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Sensitivity of dendritic cells to NK-mediated lysis depends on the inflammatory environment and is modulated by CD54/CD226-driven interactions

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ABSTRACT

Previous studies have suggested that NK cells may limit T cell responses by their ability to eradicate dendritic cells, as demonstrated by NK cell-mediated killing of dendritic cells generated from mouse bone marrow cells or human monocytes with GM-CSF. In the present study, we demonstrated that conventional dendritic cells, generated in vitro with Flt3 ligand or from spleens, were resistant to NK cell-mediated lysis. However, upon stimulation with GM-CSF, NK cells could mediate lysis of these dendritic cells. GM-CSF-stimulated Flt3 ligand dendritic cells or splenic dendritic cells increased surface expression of costimulatory molecules and known NK cell ligands. Likewise, NK cells could target dendritic cells in vivo, which could be inhibited, in part, by anti-GM-CSF antibodies. The blocking of CD54 or CD226 inhibited NK cell-mediated cytotoxicity of the GM-CSF-stimulated Flt3 ligand conventional dendritic cells. Furthermore, the CD226^{+}NKG2A^{-} subset of NK cells was selectively better at targeting GM-CSF-stimulated Flt3 ligand conventional dendritic cells. However, CD155, a known ligand for CD226, could also act as an inhibitor of NK cell-mediated lysis, as dendritic cells lacking CD155 were more sensitive to NK cell-mediated lysis than wild-type dendritic cells. We hypothesize that by only permitting a subset of NK cells to target activated dendritic cells during inflammation, this would allow the immune system to balance between dendritic cells able to drive adaptive immune responses and dendritic cells targeted for elimination by NK cells to hinder, e.g., spread of infection.

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Introduction

DCs are a variety of different cell types that have morphologic, phenotypical, or functional similarities. In both mice and humans, 2 subsets of DCs are the cDCs, including both lymphoid-tissue resident DCs and migratory DCs, and the pDCs [1]. In mice, cDCs in the spleen and lymph nodes can be subdivided further into the CD8α^{+} and CD8α^{-} populations. The former population has been suggested to be involved in cross-priming, i.e., the ability to take up antigen from the surrounding milieu and present it on MHC I to cytotoxic T cells, whereas the latter is primarily associated with MHC II antigen presentation to drive an adaptive immune response [2]. In nonlymphoid tissue, CD103^{+} DCs and CD103^{-} DCs identify similar populations of DCs. These DCs derive from a common progenitor, which is also responsible for monocyte development. Another DC population that has been described includes the Langerhans cells, which are found in the skin epithelium and are self populating. Finally, there are TiP DCs [3], which can also be generated in vitro from monocytes stimulated with GM-CSF.

DC–NK cell interactions play important roles in immune responses to malignancies and pathogen infections [4]. TLR-activated DCs not only recruit NK cells to sites of inflammation through release of chemokines, such as CCL5 and CXCL10 [5–7], but also activate NK cell-mediated cytotoxicity and cytokine production by secreting cytokines, such as IL-12, IL-15, and IL-18. Once activated, cytokines produced by the NK cells can drive Th1-adaptive immune responses [6, 8, 9]. NK cells may

Abbreviations: B6 = C57BL/6, BM = bone marrow, BMDC = bone marrow dendritic cell, CD40L = CD40 ligand, cDC = conventional dendritic cell, DC = dendritic cell, DCM = dead cell marker, Flt3L = Flt3 ligand, MFI = mean fluorescence intensity, MHC I/II = MHC class I/II, PD-1 = programmed death 1, PD-L1 = programmed death ligand 1, pDC = plasmacytoid dendritic cell, RAG2γc$^{-/-}$ = RAG2$^{-/-}$ × common γ-chain$^{-/-}$, TIGIT = T cell-Ig and -ITIM domain, TiP DC = TNF- and iNOS-producing dendritic cell

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also affect adaptive immune responses by eliminating DCs. Originally, it was proposed that NK cell activity in mixed lymphocyte cultures could affect the CTL outcome in vitro [10]. Later, NK cell-mediated killing of DCs in vitro was observed using DCs generated in vitro with GM-CSF from mouse BM cells [11] or from human monocytes [12–14].Mouse GM-CSF-derived BMDCs were eliminated in vivo primarily by the TRAIL pathway, which could affect the ensuing CTL response negatively [15]. Activation of mouse BM GM-CSF DCs by TLR ligands or IFN-γ reduced NK cell-mediated killing in vivo and in vitro by increasing the expression of Qa1 or HLA-E nonclassic MHC molecules [16, 17], which could explain why mature DCs are better at inducing T cell responses in vivo [15].

The majority of the previous studies examining NK cell–DC interactions has involved DCs derived from monocytes or BM with GM-CSF. However, it is unclear if cDCs would be sensitive to NK cell-mediated lysis under noninflammatory conditions or under what conditions they might be targeted by NK cells. In the present study, we examine whether cDCs are targeted by NK cells and what role GM-CSF plays in regulating NK cell-mediated killing of cDCs.

MATERIALS AND METHODS

Mice

B6, RAG1$^{-/-}$ [18], RAG2γc$^{-/-}$, CD80 × CD86$^{-/-}$ [19], CD112$^{-/-}$ [20], CD155$^{-/-}$ [21], and β_2m$^{-/-}$ [22] mice on a B6 background (6–10 wk old) were housed under standard conditions at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet (Stockholm, Sweden) or the Hannover Medical School (Hannover, Germany). PD-1$^{-/-}$ mice [23] were generously provided Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan). These mice were crossed to RAG1$^{-/-}$ to generate PD-1 × RAG1$^{-/-}$ mice. All procedures were performed under institutional and national guidelines (ethical numbers from Stockholm County Council, N327/12 and N147/15).

NK cell purification

Single-cell suspension from RAG1$^{-/-}$ spleens was depleted of erythrocytes, and NK cells were positively sorted using anti-DX5^{+} magnetic beads or by negative sorting using MACS separation, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in complete medium (αMEM; 10 mM HEPES, 2×10^{-5} M 2-ME, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin) with 1000 U/ml human IL-2 (PeproTech, London, United Kingdom) and cultured for 5 d at 37°C, 10% CO_2. To induce GM-CSF production, NK cells were incubated in RPMI 1640 (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA), containing 10% FCS, 10 mM HEPES, 2×10^{-5} M 2-ME, 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, with 100 ng/ml mouse IL-15 (PeproTech) and 100 ng mouse IL-18 (MBL International, Woburn, MA, USA) for 24 h before being collected, washed, and added to DCs. For isolation of NK cell subsets based on CD226 expression, NK cells were isolated as above and then sorted on MoFlo XDP cell sorter (Beckman Coulter, Brea, CA, USA). The cells were then cultured as above.

Generation of DCs in vitro

DCs were generated from Flt3L cultures, as described previously [24, 25]. In brief, BM cells were cultured in RPMI 1640 (Thermo Fisher Scientific Life Sciences) containing 100 ng/ml Flt3L (ImmunoTools, Friesoythe, Germany). After 8–9 d, the cells were harvested, followed by removal of CD11b^{-} B220^{+} cell populations (pDCs) using antibody-coated beads (Miltenyi Biotec). Cells were incubated with Flt3L, with or without 10 ng/ml GM-CSF (ImmunoTools) for 24–48 h and then collected for phenotyping or use in cytotoxicity assay. In some experiments, 1×10^{6} IL-15- and IL-18-stimulated NK cells were added to permeable Transwells (0.4 μm; Corning Costar, Corning, NY, USA) and added to 1×10^{6} DCs in the presence of Flt3L in 24-well plates. After 48 h, the DCs were collected for use in cytotoxicity assays. Anti-GM-CSF antibodies (BioLegend, San Diego, CA, USA) were added to wells at a concentration of 10 μg/ml.

GM-CSF-derived BMDCs were generated as described previously [26]. In brief, BM cells were cultured in DMEM (Thermo Fisher Scientific Life Sciences) containing 10 ng/ml recombinant GM-CSF (ImmunoTools) for 6 d. DCs were collected and purified further using anti-CD11c mAb-coated beads (Miltenyi Biotec) before incubation overnight again in GM-CSF.

