK cell apoptosis (annexin V⁺CD56⁺ cells) was also assessed by FC after culture without (“alone”) or with iDC, mDC, or DEX-induced regDC (F). Viability of iDC, mDC, and regDC generated in the presence or absence of DEX was assessed after coculture with NK cells using Zombie Green. The percentage of dead (Zombie-Green^{high}) DC was plotted (G). Expression of CD25 (H), CD69 (I), and CD56 (J) was assessed by FC on NK cells after coculture with iDC, mDC, or regDC generated in the presence of DEX. Representative zebra plots of CD25 and CD69 expression in CD3⁻CD56⁺ gated NK cells are shown (K). Representative histograms of CD56 expression on CD3⁻CD56^{dim} cells are shown (L). Thin line: NK cells cocultured with iDC; thick line: NK cells cocultured with mDC; filled gray: NK cells cocultured with regDC. Mean ± SEM of 8, 6, 3, 2, 6, 4, 4, or 7 combinations of DC and NK cells from different donors is shown in (A), (B), (C), (F), (G), (H), (I), and (J), respectively. Mean ± SEM of NK cells from 6 and 3 different donors is shown in (D) and (E), respectively. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. One-way ANOVA with Dunnett's post hoc test was used.

lation of CD56 in the CD56^{dim} NK cell population (Fig. 2J, 2L), indicating that regDC cannot induce a full activation program on NK cells as mDC. Upregulation of CD69 and CD25 was not observed when NK cells were cultured with conditioned media from mDC or regDC (data not shown), suggesting that such NK cell activation is driven by cell-to-cell contact.

As regDC secreted lower amounts of IL-12 than mDC, we supplemented regDC-derived conditioned media with this cytokine to explore whether insufficient amounts of IL-12 account for the inability of these conditioned media to trigger IFN- γ secretion by NK cells (Fig. 3A). However, addition of IL-12 could not provide NK cell-stimulating activity in the presence of regDC-derived conditioned media. Instead, supplementation of regDC-derived conditioned media with IL-18 successfully stimulated IFN- γ secretion by NK cells, although this effect was not observed when IL-18 was directly added to culture medium (Fig. 3B). Accordingly, assessment of IL-18 in cell culture supernatants confirmed that regDC secreted considerably lower amounts of IL-18 than mDC (Fig. 3C). In addition, regDC expressed higher amounts of IL-18R α (Fig. 3D), but not IL-18R β (Fig. 3E), than mDC, although NK cells cultured with regDC displayed similar amounts of both receptor subunits as compared with NK cells cocultured with mDC (Fig. 3F, 3G). Therefore, regDC actively curb IFN- γ secretion by NK cells through mechanisms mediated, at least in part, by secretion of suboptimal concentrations of IL-18.

IL-10, NK cell inhibitory receptors, and NKp46 mediate silencing of NK cell activity by regDC

To further investigate the mechanisms underlying the suppressive effect of regDC on NK cells and given that IL-10, TGF- β , and Gal-1 can be secreted by regDC and display T cell inhibitory activity (19, 20, 26), we cultured NK cells with regDC-derived conditioned media in the absence or in the presence of neutralizing mAbs against these soluble mediators. Neutralization of Gal-1,

