NK1.1<sup>+</sup> Cells and IL-22 Regulate Vaccine-Induced Protective Immunity against Challenge with *Mycobacterium tuberculosis*

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NK1.1+ Cells and IL-22 Regulate Vaccine-Induced Protective Immunity against Challenge with Mycobacterium tuberculosis

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We previously found that human NK cells lyse Mycobacterium tuberculosis-infected monocytes and alveolar macrophages and upregulate CD8+ T cell responses. We also found that human NK cells produce IL-22, which inhibits intracellular growth of M. tuberculosis, and that NK cells lyse M. tuberculosis-expanded CD4+CD25+FOXP3+ T regulatory cells (Tregs). To determine the role of NK cells during the protective immune response to vaccination in vivo, we studied the NK cell and T cell responses in a mouse model of vaccination with bacillus Calmette-Guérin (BCG), followed by challenge with virulent M. tuberculosis H37Rv. BCG vaccination increased the number of IFN-γ-producing and IL-22-producing NK cells. Depletion of NK1.1+ cells at the time of BCG vaccination increased the number of immunosuppressive Tregs (CD4+CD25hi, 95% Foxp3+) after challenge with M. tuberculosis H37Rv, and NK1.1+ cells lysed expanded but not natural Tregs in BCG-vaccinated mice. Depletion of NK1.1+ cells at the time of BCG vaccination also increased the bacillary burden and reduced T cell responses after challenge with M. tuberculosis H37Rv. IL-22 at the time of vaccination reversed these effects and enhanced Ag-specific CD4+ cell responses in BCG-vaccinated mice after challenge with M. tuberculosis H37Rv. Our study provides evidence that NK1.1+ cells and IL-22 contribute to the efficacy of vaccination against microbial challenge. The Journal of Immunology, 2012, 189: 000–000.

TBerculosis causes a staggering burden of mortality, killing 1.7 million individuals annually. Effective treatment in developing countries is hampered by the cost of antituberculosis drugs, inability to ensure completion of therapy, and rising drug resistance rates. Vaccination is an alternative cost-effective strategy that would contribute greatly to tuberculosis control. Development of an effective vaccine hinges on an improved understanding of immunity to Mycobacterium tuberculosis.

NK cells are prominent components of the innate immune response, but limited information is available on the role of NK cells in mycobacterial infection. Depletion of NK cells with Abs to NK1.1 and asialo-GM1 enhanced the growth of Mycobacterium avium in mice (1), and activated NK cells that produce IFN-γ and perforin accumulate in the lungs of M. tuberculosis-infected mice (2). NK cell-derived IFN-γ also regulates the anti-mycobacterial resistance mediated by neutrophils (3). Although depletion of NK cells did not affect the bacterial burden during M. tuberculosis infection in mice, NK cells may reduce immunopathology or favor development of protective immune responses. We found that human NK cells lyse infected monocytes and alveolar macrophages through the Nkp46 receptor and NKG2D (4, 5) and that NK cells contribute to the capacity of CD8+ T cells to produce IFN-γ and to lyse M. tuberculosis-infected monocytes (6). Human NK cells also produce IL-22, which inhibits intracellular growth of M. tuberculosis (7), and NK cells lyse M. tuberculosis-expanded CD4+ regulatory T cells (Tregs) (8). Human NK cells also produce IFN-γ when exposed to Mycobacterium bovis bacillus Calmette-Guérin (BCG) (9), and the pleural fluid of tuberculosis patients is enriched for CD56brightCD16− NK cells, which are a prominent source of IFN-γ (9). Despite these studies of human cells in response to M. tuberculosis ex vivo, the role of NK cells during an immune response to mycobacteria in vivo remains uncertain.