Purification of splenic DCs

Splenic DCs were prepared as described previously [7]. In brief, spleens from B6 mice were harvested and digested by collagenase (Liberase-CI; Roche Diagnostics, Indianapolis, IN, USA) and teased apart by repeated pipetting in PBS, 5 mM EDTA, and 5% FCS (PBS/EDTA/FCS). Splenic DCs were enriched by using anti-CD11c magnetic beads for magnetic sorting (Miltenyi Biotec). The purified splenic DCs were cultured in 100 ng/ml Flt3L, with or without 10 ng/ml GM-CSF for 24–48 h. For the purification of CD8α^{+} DCs, spleens from RAG1$^{-/-}$ mice were used, and anti-CD8α magnetic beads were used to isolate these cells. The flowthrough was then collected, and the CD11c^{+} cells were sorted as above.

Flow cytometry

All antibodies were purchased from BD Biosciences (San Jose, CA, USA), BioLegend, eBioscience (San Diego, CA, USA), or R&D Systems (Minneapolis, MN, USA). Cells were stained using standard protocols. Flow cytometry was performed on FACSCalibur, FACSort, or LSR II (BD Biosciences) or CyAn ADP LX 9 Color flow cytometer (Beckman Coulter). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Cytotoxicity assays

DCs were incubated for 1 h in the presence of $Na_2O_4Cr^{51}$ (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) and then washed thoroughly in PBS. IL-2-stimulated NK cells were then mixed with the DCs at the different E:T ratios. In antibody-blocking assays, a ratio of 10:1 was used to measure killing. Antibody was present throughout the assay. After 4 h of E:T cell coincubation, cell culture supernatants were analyzed on a γ radiation counter (Wallac Oy, Turku, Finland). Specific lysis was calculated according to the formula: % specific lysis = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. If the difference between spontaneous and maximum release was <2-fold, then these experiments were discarded. For anti-CD226 antibody blocking, we used the 3B3 clone, which we have described previously [27]. We used the following clones for anti-CD40L (MR1; BioLegend), anti-ICAM (YN1/1.7.4; BioLegend), and anti-PD-L1 (10F.G92; BioLegend) blocking.

In vivo DC rejection assay

Splenic DCs were labeled with 1 μM CFSE (target cells) or 1 μM CellTrace Violet (control cells; Thermo Fisher Scientific Life Sciences) for 10 min. Target and control cells were mixed and $1–3 \times 10^{6}$ cells coinjected intravenously via the tail vein into RAG1$^{-/-}$ mice or RAG2γc$^{-/-}$ mice as controls for NK cell-mediated killing. The injection mix was analyzed by flow cytometry for reference. Two days later, the spleens were harvested and erythrocytes depleted, and the relative percentages of target and control cells were measured by flow cytometry [28]. Rejection was estimated as the relative survival of target or cells, calculated as: % remaining target cells of labeled cells/% target cells in inoculate or % remaining control cells of labeled cells/% control cells in inoculate.

IL-18 treatment of mice

RAG1$^{-/-}$ mice were injected daily for 48 h with 2 μg recombinant mouse IL-18/mouse (MBL International). The spleens were collected and stained with anti-CD11b, -CD11c, -GR1, and -NKp46 (BioLegend) and Viability Dye eFluor

780 (eBioscience) to detect the number of DCM-positive DCs by flow cytometry. NK cell-depleted mice were treated with 200 μg/mouse anti-NK1.1 (PK136) antibody, 24 h before IL-18 treatment. Anti-GM-CSF antibody-treated mice were given 200 μg/mouse anti-GM-CSF antibody (Bio X Cell, West Lebanon, NH, USA), 24 h before IL-18 treatment. Mice were given another dose of anti-GM-CSF antibody in conjunction with the second IL-18 injection.

Statistical analysis

Statistical analyses were performed with GraphPad Prism, version 4 (GraphPad Software, La Jolla, CA, USA).

RESULTS

GM-CSF is required for NK cell-mediated killing of DCs

In contrast to previous studies examining the NK cell-mediated lysis of DCs, we generated DCs in vitro with Flt3L and tested their sensitivity to NK cells. We found that BMDCs generated with Flt3L were relatively insensitive to killing by IL-2-stimulated NK cells in vitro (**Fig. 1A**). However, when the DCs were incubated for 48 h with 10 ng/ml GM-CSF before the killing assay, we observed a significant increase in the sensitivity of these DCs to NK cell-mediated lysis (Fig. 1A). Increased sensitivity to NK cell-mediated lysis was also observed with DCs treated with 1 ng/ml GM-CSF but not at 0.1 ng/ml (data not shown).

As NK cells are a source of GM-CSF, we tested if GM-CSF derived from NK cells could also make DCs sensitive to NK cell-mediated lysis. Similar to previously published studies for human NK cells [29], we found that NK cells stimulated with IL-15 and IL-18 were efficient in producing GM-CSF (data not shown). IL-15/IL-18-stimulated NK cells were washed and added to Transwell plates, which were then cocultured with DCs cultured in Flt3L. After 48 h, these DCs were collected and used as targets for NK cells. These cocultured DCs were more susceptible to NK cell-mediated lysis than DCs cultured with Flt3L alone (Fig. 1B). This sensitivity to NK cell-mediated lysis was abrogated by the addition of anti-GM-CSF antibody to the cocultures (Fig. 1B).

As Flt3L BMDCs are similar to splenic cDCs [25], the sensitivity of splenic DCs to NK cell-mediated killing upon GM-CSF stimulation was also tested. Purified splenic DCs cultured with Flt3L and GM-CSF in vitro were more sensitive to NK cell-mediated lysis than splenic DCs cultured in Flt3L alone (Fig. 1C). It has been suggested previously that NK cells might control $CD8\alpha^+$ DC numbers in lymph nodes [30]. When splenic DCs were sorted into $CD8\alpha^+$ and $CD8\alpha^-$ populations, both populations appeared to be equally sensitive to NK cell-mediated lysis upon GM-CSF stimulation (Fig. 1D).

NK cell elimination of splenic DCs in vivo

To examine GM-CSF-stimulated DC sensitivity to NK cell-mediated killing in vivo, we first tested the relative rejection rates of splenic DCs treated with or without GM-CSF in $RAG1^{-/-}$ mice or $RAG2\gamma c^{-/-}$ mice, which lack NK cells. Forty-eight hours postinjection, we found that there was a significantly relative decrease in the number of transferred GM-CSF-stimulated splenic DCs in the $RAG1^{-/-}$ mice compared with splenic DCs cultured in Flt3L alone (**Fig. 2A**), whereas there was no difference in the recovery of both of these cells from the $RAG2\gamma c^{-/-}$ mice. This finding suggested that NK cells also selectively eliminated DCs stimulated with GM-CSF in vivo.