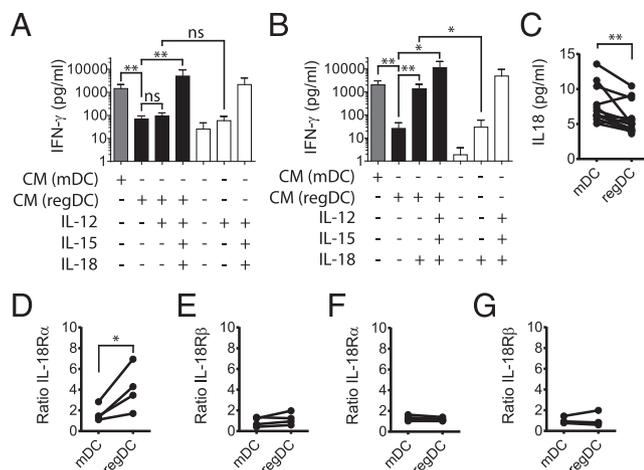


FIGURE 3. Suboptimal secretion of IL-18 by regDC contributes to restrain IFN- γ production by NK cells. NK cells were cultured with conditioned media from mDC (gray bars) or regDC generated in the presence of DEX (black bars), or with culture medium (white bars) supplemented with IL-12 (A) or IL-18 (B), and IFN- γ secretion was assessed by ELISA. As positive controls, NK cells were cultured with conditioned media from regDC or with culture medium supplemented with IL-12, IL-15, and IL-18. The concentrations of IL-18 were assessed in conditioned media from mDC and regDC by ELISA (C). mDC or regDC generated in the presence of DEX (D and E) and NK cells (F and G) were used to assess the expression of IL-18R α (D and F) and IL-18R β (E and G) chains by FC. Results were expressed as the ratio of the mean fluorescence intensity obtained with mDC or regDC compared with the mean fluorescence intensity obtained with iDC. Mean \pm SEM of NK cells from at least 6 and 3 different donors is shown in (A) and (B), respectively. Individual values from 14 different donors are shown in (C), whereas individual values from at least 4 different donors are shown in (D)–(G). One-way ANOVA with Bonferroni's post hoc test was used in (A) and (B), whereas $*p < 0.05$, $**p < 0.01$. A paired t test was used to compare the data shown in (C)–(G).

TGF- β , or IL-10 in mDC- or regDC-derived conditioned media did not alter NK cell secretion of IFN- γ induced by these DC populations (Fig. 4A and data not shown). However, neutralization of TGF- β or IL-10 during mDC–NK cell cocultures led to augmented secretion of IFN- γ by NK cells, whereas neutralization of IL-10 during regDC–NK cell cocultures rescued the ability of regDC to trigger IFN- γ secretion by NK cells (Fig. 4B). Of note, we observed a similar trend toward enhanced IFN- γ production following TGF- β blockade, although these data did not reach statistical significance. These results suggest that regDC inhibits IFN- γ secretion by NK cells through IL-10-mediated mechanisms involving direct cell-to-cell contact.

As extensively washed regDC restrained IFN- γ production by NK cells, we then studied whether NK cell receptors contribute to such effect by performing coculture experiments in the absence or in the presence of blocking mAb against different cell surface receptors or cognate ligands (Fig. 5A–C). Surprisingly, we detected substantial amounts of IFN- γ in cocultures of NK cells and regDC upon blockade of the activating receptor NKp46, but not upon blockade of other activating receptors, such as NKG2D, NKp30, or NKp44 (Fig. 5A). Such inhibitory role of NKp46 was not verified when NK cells were cocultured with mDC (Fig. 5A). The unexpected behavior of NKp46 in our experimental setting was detected in both allogeneic (Fig. 5A) and syngeneic (Fig. 5B) combinations of regDC and NK cells, suggesting a previously unrecognized function for NKp46 during interactions between NK cells and regDC. In addition, blockade of the interaction between MHC class I molecules on regDC and NK cell receptors using the W6/32 mAb induced increased IFN- γ secretion (Fig. 5C), suggesting that modulation of NK cell activity by regDC involves engagement of the NK cell inhibitory receptors by MHC class I molecules. Of note, iDC, mDC, and regDC generated by DEX expressed similar amounts of NKp46 ligands (NKp46L), excluding the possibility of a differential engagement of NKp46 by mDC versus regDC (Fig. 5D, 5E).

Previously, a loss-of-function mutation in the Ncr1 gene (encoding NKp46) was associated with silencing of the Helios transcription factor in NK cells (37). In contrast, T-bet has been shown to be a critical transcription factor involved in IFN- γ production by NK cells (38). As regDC suppressed IFN- γ secretion by NK cells through a mechanism involving NKp46, we explored whether cocultures with iDC, mDC, or regDC modulated expression of these transcription factors in NK cells (Fig. 6). We observed that the proportion of NK cells expressing Helios was similar fol-

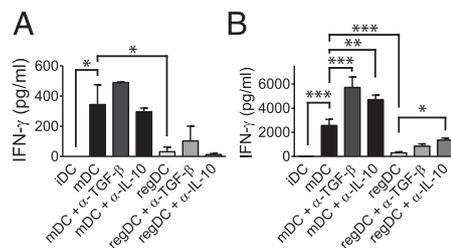


FIGURE 4. IL-10 curbs IFN- γ production during coculture of NK cells with mDC or regDC. NK cells were cultured for 18 h with conditioned media from iDC, mDC, or regDC generated in the presence of DEX (A) or with iDC, mDC, or regDC generated in the presence of DEX (B) in the absence or presence of neutralizing mAb against TGF- β or IL-10. The concentrations of IFN- γ were assessed in cell culture supernatants by ELISA. Mean \pm SEM of NK cells from at least two different donors is shown in (A), whereas mean \pm SEM of at least four combinations of DC and NK cells from different donors is shown in (B). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One-way ANOVA with Bonferroni's post hoc test was used.

