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References

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NK Cells Influence Both Innate and Adaptive Immune Responses after Mucosal Immunization with Antigen and Mucosal Adjuvant

Lindsay J. Hall, Simon Clare, and Gordon Dougan

NK cells were found to be recruited in a temporally controlled manner to the nasal-associated lymphoid tissue and the cervical lymph nodes of mice after intranasal immunization with Ag85B-early secreted antigenic target 6kDa from *Mycobacterium tuberculosis* mixed with *Escherichia coli* heat-labile toxin as adjuvant. These NK cells were activated and secreted a diverse range of cytokines and other immunomodulators. Using Ab depletion targeting anti-asialo GM1, we found evidence for altered trafficking, impaired activation, and cytokine secretion of dendritic cells, macrophages, and neutrophils in immunized NK cell-depleted mice compared with control animals. Analysis of Ag-specific immune responses revealed an attenuated Ab and cytokine response in immunized NK cell-depleted animals. Systemic administration of rIL-6 but not rIFN- γ significantly restored immune responses in mice depleted of NK cells. In conclusion, cytokine production, particularly IL-6, via NK cells and NK cell-activated immune populations plays an important role in the establishment of local innate immune responses and the consequent development of adaptive immunity after mucosal immunization. *The Journal of Immunology*, 2010, 184: 4327–4337.

In recent years, the mucosal immunization route has emerged as an attractive option for inducing both local and systemic immunity (1–3). In rodents, tissues involved in the generation of such responses after intranasal (i.n.) immunization include nasal-associated lymphoid tissue (NALT) and draining cervical lymph nodes (CLNs) (4–8). Intranasal immunization with Ag mixed with mucosal adjuvants, such as the heat-labile toxin (LT) from *Escherichia coli*, is a particularly efficient regimen to stimulate both mucosal and systemic Ag-specific responses. In addition, LT-based adjuvants can induce potent adaptive T_H1 immune responses, involving significant production of Ag-specific IFN- γ and the generation of cytotoxic T cell responses. For example, purified *Mycobacterium tuberculosis* fusion Ag85B-early secreted antigenic target 6 kDa (Ag85B-ESAT6) has been shown to induce potent Ag-specific immune responses when co-administered i.n. with an LT-based adjuvant generating significant immune responses and protection after *M. tuberculosis* challenge (9, 10).

NK cells can contribute to host resistance to viruses, bacteria and certain parasites as well as providing immune surveillance against the development of tumors (11–13). NK cells mediate their effects through cellular cytotoxicity alongside the production of a range of cytokines and other immunoregulatory mediators (14–19). Early

resistance to intracellular pathogens, including *M. tuberculosis*, can be driven by IFN- γ production and lysis of infected cells and through modulatory effects on T regulatory responses (20–22). Several studies have suggested that crosstalk between NK cells and APCs, such as dendritic cells (DCs) and macrophages (M Φ s), during the early phases of the immune response influences the type of adaptive immunity that follows (23–25). Such cellular cross talk can involve activation of both the NK cell and APC populations—the nature of such responses influencing subsequent T and B cell activities (26). Another study has highlighted the importance of NK-DC cellular interactions in vivo within the lymph nodes of mice infected with *Leishmania major* and the consequent activation of CD4⁺ T cells (27). Other evidence indicates that early IFN- γ production by NK cells can affect the characteristics of Ag-specific immune response, particularly by driving T_H1 polarization (28–31).

Because NK cells are major producers of proinflammatory cytokines and have the ability to closely interact with other innate populations, we sought to uncover and define their role in the generation of local and systemic immune responses after mucosal vaccination. We used i.n. immunization as a tool to examine local NK cell responses in both the NALT and CLN and the administration of anti-asialo GM1 (anti-AGM1) for the ablation of NK cells in vivo. In this study, we present evidence that recruitment of NK cells shortly after i.n. immunization modulates trafficking and activation of other innate cell populations to the NALT and CLN. These early local events consequently affect the magnitude of systemic Ag-specific responses and are linked to NK cell and NK cell-associated IL-6 production.

Materials and Methods

Animals and in vivo experimental protocols

Female BALB/c mice (5–6-wk-old) from Charles River (Margate, U.K.) were used in all animal experiments. All animals were given food and water ad libitum. Mice were sacrificed by cervical dislocation or exsanguination. Animal husbandry and experimental procedures were conducted according to the United Kingdom Animals (Scientific Procedures) Act of 1986. For immunizations, mice were immunized i.n. on day 0 with PBS (negative controls) or 1 μ g LT plus 25 μ g Ag85B-ESAT6 in a volume of 15 μ l per nostril. The adjuvant LT was a gift from Novartis, Siena, Italy, and purified Ag85B-ESAT6 was obtained from the Statens Serum Institute,

Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom

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Abbreviations used in this paper: Ag85B-ESAT6, Ag85B-early secreted antigenic target 6 kDa; AGM1, asialo GM1; BZ, B cell zone; CLN, cervical lymph node; DC, dendritic cell; i.n., intranasal; LT, heat-labile toxin; M Φ , macrophage; MFI, mean fluorescence intensity; NALT, nasal-associated lymphoid tissue; OD, optical density; TZ, T cell zone.

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Copenhagen, Denmark. NK cells were depleted from BALB/c mice by administration of 50 μ g functional grade rabbit anti-AGM1 Ab (Wako Chemicals, Neuss, Germany) in 200 μ l PBS i.v. or 300 μ l i.p. injection (32, 33). For the depletion of NK cells from both the NALT and CLNs, mice were depleted i.n. with 5 μ g anti-AGM1 in 10 μ l PBS. Control animals received 50 or 5 μ g of appropriate functional grade rabbit isotype control Ab (rabbit IgG, R&D Systems, Abingdon, U.K.). Injections were performed 7 days prior to Ag immunization and repeated every 3–4 d to maintain depletion. Intranasal depletions were performed 5 d prior to immunization between injections. During experiments involving administration of exogenous murine IL-6 and IFN- γ , mice were depleted of NK cells and immunized as described earlier. rIL-6 had a sp. act. of 5×10^7 U/mg, and rIFN- γ had a sp. act. of 1×10^7 U/mg (PeproTech EC, London, U.K.). The mice were then injected i.v. with 2 μ g rIL-6 or 10 μ g rIFN- γ diluted in 200 μ l PBS on day 1 and 2 postimmunization.

Immunofluorescent staining

Mice were sacrificed at 5, 24, and 72 h after i.n. immunization, and the CLNs and NALT were removed (34). The CLNs and NALT were snap-frozen in liquid nitrogen and 6- μ m sections were cut. Frozen sections were stained with primary mAb as specified in Supplemental Table 1 according to Bajénoff et al. (27). Hoechst (Invitrogen, Paisley, U.K.) was used as a nuclear counterstain.

Cytometric bead array cytokine analysis

Spleens were removed from mice 21 d postimmunization and seeded in duplicate at a concentration of 5×10^5 cells per well. Splenocytes were stimulated with either Ag85B-ESAT6 or Con A (both at 5 μ g/ml) or RPMI 1640 medium. Plates were incubated at 37°C and 5% CO₂ for 36–48 h. Cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IFN- γ , and TNF- α were analyzed using cytometric bead analysis flexi-kits (BD Biosciences, San Jose, CA), and assays were performed per the manufacturer's instructions. Samples were then analyzed on a FACSAria flow cytometer (BD Biosciences). The limit of detection was ~ 8 pg/ml for all cytokines.

ELISA analysis of cytokines

Serum samples were isolated from mice on days 1, 7, 14, and 21 postimmunization and analyzed for IL-6, IL-22, IFN- γ , and IL-10 levels. Supernatants from stimulated splenocytes (see above) were also analyzed for IL-22. ELISAs were performed per the manufacturer's instructions (Bender MedSystems, Vienna, Austria).

Flow cytometry

Single-cell suspensions from the NALT, CLNs, and spleens of individual mice were prepared to obtain a final concentration of 5×10^5 cells per well in blocking buffer (1 $\times$ PBS/1% BSA/0.05% sodium azide/1% rat, hamster, and mouse serum). To this buffer, 50 μ l of each mAb dye mix was added plus 5 μ l of the amine-reactive viability dye ViViD (Invitrogen) to determine dead cells, with incubation in the dark at 4°C for 30 min (35). The mAb used for flow cytometry are listed in Supplemental Table 1. Cells were washed twice with blocking buffer and finally resuspended in 200 μ l 1% paraformaldehyde. To perform flow cytometric analyses and measure relative fluorescence intensities a FACSAria cytometer and BD Diva software (BD Biosciences) were used. For each mouse, 20,000–200,000 events were recorded. The percentage of cells labeled with each mAb was calculated in comparison with cells stained with isotype control Ab. Background staining was controlled by labeled isotype controls (BD Biosciences, Caltag Laboratories [Burlingame, CA], and Serotec [Oxford, U.K.]) and fluorescence-minus-one. The results represent the percentage of positively stained cells in the total cell population exceeding the background staining signal. For determination of intracellular cytokine production by leukocytes at earlier time points, cells were incubated for 6 h at 37°C with BD Leukocyte Activation Cocktail with BD GolgiPlug or GolgiPlug alone (BD Biosciences). When examining later responses, cells were incubated for 36–48 h with either Ag85B-ESAT6 or Con A (both at 5 μ g/ml) or RPMI 1640 medium with GolgiPlug added for the final 6 h. Cells were then washed with staining buffer and stained at 4°C with appropriate surface mAbs listed in Supplemental Table 1. Cells were then fixed and saponin-permeabilized (Perm/Fix solution; BD Biosciences) and incubated with cytokine mAbs listed in Supplemental Table 1 or isotypic controls. After 30 min, cells were twice washed in permeabilisation buffer (BD Biosciences) and then analyzed by flow cytometry as described above.