To test whether NK cells could eliminate DCs under inflammatory conditions, we treated $RAG1^{-/-}$ mice with IL-18, injected intraperitoneally, as NK cells are known to express IL-18R and be activated by this cytokine. Furthermore, elevated IL-18 levels can be found in a number of inflammatory diseases. When the frequency of DCM^+ DCs was examined after 48 h of treatment, there was a 71% increase in the number of DCM^+ DCs in the mice treated with IL-18 when compared with control mice [7.7 ± 1.8% DCM^+ DCs, IL-18 treated ($n = 12$), vs. 4.5 ± 1.1% DCM^+ DCs from control mice ($n = 7$); $P < 0.05$, ANOVA with Bonferroni posttest (Fig. 2B and C)]. In mice depleted of NK cells and treated with IL-18, the frequency of DCM^+ DCs was significantly reduced when compared with the frequency of

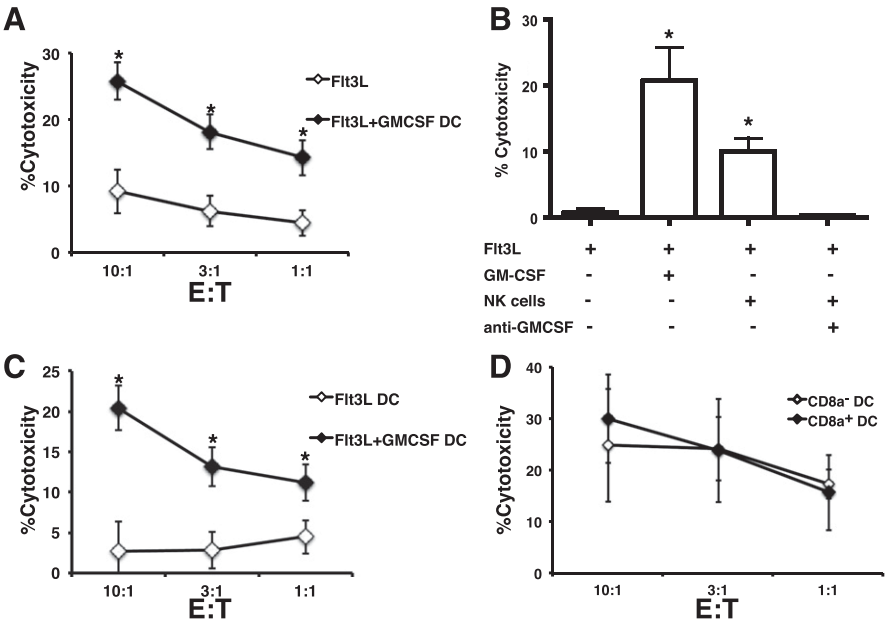

Figure 1. GM-CSF induces NK cell-mediated lysis of cDCs. (A) NK cell-mediated lysis of in vitro-derived Flt3L DCs and Flt3L DCs stimulated with GM-CSF (*$P < 0.05$, paired test, $n = 9$). (B) Flt3L-derived DCs incubated in the presence of IL-15- and IL-18-stimulated NK cells become sensitive to NK cell-mediated lysis. Addition of 10 μg/ml anti-GM-CSF antibody to the cultures reduces the sensitivity (*$P < 0.05$, ANOVA with Bonferroni posttest, $n = 3$). (C) NK cell-mediated lysis of splenic DCs stimulated Flt3L alone (open diamonds) or Flt3L and GM-CSF (filled diamonds; *$P < 0.05$, paired t test, $n = 3$). (D) Sensitivity of splenic $CD8\alpha^+$ (filled diamonds) and $CD8\alpha^-$ DC (open diamonds) to NK cell-mediated lysis following GM-CSF stimulation ($n = 3$).

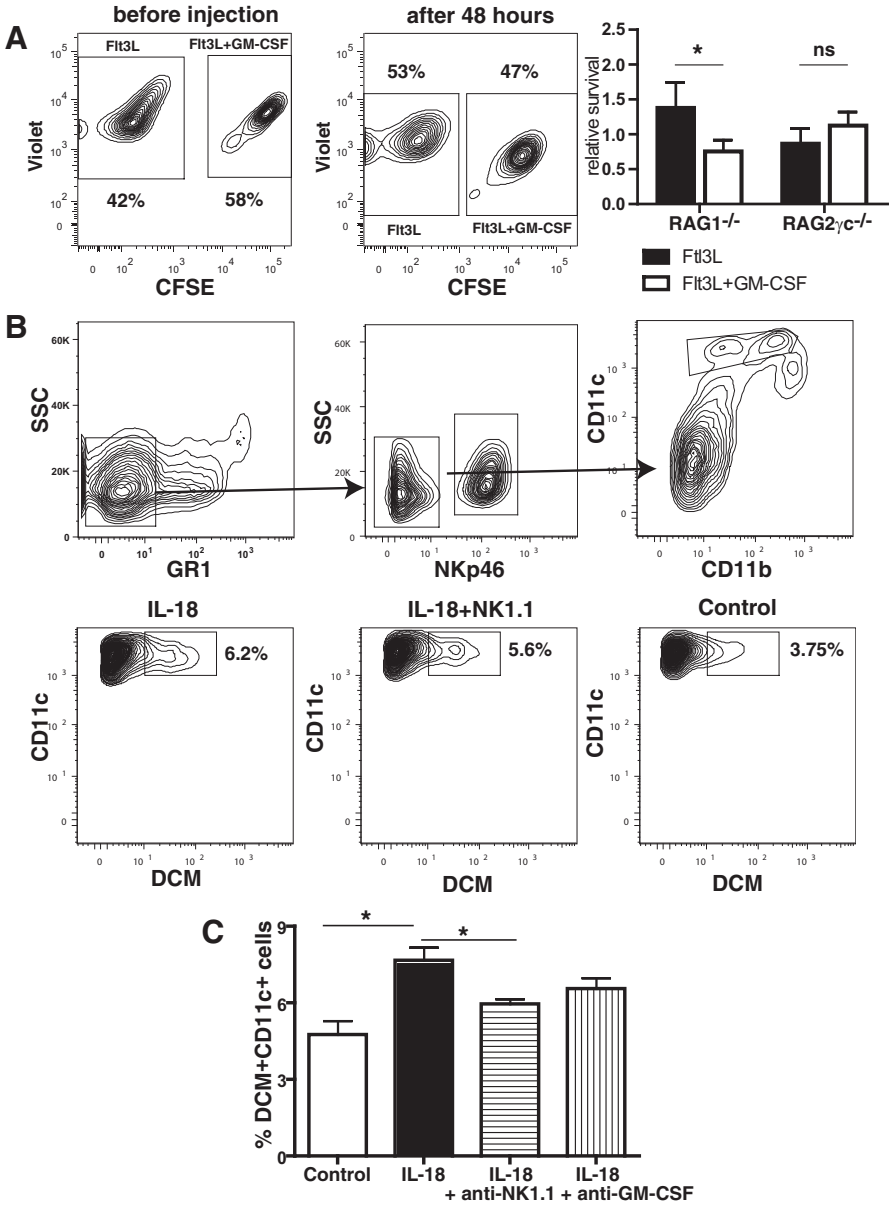

Figure 2. IL-18 induces NK cell-mediated killing of DCs in vivo. (A) GM-CSF stimulation makes in vitro-derived Flt3L DCs more sensitive to NK cell-mediated elimination in vivo. Flow cytometry plots show ratio of 1 representative experiment before injection (left) and after 48 h (middle). (Right) Combined results of 3 independent experiments (*$P < 0.01$, Student's t test, $n = 9$ for RAG1$^{-/-}$ mice; $n = 3$ for RAG2γc$^{-/-}$). (B) Gating strategy to show frequency of DCM$^+$ CD11c$^+$ DCs after injection of RAG1$^{-/-}$ mice with IL-18 (lower left) in combination with anti-NK1.1 (lower middle) or control antibody (lower right; 1 representative experiment). SSC, side scatter. (C) Frequency of DCM$^+$ CD11c$^+$ DCs after injection of IL-18, in combination with anti-NK1.1, control, or anti-GM-CSF antibody (4 independent experiments, $n = 7$–12 mice/group; *$P < 0.05$, ANOVA with Bonferroni posttest).

DCM$^+$ DCs in mice treated with IL-18 alone [5.6 ± 0.8% DCM$^+$ DCs from mice treated with IL-18 and anti-NK1.1 antibody ($n = 10$) vs. 7.7 ± 1.8% DCM$^+$ DCs from IL-18 alone-treated mice ($n = 12$); $P < 0.05$, ANOVA with Bonferroni posttest (Fig. 2C)], suggesting that NK cells were involved in the increased DC cell death. As the NK cells could not only induce the cytotoxicity of the DCs but also produce GM-CSF to make the DCs sensitive to NK cell-mediated lysis, we also treated mice with anti-GM-CSF antibody. When compared with the IL-18-treated mice, there was an ~15% reduction in the number of DCM$^+$ DCs [7.7 ± 1.8% DCM$^+$ DCs, IL-18 treated, vs. 6.5 ± 0.6% DCM$^+$ DCs from mice treated with IL-18 and anti-GM-CSF antibody ($n = 8$; Fig. 2C)]. This suggested that IL-18 production in vivo during inflammatory responses could induce NK cell-mediated lysis of DCs, which was, in part, a result of GM-CSF production.