We hypothesized that NK cells could contribute to BCG-induced protective immunity to M. tuberculosis through interactions with T cells and tested this hypothesis in a mouse model. We found that in mice depleted of NK1.1+ cells at the time of BCG vaccination, there was increased expansion of CD4+CD25+Foxp3+ cells and a greater bacillary burden in the lungs after challenge with virulent M. tuberculosis. We also found that NK1.1+ cells lyse Tregs (CD4+CD25hi, 95% Foxp3+) in vivo during BCG vaccination and that IL-22, produced by NK1.1+ cells, induces optimal protective immunity through enhancing Ag-specific T cell responses after challenge with M. tuberculosis.

Materials and Methods

Animals

All animal studies were performed on specific pathogen-free 4- to 6-wk-old female C57BL/6 mice and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Tyler.

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Abbreviations used in this article: BCG, bacillus Calmette-Guérin; PLN, peripheral lymph node; Treg, regulatory T cell.

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Abs and other reagents

For flow cytometry, we used FITC anti-NK1.1, FITC anti-CD3, FITC anti-CD4, FITC anti-CD8, FITC anti-CCR7, PE anti-CD49b, PE anti-Foxp3, PE anti-NKp46, PE anti-CD44, PE anti-CD11b, PE-Cy5 anti-CD127,allophycocyanin anti-CD117, allophycocyanin anti-CD25, and allophycocyanin anti-IL-22 (all from BioLegend). For neutralization, we used mAbs to NK1.1 or isotype control Ab (Bio X Cell). We used gamma-irradiated M. tuberculosis H37Rv and Ag85a (both from BEI Resources), the BCG Tice strain (Organon USA), and Candida Ag (Grear Laboratories). Microbeads conjugated to Abs to CD11b, CD4, or CD8 (Miltenyi Biotec) were used for cell isolation.

Flow cytometry

Surface and intracellular staining was performed as described (8). BCG vaccination and aerosol infection with M. tuberculosis H37Rv

Mice were immunized s.c. at the base of the tail with 10⁶ CFU BCG in 100 µl PBS or with PBS alone. In some cases, mice were sacrificed 1–8 d after vaccination. For other experiments, 2 mo after vaccination, mice were infected with M. tuberculosis H37Rv in an aerosol exposure chamber using published methods (2). In preliminary studies, mice were exposed to different concentrations of M. tuberculosis, and CFUs were enumerated in homogenized lungs 24 h postinfection. For further studies, we selected the concentration that deposited ~50–100 bacteria in the lungs per mouse.

Depletion of NK1.1⁺ cells and treatment with IL-22 during BCG vaccination

Mice were given 0.3 mg anti-NK1.1 or isotype control Ab intravenously on days 0, 1, and 2, relative to administration of BCG. Anti-NK1.1 reduced the percentage of splenic CD3⁺ NKp46⁺ cells from 2.1 ± 0.3% to 0.4 ± 0.09% (p = 0.02, n = 4) as measured by flow cytometry. A representative flow cytometry result is shown in Supplemental Fig. 1. Some mice received recombinant IL-22 (2 ng) with each dose of anti-NK 1.1.

Culture of lung, spleen, and lymph node cells

In some experiments, BCG-vaccinated mice, uninfected or infected with M. tuberculosis H37Rv, were sacrificed, and cells from the lungs, spleens, or peripheral lymph nodes were cultured in 24-well plates at 2 × 10⁶ cells/well in RPMI 1640 containing penicillin (Life Technologies) and 10% heat-inactivated fetal bovine serum (FBS). Cells were cultured without gamma-irradiated H37Rv (10 mg/ml), Ag85a (3 µg/ml), or Candida (3 µg/ml) at 37°C and 5% CO₂. After 72 h, culture supernatants were collected to determine cytokine levels. In other experiments, CD11b⁺ and CD4⁺ cells from spleens and peripheral lymph nodes were isolated by positive immunomagnetic selection (Miltenyi Biotec). CD4⁺ cells and CD11b⁺ cells were cultured at a ratio of 10:1, with or without gamma-irradiated H37Rv (10 µg/ml) for 72 h. Cells were either stained to identify CD4⁺CD25⁺Foxp3⁺ cells or Tregs were isolated by the Treg isolation kit (Miltenyi Biotec), using positive immunomagnetic selection for CD4⁺ cells, followed by positive selection for CD25⁺ cells. CD4⁺CD25⁺ cells were also obtained. Ninety to ninety-five percent of purified CD4⁺CD25⁺Foxp3⁺ cells were Foxp3⁺ (Supplemental Fig. 2A) and major producers of IL-10, as determined by ELISPOT assay (Supplemental Fig. 2B).