Evaluation of Ab responses

Serum samples from mice were obtained on days 14 and 21 postimmunization and analyzed for the presence of total Ig, IgG, IgG1, IgG2a, IgG2b, IgE, and IgM. Briefly, ELISA plates (Nunc Maxisorp, Rochester, NY) were coated

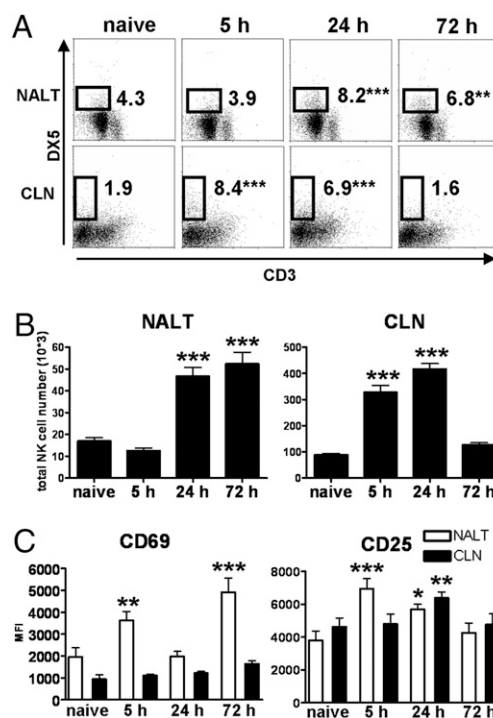


FIGURE 1. Characterization of NK cells isolated from the NALT and CLN at 5, 24, and 72 h after i.n. immunization. Cells were isolated from NALT and CLN of BALB/c mice 5, 24, and 72 h after i.n. immunization with PBS (naive mice) or 1 μ g LT + 25 μ g Ag85B-ESAT6, and stained with fluochrome-labeled mAb and analyzed by flow cytometry in which 20,000–100,000 events were recorded. *A*, Plots shown are representative of at least 10 mice, and the mean NK cell percentages are indicated. *B*, Total NK cell number is shown for each organ. Columns represent the mean $\pm$ SD. *C*, For analysis, gates were set on DX5⁺/CD3⁺ cells and the MFI of CD25 and CD69 expression was determined. Columns represent the mean MFI $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, as determined by one-way ANOVA followed by Bonferroni's multiple comparison test compared with negative control animals (i.e., PBS immunized, naive mice).

overnight at 4°C with 50 μ l of a 2 μ g/ml solution of purified Ag85B-ESAT6 in coating buffer (0.1 M Na₂HPO₄, pH 9) and then blocked with 3.0% BSA at room temperature for 1 h. Serum samples were diluted in 0.1% BSA starting at 1:100. Each plate contained control wells with preimmune sera, PBS (pH 7.4), or a known positive immune serum and incubated for 1 h at 37°C. Abs conjugated to HRP were diluted 1:1000, and plates incubated for an

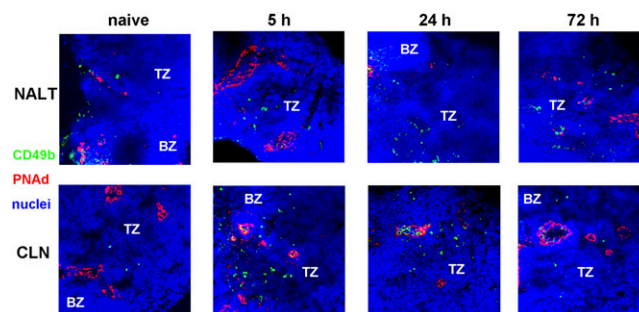


FIGURE 2. In situ visualization of NK cells within the NALT and CLN at 5, 24, and 72 h after i.n. immunization. BALB/c mice were immunized i.n. with PBS (naive) or LT plus Ag85B-ESAT6. NALT and CLNs were collected 5, 24, and 72 h postimmunization. Tissue sections from six individual mice were analyzed at each time point by confocal microscopy (original magnification $\times 28$). For NALT and CLNs, 6- μ m serial frozen sections were fixed in acetone/ethanol and stained for CD49b (green), PNA (red) and nuclei (blue). A representative picture for each group is shown. Green-stained cells indicate NK cells. BZ, B cell zone; TZ, T cell zone.

Table I. Cytokine profile of NK cells after i.n. immunization

Tissue	Cytokine	Naive	5 h	24 h	72 h
NALT	IL-10	8.5 ± 0.8	5.4 ± 0.5 ^a	3.2 ± 0.3 ^a	2.8 ± 0.4 ^a
	IFN-γ	11.5 ± 1.1	16.6 ± 1.2 ^b	19.3 ± 2.4 ^a	7.6 ± 1.4 ^b
	IL-6	2.7 ± 0.6	1.7 ± 0.3	5.8 ± 1.2 ^c	10.5 ± 2.0 ^a
	IL-22	6.9 ± 1.6	16.9 ± 2.9 ^a	18.7 ± 3.0 ^a	24.6 ± 3.2 ^a
	TNF-α	21.2 ± 2.1	12.1 ± 1.7 ^a	30.0 ± 2.8 ^b	37.4 ± 3.7 ^a
CLN	IL-10	2.2 ± 0.3	2.3 ± 0.1	2.4 ± 0.3	2.1 ± 0.2
	IFN-γ	14.5 ± 0.9	23.7 ± 2.2 ^a	12.7 ± 1.7	10.6 ± 1.2 ^b
	IL-6	2.7 ± 0.1	3.4 ± 0.4	2.2 ± 0.2	6.6 ± 1.5 ^b
	IL-22	13.1 ± 1.1	9.8 ± 0.9	22.3 ± 2.2 ^b	13.7 ± 1.6
	TNF-α	25.8 ± 3.5	36.8 ± 4.0 ^a	38.0 ± 4.9 ^a	26.7 ± 3.3

Cells were stimulated for 6 h with BD Leukocyte Activation Cocktail with BD GolgiPlug in vitro, stained with surface mAb to determine DX5⁺/CD3⁺ population, and permeabilized and stained with anticytokine fluorochrome-labeled mAb. Data represent percent of cytokine positive NK cells out of the total NK cell population ± SD. Representative dot plots are shown in Supplemental Fig. 1.

^a*p* < 0.001, as determined by one-way ANOVA followed by Bonferroni's multiple comparison test compared with negative control animals (i.e., PBS immunized, 0 h).

^b*p* < 0.05.

^c*p* < 0.01.

additional 30 min at 37°C. The level of HRP associated with each well was then determined using Sigma Fast *o*-phenylenediamine dihydrochloride (50 μl per well; Sigma-Aldrich) colorimetric substrate. The reaction was stopped after 15 min by the addition of 2.5 M H₂SO₄. The optical density (OD)₄₉₀ was determined, and the titer was expressed as the reciprocal of the dilution giving an OD of 0.2.

Statistical analysis

Experimental results were plotted and analyzed for statistical significance with Prism4 software (GraphPad, San Diego, CA).

Results

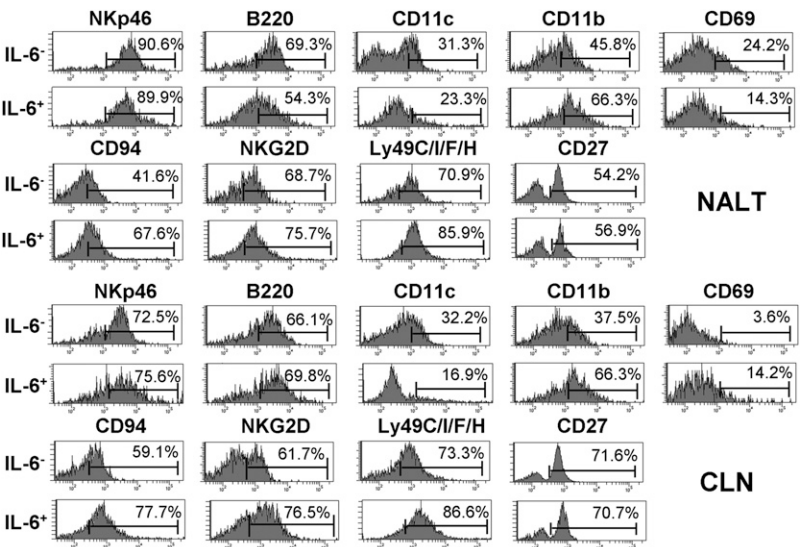
Characterization of NK cells in the NALT and CLN early after i.n. immunization

To detect any potential changes within the NK cell population shortly after i.n. immunization, CLNs and NALT were taken from mice vaccinated with a single dose of purified Ag85B-ESAT6 mixed with LT as adjuvant, and these were compared with similar tissues taken from control mice. The cell populations were then examined by flow cytometry and immunofluorescence microscopy. Groups of mice were sacrificed 5, 24, and 72 h postimmunization to determine the percentage, distribution, activation status, and cytokine profile of DX5⁺/CD3⁺ NK cells.

The overall percentages and total numbers of NK cells were significantly increased (*p* < 0.01) initially at 5 h postimmunization within the CLNs, in both tissues at 24 h and within the NALT at the final 72 h time point when compared with naive mice (Fig. 1A, 1B). We also observed significant increases (*p* < 0.001) in activation marker expression (CD69 and CD25) at all time points within the NALT, but only at 24 h within the CLNs of immunized mice (Fig. 1C). Direct in situ visualization of these cells within both tissues revealed localization to more central T cell zones from as early as 5 h post-immunization. In addition, NK cells were also detected within B cell follicles after 24 h, compared with naive mice in which where they were observed predominantly surrounding the periphery (Fig. 2).

When we examined the cytokine profile of BD Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences) in vitro stimulated NK cells, we observed an initial significant decrease (*p* < 0.001) in the percentage of TNF-α⁺ cells within the NALT of immunized mice, followed by a significant increase (*p* < 0.01) at 24 and 72 h postimmunization (Table I, Supplemental Fig. 1). Within the CLNs, significant increases (*p* < 0.001) in TNF-α⁺ NK cells were observed at 5 and 24 h. Both tissues showed significant increases (*p* < 0.01) in IFN-γ producing cells at 5 h (up to 24 h in the NALT), although at the final 72 h time point we detected significantly fewer

FIGURE 3. Characterization of surface marker expression on IL-6⁺ and IL-6[−] mouse NK subsets. BALB/c mice were immunized i.n. with 1 μg LT + 25 μg Ag85B-ESAT6 or PBS (data not shown) and NALT and CLN cells isolated at 72 h. Cells were stained with DX5, CD3, and IL-6 together with the indicated surface marker. Histograms represent expression level of the indicated surface markers on the IL-6[−] and IL-6⁺ subsets, which are gated on DX5⁺/CD3⁺ NK cells. The percentages are averaged from at least five mice and represent the mean percent positive cells for surface marker indicated above each histogram, compared with isotype control. The histograms shown are representative of at least five mice.