Identification of potential NK cell ligands on DCs stimulated with GM-CSF

GM-CSF stimulation of Flt3L DCs leads to significantly increased expression of CD54, CD80, CD86, CD112, CD155, and CD274 (PD-L1; **Fig. 3B–G**). Likewise, GM-CSF stimulation did increase the levels of CD40 and classic and nonclassic MHC I proteins on the cell surface on average; however, these were not significantly higher, as the expression of the molecules varied on the control DCs from experiment to experiment (Fig. 3A, H, and I). Similar results were also observed when splenic DCs were isolated from mice and then incubated in vitro with Flt3L, with or without GM-CSF (data not shown).

Likewise, we detected increased expression of CD54 (MFI 218 ± 44 control mice vs. 373 ± 41 IL-18-treated mice; $n = 5$–6; $P < 0.01$, Mann-Whitney test), CD80 (MFI 55 ± 7 vs. 86 ± 7;

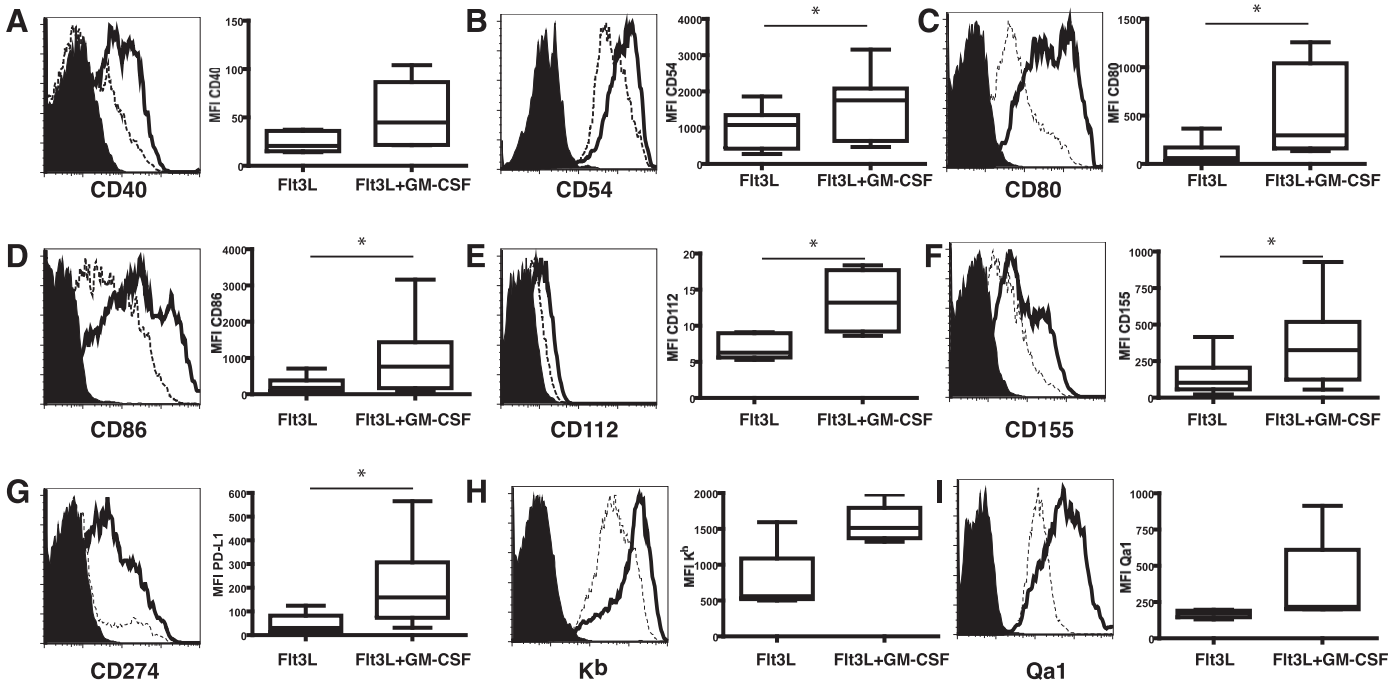

Figure 3. Expression of selected molecules on cDCs after GM-CSF stimulation. Comparison of MFI between unstimulated and GM-CSF-stimulated Flt3L DCs for (A) CD40, (B) CD54, (C) CD80, (D) CD86, (E) CD112, (F) CD155, (G) PD-L1 (CD274), (H) MHC I (K^b), and (I) Qa1. *$P < 0.05$, paired t test, n = at least 4 experiments.

$P < 0.01$), CD86 (MFI 32 ± 7 vs. 50 ± 7; $P < 0.05$), CD155 (MFI 15 ± 4 vs. 20 ± 3; $P < 0.05$), and PD-L1 (MFI 24 ± 15 vs. 76 ± 10; $P < 0.01$) in the mice treated with IL-18 when compared with mice injected with PBS alone. Expression of MHC I molecules was similar between treated and untreated mice.

PD-1–PD-L1, CD80–CD86, and CD40L do not play a role in NK cell-mediated killing of DCs

GM-CSF stimulation of DCs could increase PD-L1 expression, which has been described previously [31, 32]. However, whereas PD-1 has been identified on NK cells [33–36], we could not detect surface expression of this molecule on the IL-2-stimulated NK cells (data not shown). Furthermore, there was no significant difference in the ability of IL-2-stimulated NK cells from PD-1 × $RAG1^{-/-}$ mice to kill DCs when compared with NK cells from the $RAG1^{-/-}$ mice (**Fig. 4A**). When anti-PD-L1 antibody was added to the cytotoxicity culture, there was a slight increase in cytotoxicity of the DCs, on average, but it was not significantly different when compared with control antibodies (15.6% ± 5.1 cytotoxicity with control antibody vs. 24.0 ± 7.9% cytotoxicity with anti-PD-L1 antibody, n = 5; Fig. 4B). It is ntoteworthy that PD-L1 is also expressed on NK cells, and some studies have demonstrated that PD-L1 may form interactions with CD80 and CD86 [37]. However, even though we detected increased expression CD80 and CD86 on the GM-CSF-stimulated cDCs, we found that GM-CSF-stimulated DCs from CD80 × $CD86^{-/-}$ mice were equally sensitive to NK cell-mediated lysis as wild-type DCs (Fig. 4C). Like CD80 and CD86, CD40 has been associated with NK cell-mediated killing [38, 39] and was associated with NK cell-mediated killing of autologous human DCs [12]. However, when testing this possibility, we found that the blocking of CD40L on NK cells did not alter the killing of the GM-CSF-stimulated DCs (Fig. 4D).

CD54 and CD226 drive NK cell-mediated killing of DCs

CD54 has a critical role in NK cell-mediated killing by focusing the cytotoxic synapse and arranging granular release between the effector and target cells [40, 41]. By adding anti-CD54 antibody to the cell cultures, there was a significant reduction in cytotoxicity of the DCs stimulated with GM-CSF (15.5% ± 5.9 cytotoxicity with control antibody vs. 7.8 ± 3.7% cytotoxicity with anti-CD54 antibody, n = 4; $P < 0.05$, paired t test; **Fig. 5A**). Thus, NK cell-mediated lysis of GM-CSF-stimulated cDCs was, in part, a result of increased expression of CD54.