Culture of monocytes, CD8⁺ cells, and Treg

Mice were vaccinated with BCG. One week later, freshly isolated splenic CD8⁺ cells were isolated and cultured (2 × 10⁶ cells per well) with 2 × 10⁵ autologous monocytes per well. In some wells, 2 × 10⁵ autologous Tregs, isolated from CD4⁺ cells and monocytes cultured with gamma-irradiated M. tuberculosis H37Rv, were added. Cells were cultured for 72 h, CD8⁺ cells were isolated by positive immunomagnetic selection, and IFN-γ mRNA expression was quantified by real-time PCR.

Real-time PCR for quantification of IFN-γ mRNA

IFN-γ mRNA was quantified in CD8⁺ cells using minor modifications of our published methods (7) and using primers for murine IFN-γ (forward, 5’-AGCTCATCCGAGTGGTCCAC-3’, reverse, 5’-AGCAAGGAGTGGTGTCCCC-3’), TTTTCG-3’- β-actin (forward, 5’-CTCTCAACCCCCCACTG-3’, reverse, 5’-TGGGACACCCAGGAGATTCA-3’).

In vivo cytotoxicity assay

Mice were vaccinated with BCG. After 72 h, CD4⁺CD25⁺ cells from control and vaccinated mice were isolated from pooled spleen and lymph node cells using the Treg isolation kit (Miltenyi Biotec). CD4⁺CD25⁺ cells from BCG-vaccinated and unvaccinated mice were labeled with 5 µM (CFSE 5’-OH) and 0.5 µM (CFSE 5’-OEt) CFSE, respectively, mixed 1:1 and inoculated intravenously (6 × 10⁶ cells/mouse) into recipient C57BL/6 mice 72 h after BCG vaccination. Before adoptive transfer of CD4⁺ CD25⁺ cells, recipient mice were treated with anti-NK1.1 or isotype control Abs 0, 1, and 2 d after vaccination. Eighteen hours after transfer of CD4⁺CD25⁺ cells, CFSElow and CFSEhigh cells in spleens and lymph nodes draining the vaccination site were quantified by flow cytometry. The percent in vivo lysis of BCG-expanded CD4⁺CD25⁺ cells by NK cells was calculated as follows: 100 - 100% [target population (BCG-expanded CD4⁺CD25⁺ cells) in isotype-treated /% control CD4⁺CD25⁺ cells in isotype-treated] + (% BCG-expanded CD4⁺CD25⁺ cells in NK1.1-depleted / % control CD4⁺CD25⁺ cells in NK1.1-depleted).

Measurement of IFN-γ concentrations by ELISA

Supernatants from cultured cells were collected after 72 h and stored at −70°C until IFN-γ concentrations were measured by ELISA (BioLegend).

Frequency of lung, spleen, and lymph node cells producing IFN-γ and IL-10

Lung, spleen, and lymph node cells from BCG-vaccinated mice, with or without subsequent M. tuberculosis infection, were isolated, and cells (1 × 10⁶ to 5 × 10⁷, depending on the experiment) were placed on ELISPOT plates coated with 2 µg/ml anti–IFN-γ Ab (BioLegend) in PBS. In some experiments, NK cells were isolated from spleen and lymph node cells with a negative selection kit (Miltenyi Biotec) and placed on the ELISPOT plates. After overnight incubation at 37°C in 5% CO₂, 2 µg/ml biotinylated anti-mouse IFN-γ (BioLegend) was added. After 2 h, plates were washed with PBS, and 1 µg/ml streptavidin-conjugated alkaline phosphatase (Mabtech) was added. After 45 min, plates were washed with PBS and developed with BCIP/NBT plus substrate (R&D Systems). Plates were dried, and the numbers of IFN-γ cells were counted. The frequency of IL-10-producing cells was identified by using ELISPOT kits (BioLegend).