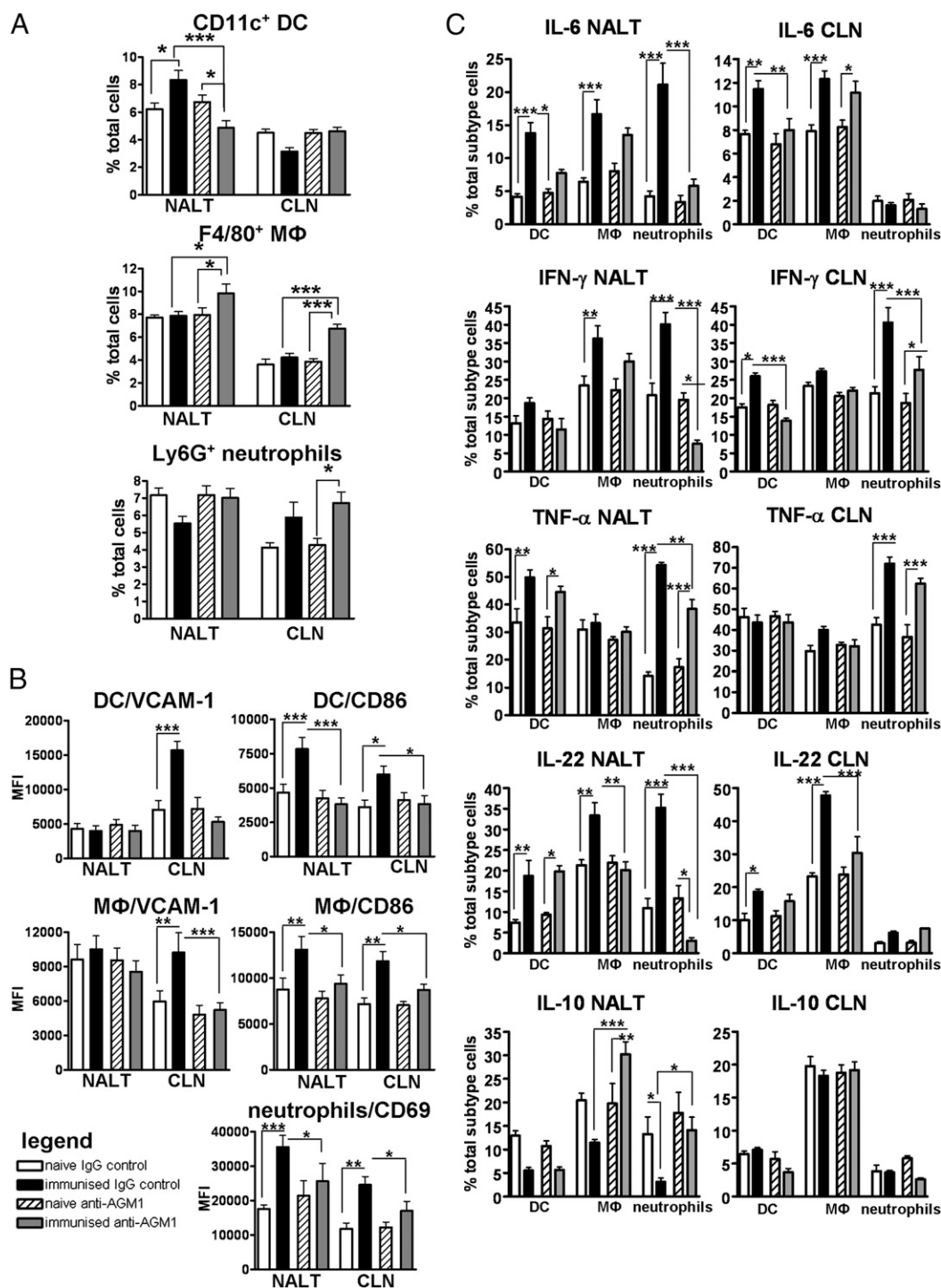


FIGURE 4. NK cell depletion impacts on other innate populations in the NALT and CLN 24 h post immunization. BALB/c mice were treated with anti-AGM1 or normal rabbit IgG on days -7 , -4 , and -2 . Mice were then immunized i.n. with PBS (naive) or $1 \mu\text{g}$ LT and $25 \mu\text{g}$ Ag85B-ESAT6 on day 0. Cells were isolated from the NALT and CLNs of naive and immunized animals 24 h after i.n. immunization, stained with fluorochrome-labeled mAb, and analyzed by flow cytometry in which 20,000–100,000 events were recorded. All data represent 10 animals per group from two independent experiments. **A**, Columns represents the mean $\pm$ SD percentage of CD11c⁺, F4/80⁺, and Ly6G⁺ cells. **B**, For analysis, gates were set on the innate subset marker positive (CD11c⁺ or F4/80⁺) cells and the MFI of VCAM-1, and CD86 expression was determined. A gate was also set around Ly6G⁺ cells, and the level of CD69 expression was determined. Data represent mean MFI $\pm$ SD. **C**, Cells were stimulated for 6 h with BD Leukocyte Activation Cocktail with BD GolgiPlug (BD Biosciences) in vitro, stained with surface mAb to determine CD11c⁺, F4/80⁺, or Ly6G⁺ populations, and then permeabilized and stained with anti-cytokine fluoro-labeled mAb. Data represent percent of cytokine positive cells out of total specific cell population $\pm$ SD. Representative dots plots are shown in Supplemental Fig. 2B. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ using one-way ANOVA followed by Bonferroni's multiple comparison test.

($p < 0.05$) IFN- γ ⁺ NK cells in both lymphoid tissues. Examining IL-6 production from NK cells revealed significantly greater ($p < 0.01$) percentages of positive cells from 24–72 h within the NALT and at 72

h within the CLNs of immunized mice. The IL-22⁺ NK cell population was significantly greater ($p < 0.01$) in the NALT at all time points tested and within the CLN at 24 h. Interestingly, when we

examined IL-10–producing NK cells we observed significantly less ($p < 0.001$) in the NALT of immunized mice when compared with similarly stimulated naive cells. We also observed similar overall cytokine profiles in unstimulated cells isolated from immunized mice (data not shown). Thus, significant changes are apparent in both the CLN and NALT NK cell populations within the first 3 d after i.n. immunization, and such changes might point to a role for such cells during mucosal vaccination.

Surface expression of NK cell receptors and markers in IL-6⁺ and IL-6[−] NK cell subsets

Interestingly, when analyzing the cytokine profile of both NALT and CLN NK cells, we observed a significant population of IL-6⁺ cells induced after i.n. immunization. To further characterize this murine NK cell population, we examined the expression patterns of a number of markers and receptors in IL-6⁺ and IL-6[−] NK cell subsets (Fig. 3). We found that IL-6–producing NK cells had significantly higher ($p < 0.05$) expression of CD11b, CD94, and Ly49C/I/F/H when compared with IL-6[−] NK cells within both tissues. These two subsets are also divergent in CD11c expression, which is significantly lower ($p < 0.05$) in the IL-6⁺ subset. In addition, NALT IL-6⁺ NK cells also had significantly lower B220 and CD69 surface expression, with significantly higher CD69 expression on the CLN IL-6⁺ subset.

Notably, we also observed significant differences in marker expression in the IL-6⁺ subset between tissues. Within the NALT, NKp46 expression was significantly higher ($p < 0.05$) than in CLN IL-6⁺ NK cells. Conversely, CD27 expression was significantly higher within the CLN subset when compared with similar IL-6⁺ NALT NK cells. Therefore, it appears that IL-6–producing NK cells represent a discrete subset of this important cellular population.

In vivo depletion of NK cells alters other innate immune cell populations within the NALT and CLN early after i.n. immunization

To detect any potential influence of NK cells on other local immune cell populations, polyclonal anti-AGM1 Ab was used to actively deplete NK cell populations from murine tissues in vivo (32, 33). Consequently, groups of mice were treated with either anti-AGM1 or an equivalent IgG control Ab; these were then immunized i.n. with either LT mixed with Ag85B-ESAT6 (immunized mice) or PBS (naive mice). Both the NALT and CLN tissues were then removed to determine the activation status, percentages, and cytokine profile of selected innate cell populations at 24 h post-immunization, when significant increases in NK cells within both these lymphoid tissues had been observed (Fig. 1). Depletion of NK cell activity was confirmed by a marked reduction in DX5⁺/CD3[−] cells in the NALT, CLNs, and spleens of anti-AGM1–treated mice, but not control mice (Supplemental Fig. 2A). Less

than 1% NK cells were consistently observed in mice treated with anti-AGM1. Importantly, we did not observe any significant reductions of other immune cell populations at earlier or later time points using anti-AGM1 (data not shown).

Depletion of NK cells before i.n. immunization resulted in quantitative differences in the NALT- and CLN-associated DC, MΦ, and neutrophil populations detected at the 24 h time point. In immunized IgG control mice, we observed significantly higher ($p < 0.05$) percentages and total numbers of NALT DCs compared with the significant decrease detected in anti-AGM1–treated immunized animals when compared with appropriate naive groups (Fig. 4A, Table II). In contrast, immunized mice treated with anti-AGM1 had significant increases ($p < 0.05$) in MΦ populations in both the NALT and CLN, compared with depleted naive mice and those. Alternatively, only total MΦ numbers were significantly increased in immunized IgG control mice. Within the CLNs we observed significant increases ($p < 0.05$) in total neutrophil number in both immunized groups; however, only NK cell-depleted mice were found to have significantly higher ($p < 0.05$) percentages of neutrophils when compared with their appropriate naive group (Fig. 4A, Table II).