We have previously reported that CD226 was involved in the cytotoxicity of GM-CSF-derived BMDCs in vitro [27]. In the present study, the addition of anti-CD226 antibody to the culture could inhibit NK cell-mediated lysis of GM-CSF-stimulated, Flt3L-derived cDCs (34.7% ±5.1 cytotoxicity with control antibody vs. 13.0 ± 8.6% cytotoxicity with anti-CD226 antibody; $P < 0.05$, paired test, n = 3; Fig. 5B). We have previously postulated that the coexpression of CD226 and NKG2A on NK cells might be a means of controlling NK cell-mediated killing of DCs [16, 27]. Therefore, we sorted NK cells into $CD226^+NKG2A^+$, $CD226^-NKG2A^-$, or $CD226^+NKG2A^-$ subpopulations and examined their cytotoxic capacity against GM-CSF-stimulated cDCs. The $CD226^+NKG2A^-$ NK cells exhibited significantly greater cytotoxicity of the GM-CSF-stimulated cDCs compared with the $CD226^+NKG2A^+$ populations at the 10:1 (24.3 ± 7.6 vs. 14.6 ± 7; $P < 0.05$, paired t test, n = 7) and 3:1 (17.3 ± 8.6 vs. 7.5 ± 5; $P < 0.05$, paired t test, n = 7; Fig. 5C) ratios. Furthermore, compared with the $CD226^-NKG2A^-$ populations, the $CD226^+NKG2A^-$

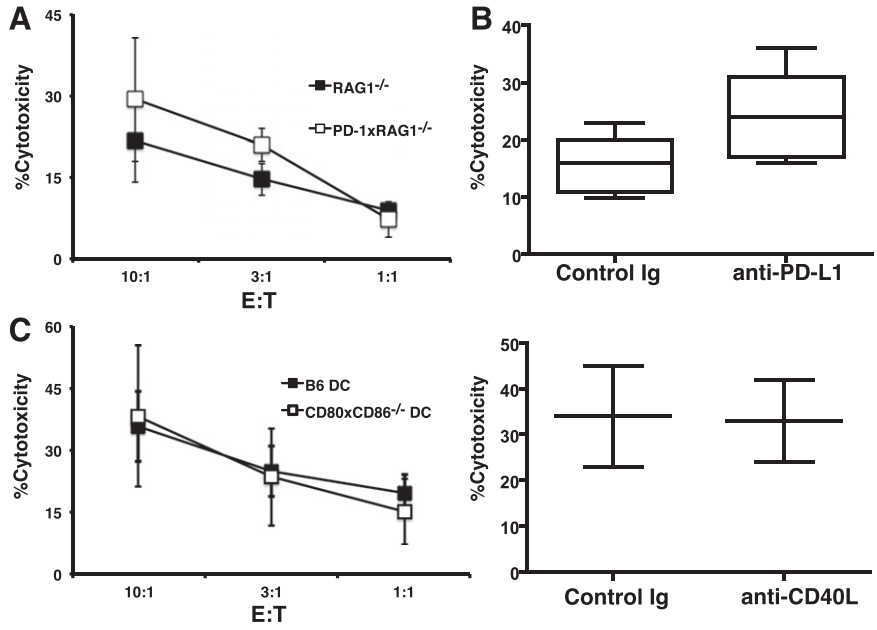

Figure 4. PD-1–PD-L1, CD80–CD86, and CD40L do not affect NK cell-mediated lysis of DCs. (A) NK cell-mediated killing of DCs by PD-1 × RAG1$^{-/-}$ NK cells (open squares; $n = 3$). (B) Anti-PD-L1 antibody does not significantly affect NK cell-mediated lysis of DCs ($n = 5$, 10:1 ratio). (C) NK cell-mediated killing of CD80 × CD86$^{-/-}$ DCs (open squares; $n = 3$) by NK cells. (D) Anti-CD40L antibody does not affect NK cell-mediated lysis of DCs ($n = 2$, 10:1 ratio).

populations were also more cytotoxic (24.3 ± 7.6 vs. 18.1 ± 10.2 and 17.3 ± 8.6 vs. 11.2 ± 7.8; $P < 0.05$, paired t test, $n = 7$; Fig. 5C).

Lack of CD155 increases sensitivity to NK cell-mediated lysis

To investigate further the mechanisms by which NK cells mediate DC lysis, we examined the involvement of CD155 and CD112, the 2 known CD226 interaction partners identified thus far [42]. To examine if CD155 up-regulation played a role in NK cell-mediated lysis of DCs, we generated DCs with Flt3L from CD155$^{-/-}$ mice. Although these cells were not as sensitive as DCs generated from β_2m$^{-/-}$ mice, there were significant differences in their NK cell sensitivity when compared with wild-type DCs (Fig. 5D and E). This surprising result may be a result of the fact

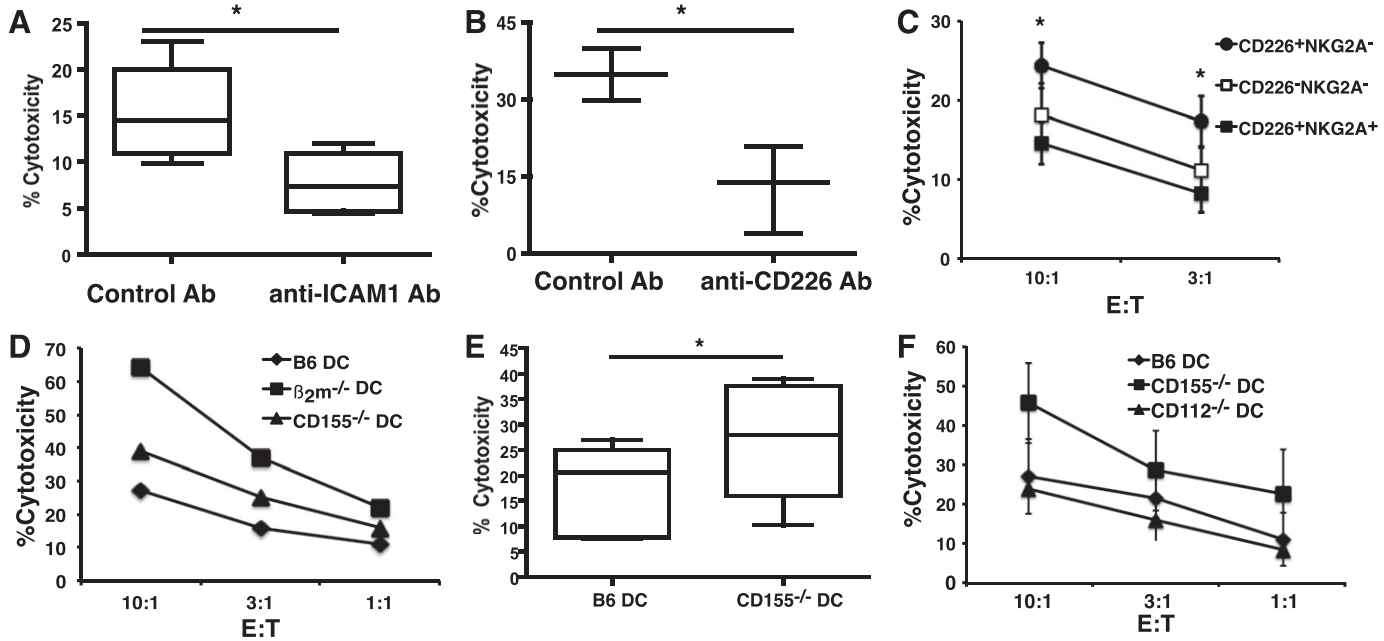

Figure 5. CD54 and CD226 induce NK cell-mediated lysis of cDCs, but CD155 may inhibit lysis. (A) Anti-CD54 antibody inhibits NK cell killing of DCs (*$P < 0.05$, paired t test, $n = 4$, 10:1 ratio). (B) The blocking of anti-CD226 inhibits NK cell-mediated lysis of DCs (*$P < 0.05$, paired t test, $n = 3$). (C) CD226 and NKG2A subsets of NK cells exhibit preferential killing of DCs (*$P < 0.05$, repeated-measures ANOVA with Bonferroni posttest, $n = 7$). (D) Lysis of CD155$^{-/-}$ DCs and β_2m$^{-/-}$ DCs by NK cells (1 representative experiment of 7). (E) Comparison of NK cell-mediated lysis of wild-type and CD155$^{-/-}$ DCs at E:T of 10:1 (*$P < 0.05$, paired t test, $n = 7$). (F) Comparison of NK cell-mediated killing of DCs from wild-type CD155$^{-/-}$ and CD112$^{-/-}$ mice ($n = 3$).

that NK cells also express CD96 and TIGIT, which are inhibitory receptors on NK cells that bind CD155 as well. However, whereas CD112 is also a ligand for CD226, we did not see any difference in DC sensitivity to NK cell-mediated lysis in mice lacking this molecule, suggesting that CD112 does not play a role in NK cell-mediated killing of DCs (Fig. 5F).