Statistical analysis

Results are shown as the mean ± SE. Comparisons between groups were performed by a paired or unpaired t test, as appropriate.

Results

BCG vaccination enhances NK cell number and production of IFN-γ and IL-22

To determine whether BCG vaccination affects NK cells, mice were either vaccinated with BCG or not vaccinated. At different time points after vaccination, spleen and peripheral lymph node (PLN) cells were isolated, and the percentages of CD3⁺NKp46⁺ or CD3⁺CD4⁺NKp46⁺ cells were measured by flow cytometry. NK cells express NKp46 but not CD3. NKT cells are CD3⁺, and in mice some NKT cells express NKp46. The percentage of CD3⁺NKp46⁺ cells in PLN increased from 3.5 ± 0.1% at baseline to 6.2 ± 0.3% 48 h after vaccination (p = 0.01, Fig. 1A), with similar increases in the spleen (p = 0.002; Fig. 1A). In contrast, the number of CD3⁺NKp46⁺ cells were similar in control and BCG-vaccinated mice (Fig. 1A), indicating that the number of NK cells but not NKT cells increases in PLN and spleen in response to BCG vaccination during the first 72 h.

We next measured the frequency of IFN-γ-producing NK cells at different time points after BCG vaccination. Purified NK cells were obtained from spleen and PLN cells by negative selection, and ELISPOT assay was performed, as outlined in Materials and Methods. The frequency of IFN-γ-producing splenic NK cells increased 4- to 7-fold from 44 ± 5 per 10⁶ cells in unvaccinated mice to 182 ± 7, 306 ± 21, and 326 ± 19 per 10⁶ cells 24, 48, and 72 h after vaccination, respectively (p < 0.001 for all time points compared with unvaccinated mice; Fig. 1B). Similarly, the frequency of IFN-γ-producing PLN NK cells increased 8- to 20-fold from 30 ± 6 per 10⁶ cells in unvaccinated mice to 296 ± 12, 256 ± 27, and 606 ± 36 per 10⁶ cells 24, 48, and 72 h after vaccination, respectively (p < 0.001 for all time points compared with unvaccinated mice; Fig. 1B).

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FIGURE 1. BCG vaccination enhances NK cell number and production of IFN-γ and IL-22. C57BL/6 mice (five mice per group) were unimmunized or immunized s.c. with 10^6 CFU BCG in 100 μl PBS. After 24, 48, and 72 h, spleen and PLN cells were isolated. (A) The percentages of CD3^+ NKp46^+ and CD3^+ NKp46^+ cells were measured by flow cytometry. (B) The frequency of IFN-γ-producing NK cells was determined by ELISPOT assay. Purified NK cells were obtained from spleen and PLN cells by negative selection and incubated overnight in triplicate wells on an ELISPOT plate to determine the frequency of IFN-γ-producing NK cells. (C) Intracellular staining was performed to detect IL-22–producing CD3^+ NKp46^+ and CD3^+ NKp46^+ cells. Surface staining was performed with anti-CD3 and anti-NKp46, and intracellular staining was performed with isotype control or anti–IL-22. Mean values and SEs are shown. (D) A representative flow cytometry result of staining for IL-22 and NKp46 after gating on CD3^− and CD3^+ spleen cells is shown.