Qualitative changes were also apparent within DC, MΦ, and neutrophil populations early after i.n. immunization in mice depleted of NK cells compared with controls (Fig. 4B). Upregulation of CD86 and VCAM-1 expression was apparent ($p < 0.01$) within both NALT- and CLN-associated DC and MΦ populations detected in immunized mice treated with IgG control Ab, compared with similarly treated naive animals (Fig. 4B). Immunization did not, however, significantly ($p > 0.05$) influence surface expression of either VCAM-1 or CD86 on DCs or MΦs in animals treated with anti-AGM1 and therefore depleted of NK cells. In addition, i.n. immunization significantly altered ($p < 0.01$) the surface expression of CD69 on neutrophils in IgG control Ab-treated animals by >70%, compared with naive mice. In contrast, CD69 mean fluorescence intensity (MFI) on Ly6G⁺ cells in mice depleted of NK cells increased by ~15% and was not significantly higher ($p > 0.05$) than in depleted naive animals (Fig. 4B).

Examining cytokine profiles of these innate populations revealed significant reductions in the number of proinflammatory cytokine positive DCs, MΦs, and neutrophils in those immunized mice treated with anti-AGM1 when compared with IgG control immunized mice (Fig. 4C, Supplemental Fig. 2B). Specifically, IgG control mice had a significantly higher ($p < 0.05$) number of IL-6⁺ NALT and CLN DCs at 24 h postimmunization when compared with mice depleted of NK cells; this was also the case for IFN-γ⁺ and IL-22⁺ CLN DCs. We observed significantly higher ($p < 0.05$) numbers of IL-6, IFN-γ, and IL-22–positive NALT MΦs and neutrophils in immunized IgG control treated mice when compared with anti-AGM1 immunized mice. Indeed, those neutrophils positive for IFN-γ and IL-22 obtained from anti-AGM1–immunized treated animals were found to

Table II. Innate cell NALT and CLN numbers in immunized control or NK cell-depleted mice

Tissue	Cell Type	Naive IgG Control	Immunized IgG Control	Naive Anti-AGM1	Immunized Anti-AGM1
NALT	DC	29.9 ± 2.1	60.1 ± 5.1 ^a	35.4 ± 2.7	31.9 ± 3.4 ^b
	MΦ	37.2 ± 1.7	56.2 ± 2.4 ^c	41.7 ± 3.4	64.4 ± 5.4 ^a
	Neutrophil	34.6 ± 2.0	39.8 ± 2.9	37.8 ± 2.8	45.9 ± 3.6
CLN	DC	252.6 ± 14.7	201.1 ± 17.5	261.1 ± 14.9	293.7 ± 18.8 ^b
	MΦ	203.2 ± 26.8	269.4 ± 23.3	225.5 ± 15.3	430.4 ± 23.3 ^{a,b}
	Neutrophil	232.4 ± 15.7	375.4 ± 56.1 ^d	249.4 ± 23.1	427.3 ± 41.3 ^c

Total cell number for each lymphoid organ is shown (1×10^3) ± SD.

^a $p < 0.001$, naive versus immunized.

^b $p < 0.001$, immunized versus immunized using one-way ANOVA followed by Bonferroni's multiple comparison test.

^c $p < 0.01$, naive versus immunized.

^d $p < 0.05$, naive versus immunized.

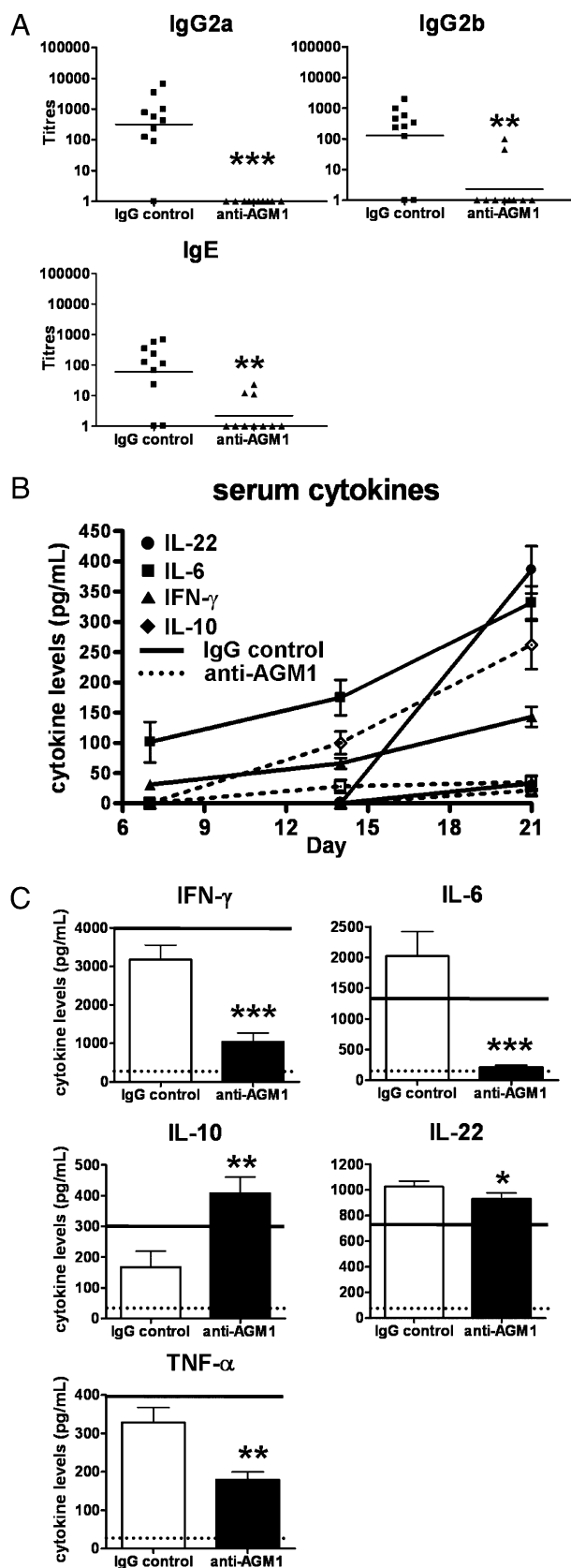


FIGURE 5. Anti-AGM1 treatment influences the development of Ag-specific Ab and cytokine responses. BALB/c mice were treated with anti-AGM1 or normal rabbit IgG on days -7 , -4 , -2 then immunized i.n. with $1 \mu\text{g}$ LT and $25 \mu\text{g}$ Ag85B-ESAT6 or PBS on day 0. A, Mice were further treated every 3–4 d and then bled on days 14 and 21 for evaluation of primary Ag-specific Ab synthesis by ELISA. Titers from day 21 are shown and reflect those seen on day 14 (data not shown). Data reflect 10 in-

be significantly less able ($p < 0.05$) to produce these cytokines than were similarly treated naive mice. Within the CLN MΦ population, we observed a significantly greater number ($p < 0.001$) of IL-22⁺ cells in control immunized mice when compared with NK cell depleted mice. In addition, the IFN-γ⁺ neutrophil subpopulation was significantly lower ($p < 0.001$) in anti-AGM1 treated immunized mice when compared IgG control immunized mice. Notably, NALT MΦs and neutrophils were found to have a significantly greater ($p < 0.05$) anti-inflammatory IL-10 subpopulation in mice immunized and depleted of NK cells when compared with immunized IgG control-treated animals. As already described for NK cells, we observed similar cytokine profiles from unstimulated innate cells isolated from immunized mice (data not shown). These data suggest that depletion of NK cells affects the trafficking of APCs within the local lymphoid tissues of the upper respiratory tract, and it additionally influences the activation and cytokine profile of other innate cellular populations shortly after i.n. immunization.

Mice depleted of NK cells have altered Ag-specific Ab and cytokine levels after mucosal immunization

To assess the effects of anti-AGM1 treatment on Ag85B-ESAT6 Ag-specific Ab production, the levels of specific total Ig, IgG, IgG1, IgG2a, IgG2b, IgE, and IgM were measured in plasma at day 14 and day 21 after i.n. immunization. Fig. 5A shows that immunized NK cell-depleted mice had no detectable ($p < 0.001$) serum IgG2a anti-Ag85B-ESAT6 titers above preimmune levels, and IgG2b levels were significantly lower ($p < 0.01$). Immunized IgG-treated control-treated mice also had significantly higher ($p < 0.01$) anti-Ag85B-ESAT6 IgE titers compared with immunized anti-AGM1 treated animals. However, anti-Ag85B-ESAT6 total Ig, IgG, IgG1, and IgM responses did not appear to be affected by anti-AGM1 treatment (data not shown). Serum collected at day 14 post-immunization gave the same overall pattern of Ab titers to those observed in day 21, albeit with slightly lower levels for all subtypes (data not shown).

Analysis of serum cytokine levels at various time points after immunization showed significantly less ($p < 0.05$) IFN-γ and IL-6 in immunized anti-AGM1 treated animals when compared with immunized IgG-treated controls from day 7 onward (Fig. 5B). However, it was not until day 21 that significantly lower ($p < 0.01$) levels of IL-22 were observed between immunized IgG control and NK cell-depleted mice. Importantly, NK cell-depleted mice were found to have significantly higher ($p < 0.01$) serum IL-10 levels (from day 14) when compared with similarly immunized IgG controls (Fig. 5B).

dividual mice per group from two independent experiments (minus background naive animal titers) expressed as total Ab titer using a cut-off of OD 0.2., with the black bar showing the geometric mean. B, Mice were also bled on days 1, 7, 14, and 21 postimmunization, and serum cytokine responses were evaluated using ELISA. Graph represents cytokine levels (pg/ml) $\pm$ SD. C, Mice were sacrificed on day 21, and spleens were removed for T-cell assays. Cytokine responses were measured upon in vitro stimulation with Ag85B-ESAT6 ($5 \mu\text{g/ml}$) for 36–48 h. Cells were also stimulated with $5 \mu\text{g/ml}$ Con A (positive control) indicated by solid black line and RPMI 1640 media (negative control) indicated by dashed line. Columns represent the mean $\pm$ SD stimulation indices of splenocytes from 10 immunized animals per group from two individual experiments (minus background naive cytokine levels). For all cytokines, we found significant difference between naive and immunized mice (data not shown). The differences in cytokine levels between immunized IgG control or anti-AGM1 treated animals is shown. ** $p < 0.01$; *** $p < 0.001$ using the Mann-Whitney U test.