DISCUSSION

We have previously hypothesized that NK cell-mediated killing of DCs is a means by which inflammatory responses induced by DCs could be limited [43]. Data that supported this hypothesis came from studies demonstrating NK cell-mediated lysis of DCs generated from monocytes or BM cells with GM-CSF, which are equivalent to the TiP DCs found in vivo and arise during inflammation [44, 45]. However, this implies that the previous studies of NK cell-mediated killing of DCs have been biased toward inflammatory monocytes rather than cDCs. In the present study, we have shown that cDCs from Flt3L BM cultures were not sensitive to NK cell-mediated lysis. Importantly, this was also observed when testing ex vivo cDCs isolated from spleen. However, upon stimulation with GM-CSF, NK cells could mediate cytotoxicity of cDCs, which was, in part, a result of up-regulation of CD54 on the surface of the DCs and of the $CD226^{+}NKG2A^{-}$ subset of NK cells. Furthermore, GM-CSF produced by NK cells could also increase the sensitivity of cDCs to NK cell-mediated lysis. In vivo, injection of the proinflammatory cytokine IL-18 also led to increased NK cell-mediated killing of cDCs. Under these conditions, anti-GM-CSF antibodies could only partially inhibit cDC death, suggesting that other proinflammatory cytokines might play a role in aiding NK cell-mediated killing of cDCs. Together, these data highlight that NK cells do not attack cDCs until an inflammatory response is induced and that specialized NK cells can play a role in cDC elimination.

In the present study, NK cell-mediated cytotoxicity of DCs was inhibited by antibodies directed against CD54 and CD226. CD54 was 1 of the first identified ligands to trigger NK cell-mediated cytotoxicity through LFA-1 [40, 41, 46], so it may not be surprising that increased expression of CD54 on activated DCs leads to their sensitivity to cytotoxicity. However, the receptors for CD54, LFA-1, and CD226 are found associated with each other in the lipid rafts on NK cells, and they work synergistically to activate NK cells [47–49]. Phosphorylation of CD226 at Ser329 leads to the association of CD226 with LFA-1, which then recruits Fyn to phosphorylate the Tyr322 of CD226 that leads to Sh2 domain-containing leukocyte protein of 76 kDa (SLP-76) and Vav-1 phosphorylation. More recently, CD226-mediated cytotoxicity was also found to be involved directly via growth factor receptor-bound protein 2 [50]. This suggests that GM-CSF induction of CD155 and CD54 expression may act as a signal for DCs to make them more vulnerable to NK cells and to aid in their elimination during inflammation.

We [27] and others [51] have previously found that $CD226^{+}$ NK cells are more cytotoxic than $CD226^{-}$ NK cells and may produce more cytokines. Furthermore, NK cells express different levels of receptors and chemokines related to their expression of CD226 [27, 51], which was further related to the maturation state of the NK cells [51]. In the present study, the $CD226^{+}NKG2A^{-}$ NK cells were the most proficient NK cell subset in killing DCs, which is in line with our previous hypothesis that the mouse NK cells may be subdivided into subpopulations of CD226 and NKG2A to regulate the ability of NK cells to kill DCs [16, 27]. Although the $CD226^{+}NKG2A^{-}$ population of NK cells is 1 of the smaller populations of NK cells, they were the best killers of GM-CSF-stimulated DCs, which could be related to the fact that these cells may be more mature [51] but also would not be inhibited from killing by nonclassic MHC I molecules on the DCs after GM-CSF treatment. Thus, our data and the study of Martinet et al. [51] suggest that expression of CD226, in combination with other receptors on NK cells, may impart NK cells for specific niche functions. In contrast to mice, all human NK cells express CD226, and like mouse NK cells, CD226 is involved in killing of human monocyte-derived DCs [52, 53]. However, human CMV infection of human monocyte-derived DCs reduces expression of CD226 ligands over time, making these DCs less sensitive to NK cell-mediated lysis. In this case, the CD94/$NKG2A^{+}$ NK cells appear to play a great role in killing these DCs [53]. Therefore, we can hypothesize that the immune system may have evolved to have different subsets of NK cells expressing CD226 and NKG2A as means to control pathogens and to regulate DC numbers during infection.

Although the blocking of CD226 could inhibit NK cell-mediated killing of DCs, it was surprising that DCs lacking CD155 were more sensitive to NK cell-mediated lysis. However, this can be explained, in part, by the complex nature of paired receptors on NK cells [54], which also express 2 additional inhibitory receptors that can bind CD155, namely CD96 and TIGIT [54]. Thus, when CD155 is up-regulated on DCs, there could be competition between the activation and inhibitory signals induced by CD155, which controls the sensitivity of the DCs to NK cell-mediated killing. Furthermore, we cannot rule out that in the absence of CD155, CD112 might also be made available for recognition by NK cells; however, it is still unclear if mouse NK cells can recognize CD112 [54]. Mice lacking TIGIT or CD96 have demonstrated their roles in autoimmunity and anti-tumor immunity [55–59], but mice lacking CD96 or TIGIT have not been used to address the role of these molecules in NK cell-mediated killing of DCs. However, it would appear that these receptors, in combination with NKG2A, act as further controls in regulating the extent of DC killing that might occur during inflammation, thus allowing a balance, whereby some DCs are eliminated, but allowing others to stimulate the adaptive immune system.

Whereas we observed that GM-CSF up-regulated costimulatory molecules, there was also increased expression of immunoinhibitory molecules, such as PD-L1, which binds to PD-1 on T cells, and antibodies to either of these molecules have been shown to have beneficial effects in anti-tumor therapy [60]. Although PD-1 has been reported on NK cells [33–36], to date, there has been little published about the effects of anti-PD-1 or -PD-L1 antibody treatments on NK cell function [34, 35]. PD-1 on NK cells has been found in inflammatory conditions, where IL-18 has been added to NK cells [35] or during chronic viral infection [36]. This suggests that during inflammatory conditions, PD-1–PD-L1 interactions might play an important role in limiting NK cell-mediated activation. In the present study, we did see increased lysis of DCs

when anti-PD-L1 antibody was added to the cultures, but it was not significantly different from control antibody treatment, which suggested that PD-L1 might only play a minor role in inhibiting NK cells from eliminating DCs. However, PD-L1 can also be expressed on activated NK cells, so it is unclear whether the antibodies are blocking PD-L1 on the NK cells, DCs, or both. As there are increasing numbers of clinical studies using anti-PD-1 and -PD-L1 antibodies [60], it will be interesting to see if there are any effects of these antibodies on NK cells from these patients.

We have previously postulated a model to explain why NK cells would target DCs for killing [43]. With the current study, we have further elucidated the mechanisms of how this model works. We propose that upon encountering a pathogen, DCs could release NK cell-activating cytokines, such as IL-12, IL-15, IL-18, and TNF, as well as chemokines, to attract NK cells to the site of infection. The combination of cytokines could induce GM-CSF and IFN-γ production, which would then initiate cytotoxic T cell function but also feedback on the DCs, including uninfected DCs, to make them sensitive to NK cells. The elimination of bystander DCs would be important in this case, as it would limit and focus T cell responses to only infected cells and make immune responses more efficient, as well as potentially eliminating DCs expressing autoantigens [61]. The infected DCs could also be targeted by NK cells to limit the infection, and the resulting debris would be processed by other DCs for presentation to T cells through (e.g., cross-priming). Furthermore, expression of PD-L1, as well as classic and nonclassic MHC I molecules, may limit the extent/speed of elimination of DCs by NK cells, which again, could allow T cells to find their antigen and become activated. Thus, by this means, the immune system can balance the elimination of infected cells while developing, at the same time, a specific adaptive immune response to a pathogen.