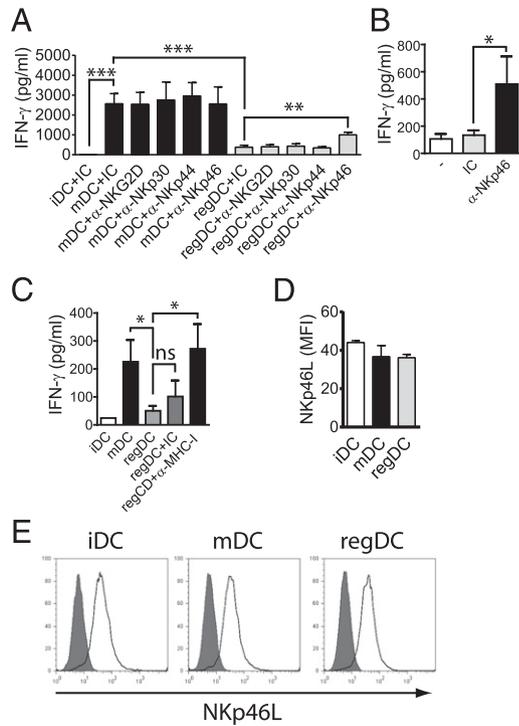


FIGURE 5. NKp46 displays inhibitory activity during the cross-talk between NK cells and regDC. NK cells were cocultured with allogeneic iDC, mDC, or regDC generated in the presence of DEX in the absence or in the presence of blocking mAb against NKG2D, NKp30, NKp44, or NKp46 (A), and IFN- γ was assessed in cell culture supernatants by ELISA. NK cells were also cocultured with syngeneic regDC generated in the presence of DEX in the absence or presence of anti-NKp46 blocking mAb (B), or NK cells were cocultured with allogeneic iDC, mDC, or regDC generated in the presence of DEX in the absence or presence of blocking mAb against MHC class I molecules (C), and IFN- γ was assessed in cell culture supernatants by ELISA. Expression of NKp46L was assessed on iDC, mDC, or regDC produced with DEX by FC, as described in *Materials and Methods* (D). Representative histograms are shown (E). Mean \pm SEM of NK cells from 4 (A), 6 (B), and 3 (C) combinations of DC and NK cells from different donors is shown. Mean \pm SEM of three DC from different donors is shown in (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. One-way ANOVA with Bonferroni's post hoc test was used.

lowing coculture with either mDC or regDC, and that these percentages were higher than the percentages of NK cells expressing Helios following coculture with iDC (Fig. 6A, 6C). However, the increased percentage of NK cells expressing Helios reached statistical significance only after coculture of NK cells with mDC, but not with iDCs. Because a similar percentage of NK cells that express Helios after coculture with mDC or with regDC was detected, it is likely that differences in Helios expression do not explain the different response of NK cells to mDC versus regDC. Conversely, we observed a trend toward an increased percentage of NK cells expressing T-bet upon coculture with mDC that was not observed when NK cells were exposed to regDC, as compared with iDC–NK cell cocultures (Fig. 6B, 6D).

Overall, our results indicate that regDC promote activation of NK cells toward an alternative phenotype characterized by low IFN- γ secretion via mechanisms involving secretion of suboptimal amounts of IL-18, synthesis of IL-10, and engagement of NKp46 and MHC class I-specific receptors. Our results contribute to unravel the mechanisms that control interactions between human regDC and NK cells and disclose a previously unanticipated regulatory function for NKp46 in the control of NK cell activation.