Spleens were also removed from mice 21 d postimmunization, and these cells were stimulated with Ag85B-ESAT6 *in vitro* to assess cytokine levels (IL-2, IFN- γ , TNF- α , IL-4, IL-6, IL-10, IL-5, and IL-22). Significantly higher levels ($p < 0.05$) of each cytokine were observed in immunized compared with naive groups (data not shown). When IgG control-treated immunized mice were compared with similar anti-AGM1-treated mice, IL-2, IL-5, and IL-4 levels were not significantly different ($p > 0.05$) (data not shown). However, IFN- γ , TNF- α , IL-6, and IL-22 were all significantly lower ($p < 0.05$) (Fig. 5C). Furthermore, immunized mice treated with anti-AGM1 had significantly higher levels of IL-10 when compared with controls (Fig. 5C). Collectively, these data argue that anti-AGM1-mediated NK cell depletion impacts the development of Ag-specific Ab and cytokine responses to Ags after *i.n.* immunization.

Depletion of NK cells also influences the number and cytokine profile of systemic immune populations after i.n. immunization

To clarify which splenic cellular populations might be associated with the differences observed in cytokine levels between immunized

IgG control and anti-AGM1 treated mice, intracellular cytokine analysis was performed (Fig. 6, Supplemental Fig. 3). Examining the NK cell population within the spleens of IgG control immunized mice, we observed a 2-fold rise in both percentage and absolute cell number (53.9 ± 2.5 SD versus 102.6 ± 4.6 SD) when compared with naive mice (Fig. 6A). NK cells positive for IL-6, IL-22, and TNF- α increased ($p < 0.001$) in immunized animals, whereas those producing IFN- γ and IL-10 were significantly lower ($p < 0.05$) than those observed in naive mice (Fig. 6B, Supplemental Fig. 3A).

Analysis of other cell populations found within the spleen showed significant changes in both percentage and total measured cell numbers in naive compared with immunized animals in both IgG control and anti-AGM1 treated mice (Fig. 6A, Table III). Importantly, when we examined cytokine production from adaptive cell populations stimulated with Ag, we noted that B cells from immunized NK cell-depleted mice produced significantly less ($p < 0.001$) IL-6 when compared with similarly stimulated IgG-treated control cells. Significantly fewer ($p < 0.05$) IL-22⁺ T cells were observed in immunized, NK cell-depleted mice when compared with IgG-treated controls. Both TNF- α and IFN- γ

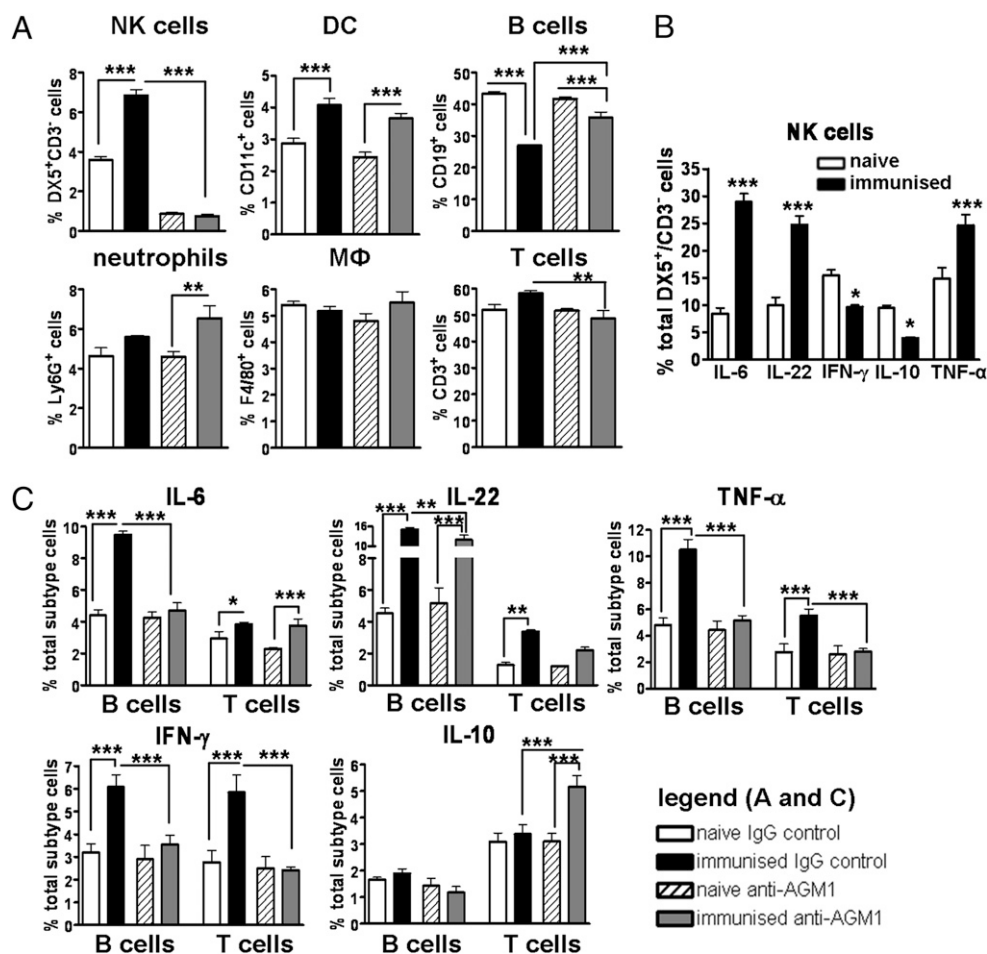


FIGURE 6. Anti-AGM1 Ab treatment alters the number and cytokine profile of systemic immune populations. BALB/c mice were treated with either anti-AGM1 or control IgG Ab and then immunized with either PBS (naive) or Ag85B-ESAT6 and LT. Mice were sacrificed on day 21, and spleens were removed for T cell assays. *A*, Splenocytes were stained with fluorochrome-labeled mAb and analyzed by flow cytometry in which 200,000 events were recorded. Data represent the mean $\pm$ SD percentage of DX5⁺CD3⁺, CD11c⁺, F4/80⁺ Ly6G⁺, CD19⁺ and CD3⁺ cells. Cytokine responses were measured upon *in vitro* stimulation with Ag85B-ESAT6 (5 μ g/ml) for 36–48 h. Cells were also stimulated with 5 μ g/ml Con A or RPMI 1640 media (data not shown). Stimulated cells were permeabilized and stained with anticytokine fluorochrome-labeled mAb to determine cytokine profiles. *B*, Data represent percent of cytokine positive NK cells out of total NK cell population $\pm$ SD in mice treated with IgG control and immunized *i.n.* Immunized mice treated with anti-AGM1 had $<1\%$ splenic NK cells; therefore, cytokine analysis was not performed. *C*, Data indicate the percent of cytokine positive adaptive cellular populations (CD19⁺ and CD3⁺) in both IgG control and anti-AGM1 naive and immunized mice. Representative dots plots are shown in Supplemental Fig. 3. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, as determined by one-way ANOVA followed by Bonferroni's multiple comparison test for immunized mice compared with appropriate naive animals and IgG control immunized versus anti-AGM1 treated immunized mice.

Table III. Total cell number for each spleen population is shown ($1 \times 10^5 \pm SD$)

Spleen	Naive IgG Control	Immunized IgG Control	Naive Anti-AGM1	Immunized Anti-AGM1
DC	43.0 $\pm$ 2.5	61.2 $\pm$ 3.2 ^a	36.5 $\pm$ 2.4	55.9 $\pm$ 2.7 ^a
MΦ	81.0 $\pm$ 2.1	77.7 $\pm$ 2.6	72.1 $\pm$ 4.3	83.8 $\pm$ 6.2
Neutrophils	69.5 $\pm$ 6.1	84.0 $\pm$ 1.0	69.0 $\pm$ 3.9	99.0 $\pm$ 9.0 ^b
B cells	649.0 $\pm$ 10.9	404.6 $\pm$ 3.0 ^a	626.1 $\pm$ 8.1	548.3 $\pm$ 32.3 ^{c,d}
T cells	781.2 $\pm$ 28.6	873.3 $\pm$ 14.1	775.0 $\pm$ 11.2	730.8 $\pm$ 44.7 ^e

^a $p < 0.001$, naive versus immunized.^b $p < 0.01$, naive versus immunized.^c $p < 0.05$, naive versus immunized.^d $p < 0.001$ immunized versus immunized using one-way ANOVA followed by Bonferroni's Multiple Comparison Test.^e $p < 0.01$.

production was also significantly impaired ($p < 0.001$) in all Ag-stimulated cells analyzed from NK cell-depleted mice compared with IgG-treated control mice. Interestingly, T cells from mice treated with anti-AGM1 and immunized exhibited significantly elevated ($p < 0.001$) percentages of IL-10⁺ cells when compared with IgG control-treated mice (Fig. 6C, Supplemental Fig. 3B). Innate populations were also examined and demonstrated significantly lower proinflammatory cytokine profiles, but significantly higher numbers of IL-10⁺ cells in NK cell-depleted immunized mice compared with IgG controls (Supplemental Fig. 3C, 3D).

Exogenous cytokines rIL-6 and rIFN- γ significantly restore Ag-specific adaptive immune responses in mice depleted of NK cells

Given the apparent requirement of endogenous IFN- γ and IL-6 for optimal production of cytokines and Abs in vivo, exogenous IFN- γ or IL-6 was administered to assess whether this would affect adaptive immune responses in immunized animals depleted of NK cells. Exogenous IL-6 or IFN- γ administration to naive mice had no significant effect ($p > 0.05$) on any of those cytokines or Abs tested (data not shown).