AUTHORSHIP

L.E.S., M.A.O., A-M.G, A.K.W., M.D.-V., and T.H. designed and performed experiments. B.J.C., G.B., M.C.I.K., and F.G.-C. designed experiments. I.R., G.B., and S.M. generated mice.

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DISCLOSURES

The authors declare no conflicts of interests.

REFERENCES

1. Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., Leenen, P. J., Liu, Y. J., MacPherson, G., Randolph, G. J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J. M., Lutz, M. B. (2010) Nomenclature of monocytes and dendritic cells in blood. *Blood* **116,** e74–e80.
2. Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H. W., Park, C. G., Steinman, R. M., Nussenzweig, M. C. (2007) Differential antigen processing by dendritic cell subsets in vivo. *Science* **315,** 107–111.
3. Shortman, K., Liu, Y. J. (2002) Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol.* **2,** 151–161.
4. Degli-Esposti, M. A., Smyth, M. J. (2005) Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat. Rev. Immunol.* **5,** 112–124.
5. Buentke, E., Heffler, L. C., Wilson, J. L., Wallin, R. P., Löfman, C., Chambers, B. J., Ljunggren, H. G., Scheynius, A. (2002) Natural killer and dendritic cell contact in lesional atopic dermatitis skin—Malassezia-influenced cell interaction. *J. Invest. Dermatol.* **119,** 850–857.
6. Martín-Fontecha, A., Thomsen, L. L., Brett, S., Gerard, C., Lipp, M., Lanzavecchia, A., Sallusto, F. (2004) Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat. Immunol.* **5,** 1260–1265.
7. Persson, C. M., Chambers, B. J. (2010) Plasmacytoid dendritic cell-induced migration and activation of NK cells in vivo. *Eur. J. Immunol.* **40,** 2155–2164.
8. Korsgren, M., Persson, C. G., Sundler, F., Bjerke, T., Hansson, T., Chambers, B. J., Hong, S., Van Kaer, L., Ljunggren, H. G., Korsgren, O. (1999) Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. *J. Exp. Med.* **189,** 553–562.
9. Yuan, D., Wilder, J., Dang, T., Bennett, M., Kumar, V. (1992) Activation of B lymphocytes by NK cells. *Int. Immunol.* **4,** 1373–1380.
10. Shah, P. D., Gilbertson, S. M., Rowley, D. A. (1985) Dendritic cells that have interacted with antigen are targets for natural killer cells. *J. Exp. Med.* **162,** 625–636.
11. Chambers, B. J., Salcedo, M., Ljunggren, H. G. (1996) Triggering of natural killer cells by the costimulatory molecule CD80 (B7-1). *Immunity* **5,** 311–317.
12. Carbone, E., Terrazzano, G., Ruggiero, G., Zanzi, D., Ottaiano, A., Manzo, C., Kärre, K., Zappacosta, S. (1999) Recognition of autologous dendritic cells by human NK cells. *Eur. J. Immunol.* **29,** 4022–4029.
13. Wilson, J. L., Heffler, L. C., Charo, J., Scheynius, A., Bejarano, M. T., Ljunggren, H. G. (1999) Targeting of human dendritic cells by autologous NK cells. *J. Immunol.* **163,** 6365–6370.
14. Parajuli, P., Nishioka, Y., Nishimura, N., Singh, S. M., Hanibuchi, M., Nokihara, H., Yanagawa, H., Sone, S. (1999) Cytolysis of human dendritic cells by autologous lymphokine-activated killer cells: participation of both T cells and NK cells in the killing. *J. Leukoc. Biol.* **65,** 764–770.
15. Hayakawa, Y., Screpanti, V., Yagita, H., Grandien, A., Ljunggren, H. G., Smyth, M. J., Chambers, B. J. (2004) NK cell TRAIL eliminates immature dendritic cells in vivo and limits dendritic cell vaccination efficacy. *J. Immunol.* **172,** 123–129.
16. Persson, C. M., Assarsson, E., Vahlne, G., Brodin, P., Chambers, B. J. (2008) Critical role of Qa1b in the protection of mature dendritic cells from NK cell-mediated killing. *Scand. J. Immunol.* **67,** 30–36.
17. Della Chiesa, M., Vitale, M., Carlomagno, S., Ferlazzo, G., Moretta, L., Moretta, A. (2003) The natural killer cell-mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. *Eur. J. Immunol.* **33,** 1657–1666.
18. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., Papaioannou, V. E. (1992) RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68,** 869–877.
19. Borriello, F., Sethna, M. P., Boyd, S. D., Schweitzer, A. N., Tivol, E. A., Jacoby, D., Strom, T. B., Simpson, E. M., Freeman, G. J., Sharpe, A. H. (1997) B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity* **6,** 303–313.
20. Mueller, S., Rosenquist, T. A., Takai, Y., Bronson, R. A., Wimmer, E. (2003) Loss of nectin-2 at Sertoli-spermatid junctions leads to male infertility and correlates with severe spermatozoan head and midpiece malformation, impaired binding to the zona pellucida, and oocyte penetration. *Biol. Reprod.* **69,** 1330–1340.
21. Maier, M. K., Seth, S., Czeloth, N., Qiu, Q., Ravens, I., Kremmer, E., Ebel, M., Müller, W., Pabst, O., Förster, R., Bernhardt, G. (2007) The adhesion receptor CD155 determines the magnitude of humoral immune responses against orally ingested antigens. *Eur. J. Immunol.* **37,** 2214–2225.
22. Koller, B. H., Marrack, P., Kappler, J. W., Smithies, O. (1990) Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8+ T cells. *Science* **248,** 1227–1230.
23. Nishimura, H., Minato, N., Nakano, T., Honjo, T. (1998) Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int. Immunol.* **10,** 1563–1572.
24. Gilliet, M., Boonstra, A., Paturel, C., Antonenko, S., Xu, X. L., Trinchieri, G., O'Garra, A., Liu, Y. J. (2002) The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **195,** 953–958.
25. Naik, S. H., Proietto, A. I., Wilson, N. S., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. H., O'Keeffe, M., Shao, Q. X., Chen, W. F., Villadangos, J. A., Shortman, K., Wu, L. (2005) Cutting edge: generation