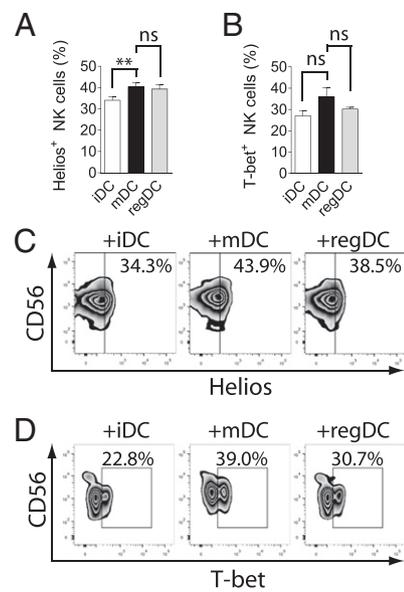


FIGURE 6. Expression of Helios and T-bet transcription factors in NK cells upon interaction with different DC. NK cells were cocultured with allogeneic iDC, mDC, or regDC generated in the presence of DEX, and the expression of Helios (A) and T-bet (B) was assessed by FC in CD3⁺CD56⁺ gated cells. Mean \pm SEM from four independent experiments performed with NK cells from different donors is shown. Representative zebra plots for Helios (C) and T-bet (D) in CD56⁺ NK cells are also shown. One-way ANOVA with Dunnett's post hoc test was used.

Discussion

Following the seminal work describing the functional consequences of the interaction between DC and NK cells (39), many soluble mediators and cell surface receptors that participate in this cross-talk have been characterized (1, 2, 6, 7, 16, 40–50). It has been demonstrated that NK cells and DC establish a cross-talk that promotes their reciprocal activation, strengthening the secretion of proinflammatory cytokines such as IL-12, IL-15, and IFN- γ , endowing DC with the ability to skew the adaptive immune response toward a Th1- and CTL-mediated profile (1, 2, 7, 16, 51, 52). Also, iDC that remain refractory to maturation can be lysed by NK cells, a phenomenon that seems to function as an editing process through which NK cells check proper maturation of DC in response to pathogens and avoid the delivery of anergy-inducing signals to T cells (7). Expression of CD94/NKG2A has been involved in the acquisition of DC resistance to NK cell-mediated lysis (53).

Regulatory DC play key roles as they are expanded by tumors to promote immune tolerance (13, 19), and ex vivo-generated regDC have been proposed as useful tools for the treatment of patients with autoimmune diseases (21) and for inducing graft-specific tolerance in transplanted patients (22). As they can be generated in vitro by DEX, regDC may also become expanded in patients chronically treated with glucocorticoids (11). Importantly, the effect of the outcome of the interaction between regDC and NK cells has not yet been explored. In this work, we found that regDC generated by DEX, VitD3, or the combination of both expressed less costimulatory molecules and were poor producers of IL-12 when compared with mDC. These regDC, as opposed to mDC, were unable to trigger IFN- γ secretion by NK cells. Because regDC generated by DEX displayed less CD86, showed more robust inhibition of NK cell-derived IFN- γ secretion than other regDC, and displayed similar amounts of CCR7 than mDC (indicating their similar capacity to migrate to secondary lymph nodes), most experiments in this study were performed with regDC generated by

DEX treatment. Using DEX-induced regDC, we demonstrated that their inability to trigger IFN- γ secretion by NK cells was mediated by soluble factors and cell surface receptors. Although IL-12 is one of the major cytokines produced by mDC and involved in NK cell stimulation (1, 34, 54, 55), we found that the capacity of regDC to suppress IFN- γ production by NK was not due to insufficient amounts of IL-12 in the culture media. However, we found that secretion of insufficient amounts of IL-18 by regDC actively contributes to their inhibitory activity on NK cells. Remarkably, priming of NK cells by IL-18 has been shown to be critical for helper NK cell function such as production of iDC-attracting chemokines CCL3 and CCL4, which attract iDC in a CCR5-dependent manner and induce production of CXCL9, CXCL10, and CCL5 by DC, promoting subsequent recruitment of type 1 effector CD8⁺ T cells (56). In addition, activation of IL-18–primed helper NK cells by cytokines such as IFN- α , IL-15, IL-12, and IL-2 stimulates DC-mediated production of CCL19 and CCR7-mediated recruitment of naive CD8⁺ T cells into lymph nodes (57). Therefore, it has been suggested that the therapeutic use of combinatorial adjuvants containing IL-18 may facilitate the induction of antitumor immune responses. Accordingly, our observation that regDC secrete suboptimal doses of IL-18 that in turn prevents NK cell–mediated IFN- γ secretion may constitute one of the mechanisms employed by tumor-expanded regDC to facilitate tumor immune escape. Of note, regDC expressed higher amounts of IL-18R α than mDC, which might indicate their ability to recapture secreted IL-18, influencing the bioavailability of IL-18 for NK cells.