Next, we used intracellular staining to detect IL-22–producing CD3^− NKp46^+ NK cells or CD3^+ NKp46^+ NKT cells in splenocytes after BCG vaccination. The percentages of CD3^− NKp46^+ IL-22^+ and CD3^+ NKp46^+ IL-22^+ cells increased from 0.05 ± 0.1% to 1.15 ± 0.2% and 0.08 ± 0.05% to 0.44 ± 0.1% 48 h after vaccination (p = 0.05, Fig. 1C). This suggests that the majority of NKp46^+ IL-22–producing cells upon BCG vaccination are NK cells and not NKT cells. A representative flow cytometry result is shown in Fig. 1D.

NK cells reduce expansion of CD4^+ CD25^+ Foxp3^+ T cells after BCG immunization

To determine the effect of NK cells on T cell responses after BCG immunization, we immunized mice s.c. with 10^6 CFU BCG. Some BCG-immunized mice were also treated with three doses of anti-NK1.1 or with isotype control Ab. Anti-NK1.1 reduced the percentage of CD3^− NKp46^+ cells from 2.1 ± 0.3% to 0.4 ± 0.09% (p = 0.02, n = 4) in spleens, and a similar decrease was seen in PLNs, as measured by flow cytometry. A representative flow cytometry result is shown in Supplemental Fig. 1. To evaluate interactions between NK1.1^+ cells and Tregs, we quantified CD4^+ CD25^+ Foxp3^+ cells by flow cytometry. One week after BCG vaccination, more CD4^+ CD25^+ Foxp3^+ cells were present in the spleen compared with unvaccinated mice (890 ± 66 versus 570 ± 34 cells per 10^6 CD4^+ cells, respectively; p < 0.02, Fig. 2A). Depleting NK1.1^+ cells at the time of BCG vaccination further increased the number of CD4^+ CD25^+ Foxp3^+ cells to 1095 ± 50 per 10^6 CD4^+ cells versus 838 ± 61 per 10^6 CD4^+ cells for treatment with the isotype control Ab (Fig. 2A, p = 0.01). Similar effects were noted in the PLNs (Fig. 2A).

One week after immunization, we also determined the capacity of CD4^+ cells to expand into CD4^+ CD25^+ Foxp3^+ cells in the presence of gamma-irradiated M. tuberculosis H37Rv. CD4^+ and CD11b^+ cells from spleens and PLNs were isolated and cultured, with or without gamma-irradiated M. tuberculosis H37Rv for 5 d, as outlined in Materials and Methods. BCG vaccination induced expansion of splenic CD4^+ CD25^+ Foxp3^+ cells to 1020 ± 60 per 10^6 CD4^+ cells compared with 510 ± 60 per 10^6 CD4^+ cells in control mice. Depletion of NK1.1^+ cells with Ab further increased expansion of CD4^+ CD25^+ Foxp3^+ cells to 1400 ± 63 per 10^6 CD4^+ cells compared with 930 ± 76 per 10^6 CD4^+ cells after treatment with isotype control Ab (Fig. 2B, p = 0.003). Similar findings were noted in PLNs (Fig. 2B) where BCG vaccination induced expansion of splenic CD4^+ CD25^+ Foxp3^+ cells to 700 ± 40 per 10^6 CD4^+ cells compared with 480 ± 36 per 10^6 CD4^+ cells in control mice. Depletion of NK1.1^+ cells further increased expansion of CD4^+ CD25^+ Foxp3^+ cells to 1210 ± 75 per 10^6 CD4^+ cells compared with 778 ± 23 per 10^6 CD4^+ cells after treatment with isotype control Ab (Fig. 2B, p = 0.001). A representative flow cytometry result is shown in Fig. 2C.