of splenic CD8+ and CD8− dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* **174,** 6592–6597.
26. Norbury, C. C., Chambers, B. J., Prescott, A. R., Ljunggren, H. G., Watts, C. (1997) Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur. J. Immunol.* **27,** 280–288.
27. Seth, S., Georgoudaki, A. M., Chambers, B. J., Qiu, Q., Kremmer, E., Maier, M. K., Czeloth, N., Ravens, I., Foerster, R., Bernhardt, G. (2009) Heterogeneous expression of the adhesion receptor CD226 on murine NK and T cells and its function in NK-mediated killing of immature dendritic cells. *J. Leukoc. Biol.* **86,** 91–101.
28. Oberg, L., Johansson, S., Michaëlsson, J., Tomasello, E., Vivier, E., Kärre, K., Höglund, P. (2004) Loss or mismatch of MHC class I is sufficient to trigger NK cell-mediated rejection of resting lymphocytes in vivo—role of KARAP/DAP12-dependent and -independent pathways. *Eur. J. Immunol.* **34,** 1646–1653.
29. Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E., Caligiuri, M. A. (2001) Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* **97,** 3146–3151.
30. Giroux, M., Yurchenko, E., St-Pierre, J., Piccirillo, C. A., Perreault, C. (2007) T Regulatory cells control numbers of NK cells and CD8alpha+ immature dendritic cells in the lymph node paracortex. *J. Immunol.* **179,** 4492–4502.
31. Yamazaki, T., Akiba, H., Iwai, H., Matsuda, H., Aoki, M., Tanno, Y., Shin, T., Tsuchiya, H., Pardoll, D. M., Okumura, K., Azuma, M., Yagita, H. (2002) Expression of programmed death 1 ligands by murine T cells and APC. *J. Immunol.* **169,** 5538–5545.
32. Gaudreau, S., Guindi, C., Ménard, M., Benabdallah, A., Dupuis, G., Amrani, A. (2010) GM-CSF induces bone marrow precursors of NOD mice to skew into tolerogenic dendritic cells that protect against diabetes. *Cell. Immunol.* **265,** 31–36.
33. Alvarez, I. B., Pasquinelli, V., Jurado, J. O., Abbate, E., Musella, R. M., de la Barrera, S. S., García, V. E. (2010) Role played by the programmed death-1–programmed death ligand pathway during innate immunity against Mycobacterium tuberculosis. *J. Infect. Dis.* **202,** 524–532.
34. Benson, Jr., D. M., Bakan, C. E., Mishra, A., Hofmeister, C. C., Efebera, Y., Becknell, B., Baiocchi, R. A., Zhang, J., Yu, J., Smith, M. K., Greenfield, C. N., Porcu, P., Devine, S. M., Rotem-Yehudar, R., Lozanski, G., Byrd, J. C., Caligiuri, M. A. (2010) The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* **116,** 2286–2294.
35. Terme, M., Ullrich, E., Aymeric, L., Meinhardt, K., Desbois, M., Delahaye, N., Viaud, S., Ryffel, B., Yagita, H., Kaplanski, G., Prévost-Blondel, A., Kato, M., Schultze, J. L., Tartour, E., Kroemer, G., Chaput, N., Zitvogel, L. (2011) IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res.* **71,** 5393–5399.
36. Golden-Mason, L., Klarquist, J., Wahed, A. S., Rosen, H. R. (2008) Cutting edge: programmed death-1 expression is increased on immunocytes in chronic hepatitis C virus and predicts failure of response to antiviral therapy: race-dependent differences. *J. Immunol.* **180,** 3637–3641.
37. Butte, M. J., Keir, M. E., Phamduy, T. B., Sharpe, A. H., Freeman, G. J. (2007) Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* **27,** 111–122.
38. Carbone, E., Ruggiero, G., Terrazzano, G., Palomba, C., Manzo, C., Fontana, S., Spits, H., Kärre, K., Zappacosta, S. (1997) A new mechanism of NK cell cytotoxicity activation: the CD40–CD40 ligand interaction. *J. Exp. Med.* **185,** 2053–2060.
39. Martín-Fontecha, A., Assarsson, E., Carbone, E., Kärre, K., Ljunggren, H. G. (1999) Triggering of murine NK cells by CD40 and CD86 (B7-2). *J. Immunol.* **162,** 5910–5916.
40. Chong, A. S., Boussy, I. A., Jiang, X. L., Lamas, M., Graf, Jr., L. H. (1994) CD54/ICAM-1 is a costimulator of NK cell-mediated cytotoxicity. *Cell. Immunol.* **157,** 92–105.
41. Topham, N. J., Hewitt, E. W. (2009) Natural killer cell cytotoxicity: how do they pull the trigger? *Immunology* **128,** 7–15.
42. Georgiev, H., Danisch, S., Chambers, B. J., Shibuya, A., Forster, R., Bernhardt, G. (2014) To the editor: TIGIT versus CD226: hegemony or coexistence? *Eur. J. Immunol.* **44,** 307–308.
43. Chambers, B. J., Ljunggren, H. G. (1999) NK cells. In *Dendritic Cells* (M. T. Lotze, A. W. Thomson, eds.), Academic Press, San Diego, 257–268.
44. Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A., Pamer, E. G. (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* **19,** 59–70.
45. Naik, S. H., Metcalf, D., van Nieuwenhuijze, A., Wicks, I., Wu, L., O'Keeffe, M., Shortman, K. (2006) Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat. Immunol.* **7,** 663–671.
46. Barber, D. F., Faure, M., Long, E. O. (2004) LFA-1 contributes an early signal for NK cell cytotoxicity. *J. Immunol.* **173,** 3653–3659.
47. Shirakawa, J., Wang, Y., Tahara-Hanaoka, S., Honda, S., Shibuya, K., Shibuya, A. (2006) LFA-1-dependent lipid raft recruitment of DNAM-1 (CD226) in CD4+ T cell. *Int. Immunol.* **18,** 951–957.
48. Shirakawa, J., Shibuya, K., Shibuya, A. (2005) Requirement of the serine at residue 329 for lipid raft recruitment of DNAM-1 (CD226). *Int. Immunol.* **17,** 217–223.
49. Shibuya, K., Shirakawa, J., Kameyama, T., Honda, S., Tahara-Hanaoka, S., Miyamoto, A., Onodera, M., Sumida, T., Nakauchi, H., Miyoshi, H., Shibuya, A. (2003) CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation. *J. Exp. Med.* **198,** 1829–1839.
50. Zhang, Z., Wu, N., Lu, Y., Davidson, D., Colonna, M., Veillette, A. (2015) DNAM-1 controls NK cell activation via an ITT-like motif. *J. Exp. Med.* **212,** 2165–2182.
51. Martinet, L., Ferrari De Andrade, L., Guillerey, C., Lee, J. S., Liu, J., Souza-Fonseca-Guimaraes, F., Hutchinson, D. S., Kolesnik, T. B., Nicholson, S. E., Huntington, N. D., Smyth, M. J. (2015) DNAM-1 expression marks an alternative program of NK cell maturation. *Cell Reports* **11,** 85–97.
52. Pende, D., Bottino, C., Castriconi, R., Cantoni, C., Marcenaro, S., Rivera, P., Spaggiari, G. M., Dondero, A., Carnemolla, B., Reymond, N., Mingari, M. C., Lopez, M., Moretta, L., Moretta, A. (2005) PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis. *Mol. Immunol.* **42,** 463–469.
53. Magri, G., Muntasell, A., Romo, N., Sáez-Borderías, A., Pende, D., Geraghty, D. E., Hengel, H., Angulo, A., Moretta, A., López-Botet, M. (2011) NKp46 and DNAM-1 NK-cell receptors drive the response to human cytomegalovirus-infected myeloid dendritic cells overcoming viral immune evasion strategies. *Blood* **117,** 848–856.
54. Martinet, L., Smyth, M. J. (2015) Balancing natural killer cell activation through paired receptors. *Nat. Rev. Immunol.* **15,** 243–254.
55. Chan, C. J., Martinet, L., Gilfillan, S., Souza-Fonseca-Guimaraes, F., Chow, M. T., Town, L., Ritchie, D. S., Colonna, M., Andrews, D. M., Smyth, M. J. (2014) The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat. Immunol.* **15,** 431–438.
56. Stanietsky, N., Mandelboim, O. (2010) Paired NK cell receptors controlling NK cytotoxicity. *FEBS Lett.* **584,** 4895–4900.
57. Stanietsky, N., Rovis, T. L., Glasner, A., Seidel, E., Tsukerman, P., Yamin, R., Enk, J., Jonjic, S., Mandelboim, O. (2013) Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur. J. Immunol.* **43,** 2138–2150.
58. Levin, S. D., Taft, D. W., Brandt, C. S., Bucher, C., Howard, E. D., Chadwick, E. M., Johnston, J., Hammond, A., Bontadelli, K., Ardourel, D., Hebb, L., Wolf, A., Bukowski, T. R., Rixon, M. W., Kuijper, J. L., Ostrander, C. D., West, J. W., Bilsborough, J., Fox, B., Gao, Z., Xu, W., Ramsdell, F., Blazar, B. R., Lewis, K. E. (2011) Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur. J. Immunol.* **41,** 902–915.
59. Joller, N., Hafler, J. P., Brynedal, B., Kassam, N., Spoerl, S., Levin, S. D., Sharpe, A. H., Kuchroo, V. K. (2011) Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J. Immunol.* **186,** 1338–1342.
60. Topalian, S. L., Drake, C. G., Pardoll, D. M. (2012) Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr. Opin. Immunol.* **24,** 207–212.
61. Morandi, B., Mortara, L., Chiossone, L., Accolla, R. S., Mingari, M. C., Moretta, L., Moretta, A., Ferlazzo, G. (2012) Dendritic cell editing by activated natural killer cells results in a more protective cancer-specific immune response. *PLoS One* **7,** e39170.

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