Administration of rIFN- γ to IgG control-treated, immunized mice significantly decreased ($p < 0.05$) titers of IgG1 compared with immunized NK cell-depleted mice also receiving rIFN- γ , although we did not observe a significant difference ($p > 0.05$) in comparison with similar mice receiving no cytokine (Fig. 7A). IgM titers were significantly lower in immunized mice that were depleted of NK cells and administered exogenous IFN- γ in comparison with IgG treated control mice also treated with this cytokine, whereas the difference between immunized mice given only PBS and those treated with anti-AGM1 and given rIFN- γ was not significantly different ($p > 0.05$; Fig. 7A). In regard to cytokine levels, administration of rIFN- γ on days 1 and 2 post-immunization significantly decreased ($p < 0.01$) detectable levels of IL-10 in mice treated with anti-AGM1 when compared with those that had just received PBS. Interestingly, the levels of IL-10 in rIFN- γ -treated immunized mice depleted of NK cells were not significantly different ($p > 0.05$) from those in immunized IgG control mice (Fig. 7B). Furthermore, rIFN- γ administration modestly decreased induction of IL-4 in both IgG control and anti-AGM1 treated mice, although this was not found to be significantly different ($p > 0.05$) (data not shown). Administration of exogenous IFN- γ also significantly increased ($p < 0.05$) levels of IFN- γ in mice treated with IgG control Ab compared with mice receiving no cytokines; however, there was no effect of rIFN- γ treatment in mice depleted of NK cells (Fig. 7B).

Administration of rIL-6 had significant effects on Ab subtype titers in NK cell-depleted animals. For example, subclasses IgG2a, IgG2b, and IgE levels were significantly increased ($p < 0.05$) in

animals treated with anti-AGM1 and rIL-6 in comparison with depleted mice that received no exogenous IL-6. Indeed, titers of these Abs were observed to be no longer significantly different ($p > 0.05$) from those in immunized IgG control animals (Fig. 7A). The addition of exogenous IL-6, as with rIFN- γ , attenuated induction of IL-10 by stimulated splenocytes from anti-AGM1-treated mice (Fig. 7B). In contrast to rIFN- γ , rIL-6 also resulted in marked up-regulation of systemic IFN- γ and IL-6 with no significant effect on IL-2, IL-5, or IL-4 (Fig. 7B and data not shown). Indeed, administration of rIL-6 to NK cell depleted immunized mice restored production of IFN- γ and TNF- α to levels that were no longer significantly different from those observed in immunized animals treated with control IgG. Notably, IgG control mice receiving rIL-6 still had significantly more IL-6 and IL-10 than those immunized mice treated with anti-AGM1 and rIL-6 (Fig. 7B).

Discussion

Many different cell types work in concert to mount a mucosal immune response against a particular Ag. In this study, we provide evidence that NK cells play a key role in the modulation of innate immune responses and the consequent development of Ag-specific immune responses after mucosal immunization.

Experimental evidence indicates that NK cells play an important role during the induction of adaptive immune responses, in addition to their function in the innate immune response. As early as 5 h after i.n. immunization, we observed a significant influx of NK cells within the CLNs and at 24 h within the NALT, as well as localization of these cells to T and B cell areas. CD25 and CD69 expression levels suggested a heightened activation status of these NK cells, which was confirmed when we detected significant increases in the percentage of proinflammatory cytokine-positive NK cells in both lymphoid organs at these early time points. IFN- γ and TNF- α production via NK cells has been very well documented, and several recent studies have highlighted the role that NK cells play in IL-22 production (36, 37). In particular, human tonsil NK cells have been found to produce significant levels of this cytokine, which is of interest because both tonsils and adenoids are the human equivalent of rodent NALT (38). Notably, the role that NK cells play in the production of IL-6 has not been evaluated properly. After mucosal immunization, we observed significant increases in IL-6⁺ NK cells in both lymphoid tissues. When we examined a number of surface markers on this subset, expression patterns appeared to distinguish these particular NK cells from those not producing IL-6. The similar expression levels of important surface markers, including NKp46, CD27, and NKG2D, suggest that NK cells capable of producing IL-6 arise from similar NK cell precursors as other cytokine functional NK cells. However, a number of markers tested gave differential expression (e.g., B220, CD11c, CD11b) on the IL-6⁺ subset compared with IL-6⁻

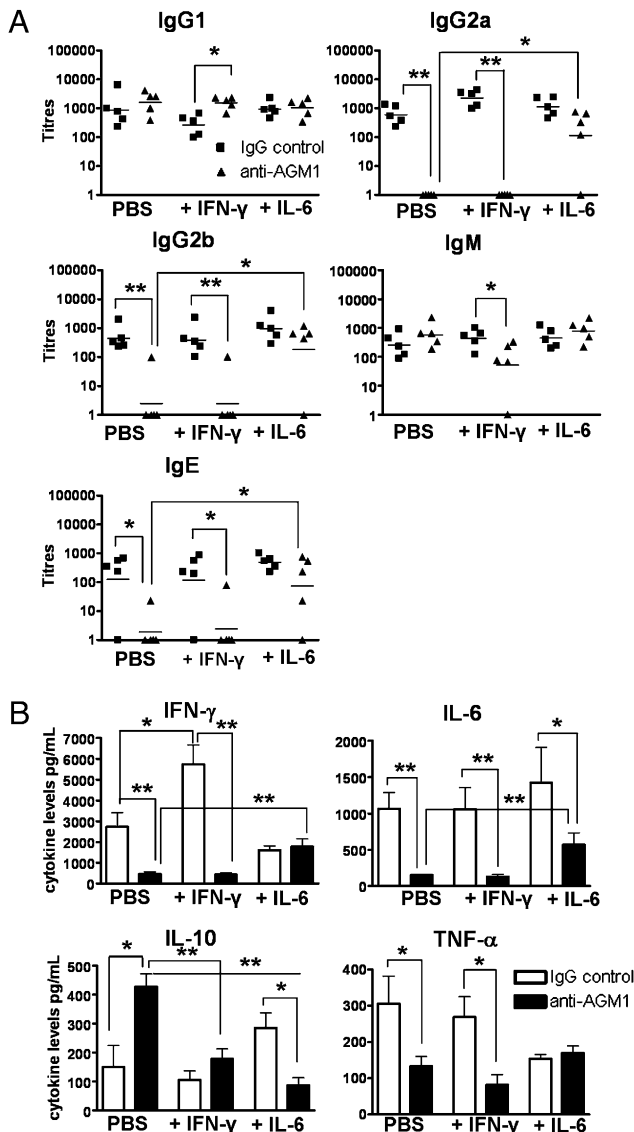


FIGURE 7. Effect of rIL-6 or rIFN- γ on Ab titers and cytokine levels in immunized mice depleted of NK cells. Animals were treated with either anti-AGM1 or rabbit IgG control Ab and were then immunized i.n. with LT and Ag85B-ESAT6. At days 1 and 2 postimmunization mice were further treated with rIL-6, rIFN- γ , or PBS (control). **A**, Mice were then bled on day 21 for evaluation of primary Ag-specific Ab synthesis by ELISA. Data reflect five individual mice per group (minus background naive animal titers) expressed as total Ab titer using a cut off of OD 0.2., with the black bar showing the geometric mean. **B**, On day 21, spleens were removed for T cell assays and cytokine responses were measured upon in vitro stimulation with Ag85B-ESAT6 (5 μ g/ml) for 36–42 h. Cells were also stimulated with 5 μ g/ml Con A (positive control) and RPMI 1640 media (negative control; see Fig. 5 for average values). Columns represent the mean $\pm$ SD from five individual mice. * p < 0.05; ** p < 0.01 using a Kruskal-Wallis test followed by Dunn's multiple comparison test.

NK cells, suggesting functional distinctions other than the ability to produce IL-6. The significantly higher number of these IL-6 NK cells with other cytokine-positive NK cells within both the NALT and CLN shortly after i.n. immunization might be expected to impact on other innate cellular populations. When we compared immunized control and depleted NK cell mice, we observed a different trafficking picture for APCs within both the NALT and CLN and in neutrophils within the CLNs in mice depleted of NK cells. These differences might result from the reduced production

of proinflammatory cytokines early after immunization because of the absence of NK cells. In addition, decreased production of inflammatory cytokines in mice treated with anti-AGM1 would also be expected to impact on the activation status and consequent cytokine production of the leukocytes present within these tissues. Correspondingly, we observed that both activation markers and proinflammatory cytokine profiles were significantly attenuated in animals depleted of NK cells.

The mechanisms by which NK cells influence later adaptive immune responses may be through the observed modulation of other innate cellular populations and/or the contribution of inflammatory mediators by NK cells themselves. Indeed, the magnitude of adaptive immune responses, including both Ab and cytokine responses, is decreased in NK cell-depleted mice. To explore the mechanisms underlying these diminished Ag-specific immune responses of NK cell-depleted mice, we checked the number and cytokine profiles of stimulated splenic innate and adaptive immune populations from immunized NK cell-depleted mice and control mice. Our data revealed that NK cell depletion impacts directly on adaptive immune populations as observed by impaired B and T cell cytokine production in response to antigenic stimulation. This is probably due to the absence of NK cell-derived inflammatory mediators available within the local lymphoid tissues early during the immune response. Investigators have shown that DCs and M Φ s require help from NK cells and NK cell-derived cytokines for maturation and proper functioning of APCs (39, 40). A recent study has shown that DCs isolated from NK cell depleted mice could not be loaded with Ag and were incapable of activating memory specific lymphocytes (41). Cytokine production by these activated lymphocytes modulates other splenic populations, including DCs, M Φ s, neutrophils, and NK cells in control animals, which when might account for the significantly attenuated overall levels of IL-6 and IFN- γ observed in immunized NK cell-depleted mice. Specifically, observed differences in innate populations, including the high number of APC IFN- γ ⁺ cells, are probably caused by an activation feedback loop from Ag-stimulated B and T cells.