The data above suggest that NK1.1^+ cells inhibit expansion of CD4^+ CD25^+ Foxp3^+ Tregs, which can in turn upregulate T cell effector function. Therefore, we evaluated the effect of NK1.1^+ cell depletion on IFN-γ production by splenocytes and lymph node cells. BCG vaccination increased M. tuberculosis-stimulated IFN-γ production by PLN cells, but depletion of NK1.1^+ cells decreased IFN-γ concentrations by 60% from 11.5 ± 0.5 ng/ml in isotype-treated mice to 4.9 ± 0.1 ng/ml (Fig. 2D, p = 0.057). NK1.1^+ cell depletion had similar but more modest effects on IFN-γ production by splenocytes, which fell from 15.9 ± 0.4 ng/ml in isotype-treated mice to 14.3 ± 0.3 ng/ml (Fig. 2D, p = 0.03).

NK1.1^+ cells inhibit expansion of Tregs after BCG vaccination and challenge with M. tuberculosis H37Rv

To determine if NK1.1^+ cells affected Tregs during infection with M. tuberculosis H37Rv in vivo, we evaluated the response of mice to aerosol challenge with M. tuberculosis H37Rv after vaccination with BCG. Mice were either unimmunized or immunized s.c. with
BCG. Some immunized mice were also treated with anti-NK1.1 or isotype control Ab, as outlined in Materials and Methods. After 60 d, mice were challenged with M. tuberculosis H37Rv by aerosol. Thirty days postinfection, CD4+CD25+Foxp3+ cells in the lungs were quantified by flow cytometry. After infection with M. tuberculosis H37Rv, the number of Tregs per lung in unvaccinated mice rose 50-fold from $4.1 \times 10^3$ to $2.1 \times 10^5$ ($p = 0.02$, Fig. 3A). Depletion of NK1.1+ cells at the time of BCG vaccination further increased the number of Tregs after challenge with M. tuberculosis H37Rv to $5.6 \times 10^5$ cells per lung compared with $1.1 \times 10^5$ cells per lung with use of isotype control Abs (Fig. 3A, $p = 0.0002$). A representative flow cytometry result is shown in Fig. 3B.

M. tuberculosis H37Rv-expanded CD4+CD25hi cells are immunosuppressive

To determine whether the CD4+CD25hiFoxp3+ cells that expand in response to M. tuberculosis H37Rv are functional Tregs, we evaluated their capacity to inhibit IFN-γ production by CD8+ cells in response to M. tuberculosis H37Rv. From mice that had been vaccinated with BCG 1 wk previously, we isolated CD4+ and CD11b+ cells and cultured them with gamma-irradiated M. tuberculosis H37Rv. After 72 h, the CD4+CD25hi cells and CD4+CD25− cells were isolated and cultured in Transwells, within large wells containing CD8+ and CD11b+ cells from BCG-vaccinated mice and gamma-irradiated M. tuberculosis H37Rv. After 72 h, the Transwells were removed, and IFN-γ mRNA was quantified in the cells in the large wells by real-time PCR. Values were normalized, so that the value of IFN-γ mRNA expression by CD8+ and CD11b+ cells in the absence of M. tuberculosis H37Rv was set to 1.0. M. tuberculosis H37Rv induced a 14-fold increase in IFN-γ mRNA expression by CD8+ cells (Fig. 3C). CD4+CD25hi cells inhibited IFN-γ mRNA expression by CD8+ T cells to a much greater extent than CD4+CD25− cells ($3.6 \pm 0.2$ versus $10.5 \pm 0.5$, $p = 0.004$, Fig. 3C). Therefore, the CD4+CD25hi BCG-expanded cells inhibit T cell responses and function as Tregs.