Notably, exposure of NK cells to regDC did not affect the viability of any of these cells. Conversely, regDC induced activation of NK cells toward an alternative phenotype, as judged by the upregulation of CD25 and CD69 without inducing the upregulation of CD56 in the CD56^{dim} NK cell subpopulation (as did mDC). Although upregulation of CD69 did not reach statistical significance, it should be kept in mind that NK cells were analyzed after an overnight culture with DC, whereas full upregulation of CD69 usually requires 48–72 h of stimulation. In addition, we demonstrated that IL-10 and TGF- β restrain IFN- γ secretion by NK cells during their cross-talk with mDC, but that only IL-10 contributes to prevent IFN- γ secretion by NK cells upon interacting with regDC. We speculate that IL-10 most likely acts through an autocrine/paracrine effect on regDC, as blockade of IL-10 only restored IFN- γ secretion when NK cells were cocultured with regDC, but not when NK cells were cultured with conditioned media derived from regDC.

Moreover, engagement of NK cell inhibitory receptors that recognize MHC class I molecules also contributes to thwart IFN- γ production by NK cells upon interaction with regDC. We can speculate that inhibitory KIRs (KIR2/3DLs), CD94/NKG2A, or some members of the Ig-like transcript family such as ILT2-6 could be involved in this response, as they recognize MHC class I molecules (58–60). Unexpectedly, blockade of the activating receptor NKp46 restored IFN- γ secretion by NK cells upon contacting regDC, which unveils a previously unrecognized regulatory function for this receptor during regDC–NK cell cross talk. Such effect was not due to a differential engagement of NKp46 on NK cells upon contact with mDC versus regDC, as expression of NKp46L was identical in both cell types. Previously, a minor stimulatory role for NKp46 during the cross-talk between NK cells and DC matured in the presence of IL-1, IL-6, TNF- α , and PGE₂ (3) or TNF (5) was observed. Others found that NKp46 and NKG2D play a role in the cross-talk between NK cells and virus-infected, but not uninfected, human DC (61, 62). The discrepancies with our results could be due to the fact that we used resting NK cells cultured for 18 h with DC, whereas, in some of these

studies, activated NK cells cultured with IL-2 (3, 5) or NK cells exposed to virus-infected DC (61, 62) were used. Moreover, the identification of mutant mice with a loss-of-function mutation in the gene encoding NKp46 (*Ncr1*), which showed increased resistance to viral infections associated with hyperresponsive NK cells, enabled the assignment of a previously unrecognized inhibitory role of NKp46 in setting the threshold of NK cell responsiveness and a negative role for the Helios transcription factor in this process (37). However, in our experimental setting, we did not observe differences in the percentage of NK cells that express Helios after coculture with mDC or regDC, making unlikely that the silencing role of NKp46 on IFN- γ production in human NK cells during their cross-talk with regDC may involve changes in the expression of Helios. However, we found a tendency of regDC, but not mDC, to trigger lower percentages of NK cells expressing T-bet, a transcription factor required for IFN- γ production (38), suggesting that a T-bet–regulated pathway may underlie the regDC–NK cell inhibitory crosstalk.

Overall, our results identify a novel regulatory circuit activated during the cross-talk between NK cells and regDC. Although the interaction between mDC and NK cells is pivotal for the promotion of Th1- and CTL-mediated immune responses (1–3, 16), regDC are relevant for the homeostasis of the immune response (20), they become expanded during tumor growth and contribute to tumor-immune escape (13, 17–19), and constitute promising tools for immunotherapy to harness excessive proinflammatory immune responses during autoimmune diseases (21) and transplant rejection (22). Our results indicate that such functions may partially involve the ability of regDC to activate NK cells toward an alternative phenotype characterized by upregulated expression of CD25 and CD69, but not of CD56, and presumably the induction of lower percentages of T-bet⁺ NK cells, leading to a NK cell phenotype with impaired ability to produce IFN- γ . The mechanisms underlying this effect entail the production of suboptimal secretion of IL-18 by regDC, as well as the activation of a regulatory pathway involving IL-10, MHC class I–specific inhibitory receptors, and engagement of NKp46. Accordingly, NKp46 displays a regulatory function during the interplay between regDC and NK cells that, integrated with other immune inhibitory pathways, may contribute to the resolution of inflammatory responses and evasion of antitumor responses, thus highlighting the relevance of NK cell plasticity at the crossroads of immune tolerance and activation.

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Disclosures

The authors have no financial conflicts of interests.

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