Finally, we have shown that adaptive immunity can be restored by the administration of rIL-6, but not rIFN- γ shortly after i.n. immunization in NK cell-depleted mice. IL-6 is a pleiotropic cytokine involved in the differentiation and proliferation of T and B cells and acts in conjunction with TNF- α and IL-1 to initiate early inflammatory responses (42). We found that rIL-6 significantly restored T_H1 type immune responses to levels seen in control animals, through increased production of IFN- γ , TNF- α , and IL-6, reduced levels of IL-10, and high titers of the subclasses IgG2a, IgG2b, and IgE. Several studies have shown that human NK cells produce IL-6. In this study, we have shown for the first time that this is also the case for murine NK cells (43, 44). Other cell populations involved in the secretion of IL-6 include APCs and neutrophils, during early immune responses, and lymphocytes at later time points (42). In this study, we observed that both APCs and neutrophils were significantly less activated and consequently produced less IL-6 in both the NALT and CLNs of immunized mice depleted of NK cells. Previous studies have shown that NK cells enhance IL-6 production, by M Φ s and DCs, during early immune responses (45, 46). Other studies concentrating on *M. tuberculosis* have shown that IL-6 is required for early IFN- γ production in the lungs of mice infected with *M. tuberculosis*, and that IL-6 participates in the induction of protective T_H1 T cell responses during vaccination (47–49). Interestingly, several recent studies have focused on the role of NK cells in IL-22 production (37). As described by Cella et al. (38) for tonsil NK cells, we observed IL-22⁺ NK cells within both the NALT and CLNs of mice immunized i.n. Recent studies have shown that IL-22 is

crucial for IL-6 production in various disease states (50, 51). This finding suggests that IL-22 production via NALT and CLN NK cells shortly after i.n. immunization might contribute to IL-6 production, and its absence in NK cell-depleted mice might account for the attenuated IL-6 levels. It therefore appears that IL-6, and possibly IL-22 production via NK cells and NK cell activated innate populations, is a crucial requirement for the successful development of T_H1 type adaptive immune responses after mucosal vaccination. Notably, although immunized mice depleted of NK cells and administered rIL-6 were no longer considered statistically different from control immunized mice, both cytokine levels and Ab titers were still modestly reduced in anti-AGM1-treated animals. It is therefore tempting to speculate that the release of other inflammatory mediators or possible cell-cell interactions via NK cells are required for optimal development of Ag-specific immune responses.

In conclusion, our results underscore the pivotal function of NK cells after mucosal immunization and define their role in the subsequent development of both innate and adaptive immune responses. These new data indicate a central and modulating role for the increased numbers of NK cells observed in the NALT and CLN shortly after i.n. immunization, with direct effects on other local innate immune populations. We also propose that NK cell activity, specifically through production of IL-6 via NK cells or through other NK cell activated populations, may be one of the factors governing the development of Ag-specific cytokine and Ab responses, particularly the T_H1 response, after mucosal vaccination. These new data expand the role of NK cells beyond tumor and microbe defense and position NK cells as important regulators of vaccination immunity.

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Disclosures

The authors have no financial conflicts of interest.

References

- Partidos, C. D. 2000. Intranasal vaccines: forthcoming challenges. *Pharm. Sci. Technol. Today* 3: 273–281.
- Slütter, B., N. Hagens, and W. Jiskoot. 2008. Rational design of nasal vaccines. *J. Drug Target.* 16: 1–17.
- Silin, D. S., O. V. Lyubomska, V. Jirathitikal, and A. S. Bourinbaiar. 2007. Oral vaccination: where we are? *Expert Opin. Drug Deliv.* 4: 323–340.
- Wu, H. Y., and M. W. Russell. 1997. Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system. *Immunol. Rev.* 16: 187–201.
- Kiyono, H., and S. Fukuyama. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4: 699–710.
- Zuercher, A. W., S. E. Coffin, M. C. Thurnheer, P. Fundova, and J. J. Cebra. 2002. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. *J. Immunol.* 168: 1796–1803.
- Davis, S. S. 2001. Nasal vaccines. *Adv. Drug Deliv. Rev.* 51: 21–42.
- Bienstock, J., and M. R. McDermott. 2005. Bronchus- and nasal-associated lymphoid tissues. *Immunol. Rev.* 206: 22–31.
- Dietrich, J., C. Andersen, R. Rappuoli, T. M. Doherty, C. G. Jensen, and P. Andersen. 2006. Mucosal administration of Ag85B-ESAT-6 protects against infection with *Mycobacterium tuberculosis* and boosts prior bacillus Calmette-Guérin immunity. *J. Immunol.* 177: 6353–6360.
- Hall, L. J., S. Clare, D. Pickard, S. O. Clark, D. L. Kelly, M. A. El Ghany, C. Hale, J. Dietrich, P. Andersen, P. D. Marsh, and G. Dougan. 2009. Characterisation of a live *Salmonella* vaccine stably expressing the *Mycobacterium tuberculosis* Ag85B-ESAT6 fusion protein. *Vaccine* 27: 6894–6904.
- Yang, Q., S. R. Goding, M. E. Hokland, and P. H. Basse. 2006. Antitumor activity of NK cells. *Immunol. Rev.* 36: 13–25.
- Roetynck, S., M. Baratin, S. Johansson, C. Lemmers, E. Vivier, and S. Ugolini. 2006. Natural killer cells and malaria. *Immunol. Rev.* 214: 251–263.
- Loddo, M. B., and L. L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Curr. Opin. Immunol.* 18: 391–398.
- Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol.* 9: 24–34.
- Scharton-Kersten, T. M., and A. Sher. 1997. Role of natural killer cells in innate resistance to protozoan infections. *Curr. Opin. Immunol.* 9: 44–51.
- Yokoyama, W. M. 2008. Mistaken notions about natural killer cells. *Nat. Immunol.* 9: 481–485.
- Kim, S., K. Iizuka, H. L. Aguila, I. L. Weissman, and W. M. Yokoyama. 2000. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 97: 2731–2736.
- Vivier, E., E. Tomasello, M. Baratin, T. Walzer, and S. Ugolini. 2008. Functions of natural killer cells. *Nat. Immunol.* 9: 503–510.
- Hamerman, J. A., K. Ogasawara, and L. L. Lanier. 2005. NK cells in innate immunity. *Curr. Opin. Immunol.* 17: 29–35.
- Roy, S., P. F. Barnes, A. Garg, S. Wu, D. Cosman, and R. Vankayalapati. 2008. NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J. Immunol.* 180: 1729–1736.
- Feng, C. G., M. Kaviratne, A. G. Rothfuchs, A. Cheever, S. Hieny, H. A. Young, T. A. Wynn, and A. Sher. 2006. NK cell-derived IFN- γ differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. *J. Immunol.* 177: 7086–7093.
- Vankayalapati, R., B. Wize, S. E. Weis, H. Safi, D. L. Lakey, O. Mandelboim, B. Samten, A. Porgador, and P. F. Barnes. 2002. The NKp46 receptor contributes to NK cell lysis of mononuclear phagocytes infected with an intracellular bacterium. *J. Immunol.* 168: 3451–3457.
- Ferlazzo, G., B. Morandi, A. D'Agostino, R. Meazza, G. Melioli, A. Moretta, and L. Moretta. 2003. The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur. J. Immunol.* 33: 306–313.
- Walzer, T., M. Dalod, E. Vivier, and L. Zitvogel. 2005. Natural killer cell-dendritic cell crosstalk in the initiation of immune responses. *Expert Opin. Biol. Ther.* 5(Suppl 1): S49–S59.
- Kalinski, P., R. B. Mailliard, A. Giermasz, H. J. Zeh, P. Basse, D. L. Bartlett, J. M. Kirkwood, M. T. Lotze, and R. B. Herberman. 2005. Natural killer-dendritic cell cross-talk in cancer immunotherapy. *Expert Opin. Biol. Ther.* 5: 1303–1315.
- Raulet, D. H. 2004. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* 5: 996–1002.
- Bajénoff, M., B. Bréart, A. Y. Huang, H. Qi, J. Cazareth, V. M. Braud, R. N. Germain, and N. Glaichenhaus. 2006. Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J. Exp. Med.* 203: 619–631.
- Moretta, A., E. Marcano, S. Parolini, G. Ferlazzo, and L. Moretta. 2008. NK cells at the interface between innate and adaptive immunity. *Cell Death Differ.* 15: 226–233.
- Fearon, D. T., and R. M. Locksley. 1996. The instructive role of innate immunity in the acquired immune response. *Science* 272: 50–53.
- O'Leary, J. G., M. Goodarzi, D. L. Drayton, and U. H. von Andrian. 2006. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* 7: 507–516.
- Martín-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN- γ for T(H)1 priming. *Nat. Immunol.* 5: 1260–1265.
- Stitz, L., J. Baenziger, H. Pircher, H. Hengartner, and R. M. Zinkernagel. 1986. Effect of rabbit anti-asialo GM1 treatment in vivo or with anti-asialo GM1 plus complement in vitro on cytotoxic T cell activities. *J. Immunol.* 136: 4674–4680.
- Harshan, K. V., and P. R. Gangadharam. 1991. In vivo depletion of natural killer cell activity leads to enhanced multiplication of *Mycobacterium avium* complex in mice. *Infect. Immun.* 59: 2818–2821.
- Asanuma, H., A. H. Thompson, T. Iwasaki, Y. Sato, Y. Inaba, C. Aizawa, T. Kurata, and S. I. Tamura. 1997. Isolation and characterization of mouse nasal-associated lymphoid tissue. *J. Immunol. Methods* 202: 123–131.
- Perfetto, S. P., P. K. Chattopadhyay, L. L. Moreaux, N. Nguyen, D. Ambrozak, R. A. Koup, and M. Roederer. 2006. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J. Immunol. Methods* 313: 199–208.
- Yokoyama, W. M., S. Kim, and A. R. French. 2004. The dynamic life of natural killer cells. *Annu. Rev. Immunol.* 22: 405–429.
- Malmberg, K. J., and H. G. Ljunggren. 2009. Spotlight on IL-22-producing NK cell receptor-expressing mucosal lymphocytes. *Nat. Immunol.* 10: 11–12.
- Cella, M., A. Fuchs, W. Vermi, F. Facchetti, K. Otero, J. K. Lennerz, J. M. Doherty, J. C. Mills, and M. Colonna. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* 457: 722–725.
- Vitale, M., M. Della Chiesa, S. Carlomagno, D. Pende, M. Aricò, L. Moretta, and A. Moretta. 2005. NK-dependent DC maturation is mediated by TNF α and IFN γ released upon engagement of the NKp30 triggering receptor. *Blood* 106: 566–571.
- Lapaque, N., T. Walzer, S. Méresse, E. Vivier, and J. Trowsdale. 2009. Interactions between human NK cells and macrophages in response to *Salmonella* infection. *J. Immunol.* 182: 4339–4348.
- Yoshida, O., F. Akbar, T. Miyake, M. Abe, B. Matsura, Y. Hiasa, and M. Onji. 2008. Impaired dendritic cell functions because of depletion of natural killer cells disrupt antigen-specific immune responses in mice: restoration of adaptive immunity in natural killer-depleted mice by antigen-pulsed dendritic cell. *Clin. Exp. Immunol.* 152: 174–181.
- Hirano, T. 1992. Interleukin-6 and its relation to inflammation and disease. *Clin. Immunol. Immunopathol.* 62: S60–S65.

43. Schmidt, K. N., B. Leung, M. Kwong, K. A. Zarembek, S. Satyal, T. A. Navas, F. Wang, and P. J. Godowski. 2004. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J. Immunol.* 172: 138–143.
44. Horikawa, M., M. Hasegawa, K. Komura, I. Hayakawa, K. Yanaba, T. Matsushita, K. Takehara, and S. Sato. 2005. Abnormal natural killer cell function in systemic sclerosis: altered cytokine production and defective killing activity. *J. Invest. Dermatol.* 125: 731–737.
45. Della Chiesa, M., C. Romagnani, A. Thiel, L. Moretta, and A. Moretta. 2006. Multidirectional interactions are bridging human NK cells with plasmacytoid and monocyte-derived dendritic cells during innate immune responses. *Blood* 108: 3851–3858.
46. Godshall, C. J., M. J. Scott, P. T. Burch, J. C. Peyton, and W. G. Cheadle. 2003. Natural killer cells participate in bacterial clearance during septic peritonitis through interactions with macrophages. *Shock* 19: 144–149.
47. Saunders, B. M., A. A. Frank, I. M. Orme, and A. M. Cooper. 2000. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to *Mycobacterium tuberculosis* infection. *Infect. Immun.* 68: 3322–3326.
48. Leal, I. S., M. Flórido, P. Andersen, and R. Appelberg. 2001. Interleukin-6 regulates the phenotype of the immune response to a tuberculosis subunit vaccine. *Immunology* 103: 375–381.
49. Leal, I. S., B. Smedegård, P. Andersen, and R. Appelberg. 1999. Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine. *Infect. Immun.* 67: 5747–5754.
50. Ma, H. L., S. Liang, J. Li, L. Napierata, T. Brown, S. Benoit, M. Senices, D. Gill, K. Dunussi-Joannopoulos, M. Collins, et al. 2008. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J. Clin. Invest.* 118: 597–607.
51. Aujla, S. J., Y. R. Chan, M. Zheng, M. Fei, D. J. Askew, D. A. Pociask, T. A. Reinhart, F. McAllister, J. Edeal, K. Gaus, et al. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* 14: 275–281.

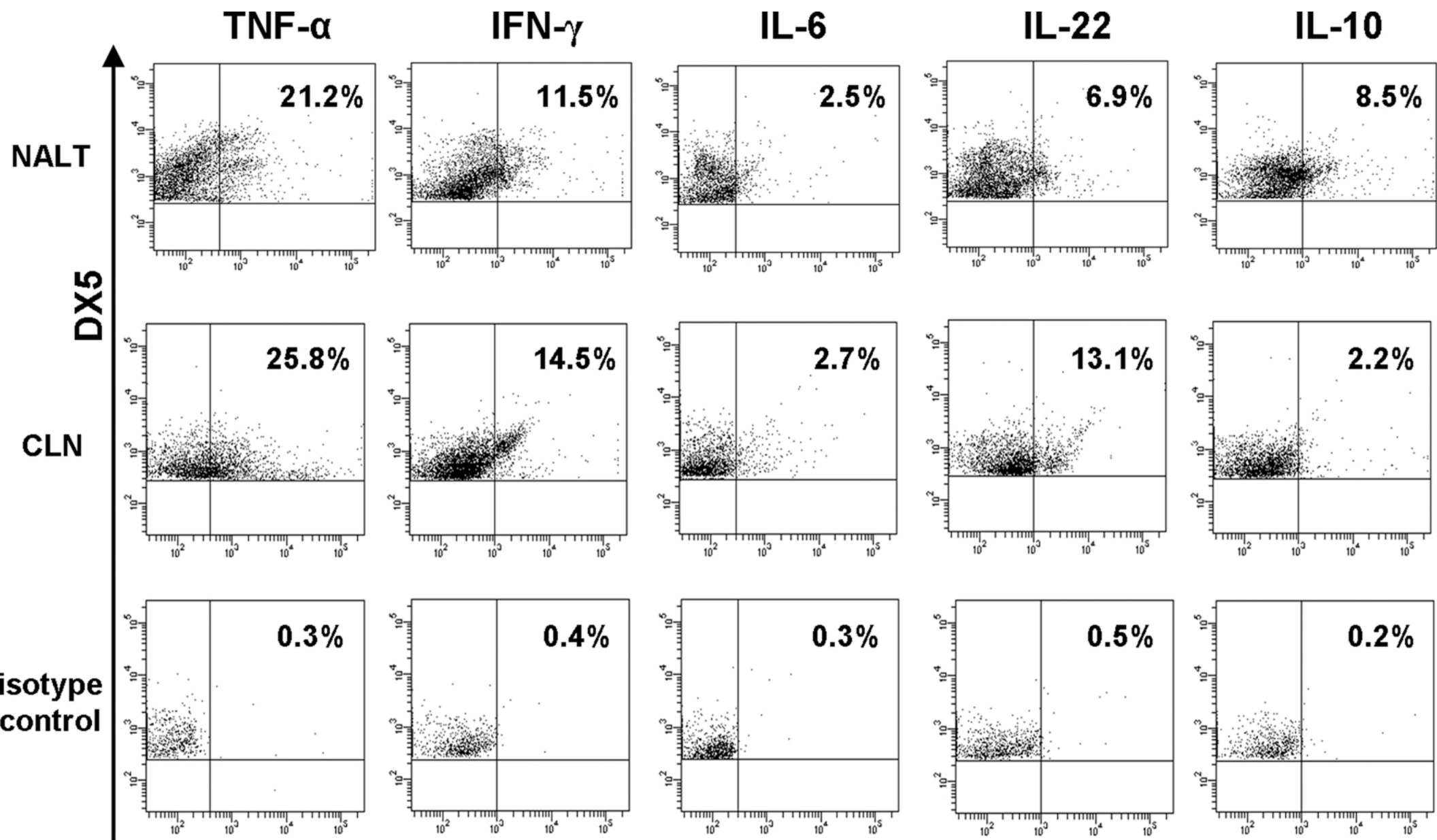


Figure S1: Representative dot plots of cytokine staining in naïve NALT and CLN NK cells after 6 h BD Activation Cocktail plus GolgiPlug stimulation *in vitro*. Cells were gated as DX5⁺/CD3⁻. In the parentheses are the percentages of the cells stained for investigative cytokines.

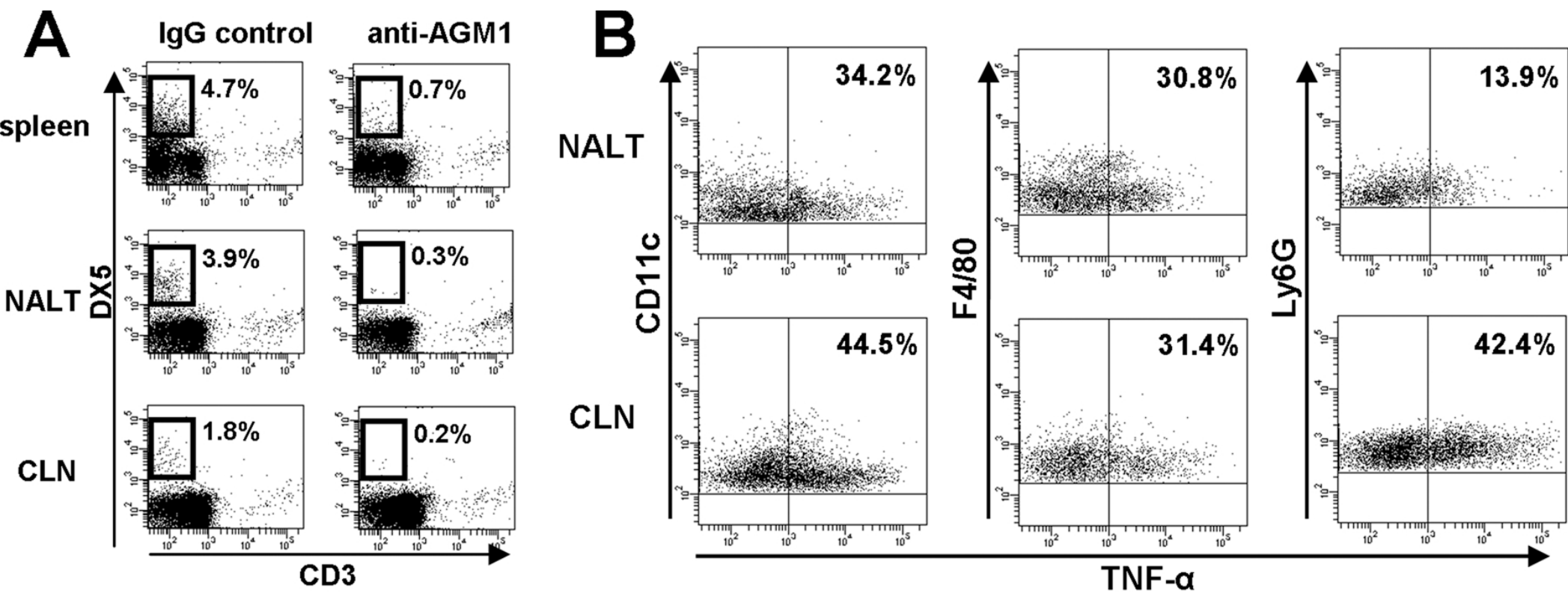


Figure S2: (A) Proportions of NK cells in the spleen, NALT and CLN of naïve mice after anti-AGM1 Ab treatment. Numbers in dot plots represent percentages of each population. **(B)** Representative dot plots of TNF- α cytokine staining in naïve NALT and CLN leukocytes after 6 h BD Activation Cocktail plus GolgiPlug stimulation *in vitro*. Cells were gated as CD11c⁺FSC^{high}, F4/80⁺/FSC^{high} or Ly6G⁺/SSC^{high}. In the parentheses are the percentages of the cells stained for investigative cytokines.