In the above studies, we used CD4+CD25hi cells that were generated in vitro. To confirm that CD4+CD25hi cells that were generated during M. tuberculosis H37Rv challenge after BCG vaccination were also immunosuppressive, we cultured in Transwells CD4+CD25hi cells from M. tuberculosis H37Rv-infected mice that had been previously BCG-immunized and treated with either isotype control or anti-NK1.1 Abs. CD4+CD25hi cells from both groups of mice inhibited IFN-γ mRNA expression by CD8+ cells, which fell from $3.8 \pm 0.3$ to $0.3 \pm 0.1$ (Fig. 3D, $p = 0.01$) and from $4 \pm 0.2$ to $1.3 \pm 0.3$ (Fig. 3D, $p = 0.04$).
NK1.1+ cells inhibit expansion of immunosuppressive Tregs after BCG vaccination and challenge with M. tuberculosis H37Rv. C57BL/6 mice (three to eight mice per group) were immunized or immunized s.c. with 10^6 CFU BCG in 100 μl PBS. Some BCG-vaccinated mice were treated with anti-NK1.1 or isotype control Ab (0.3 mg per mouse 0, 24, and 48 h after vaccination). Sixty days after BCG vaccination, mice were challenged with 50–100 CFU M. tuberculosis H37Rv by aerosol. (A) Thirty days postinfection, CD4+CD25^hiFoxp3^+ T cells in the lungs were measured by flow cytometry. (B) A representative flow cytometry result of lung cells is shown. We gated on lung CD4+ cells and then gated on CD25^+ and Foxp3^+ expressing cells. (C) M. tuberculosis H37Rv-expanded CD4+CD25^hi cells are immunosuppressive. Three C57BL/6 mice were immunized s.c. with 10^6 CFU BCG. One week later, CD4+ and CD11b+ cells from spleens and PLNs were isolated and cultured with gamma-irradiated M. tuberculosis H37Rv. After 72 h, CD4+CD25^hi cells and CD4+CD25^+ cells were isolated and cultured in Transwells, within large wells containing CD8+ and CD11b+ cells obtained from mice 1 wk after BCG vaccination and gamma-irradiated M. tuberculosis H37Rv. After 72 h, the Transwells were removed, and IFN-γ mRNA was quantified in the cells in the large wells by real-time PCR. (D) CD4+CD25^hi cells were isolated and cultured in Transwells, within large wells containing CD8+ and CD11b+ cells obtained from mice 1 wk after BCG vaccination and gamma-irradiated M. tuberculosis H37Rv. After 72 h, the Transwells were removed, and IFN-γ mRNA was quantified in the cells in the large wells by real-time PCR. Mean values and SEs are shown. (E) C57BL/6 mice (eight mice per group) were immunized s.c. with 10^6 CFU BCG. Some BCG-vaccinated mice were treated with anti-NK1.1 or isotype control Ab (0.3 mg per mouse 0, 24, and 48 h after vaccination). Sixty days after BCG vaccination, mice were challenged with 50–100 CFU M. tuberculosis H37Rv by aerosol. Thirty days postinfection, lungs were homogenized and plated on 7H11 agar with THC, and CFUs per lung were counted after 3 wk.

We determined the effects of NK1.1+ cell depletion on the bacterial burdens in the lungs. BCG vaccination reduced CFUs per lung in M. tuberculosis H37Rv-infected mice from 2.5 ± 0.3 × 10^6 to 0.3 ± 0.04 × 10^6 (p = 0.0002, Fig. 3E). Depletion of NK1.1+ cells at the time of vaccination reduced the protective effect of BCG vaccination, with CFUs increasing from 0.3 ± 0.03 × 10^6 to 1.2 ± 0.2 × 10^6 CFU per lung (p = 0.007, Fig. 3E). These findings suggest that NK1.1+ cells during BCG vaccination inhibit expansion of immunosuppressive Tregs and limit bacillary replication.

NK1.1+ cells lyse expanded Tregs during the response to BCG vaccination in vivo

Previously, we found that activated human NK cells lyse M. tuberculosis H37Rv-expanded Tregs but not natural Tregs in vitro (6), and our above data demonstrate that depletion of NK1.1+ cells at the time of vaccination increases the number of immunosuppressive Tregs and reduces the protective efficacy of BCG vaccination against challenge with M. tuberculosis H37Rv. To determine if this is due to NK1.1+ cell lysis of Tregs in vivo, we performed an in vivo cytotoxicity assay by adoptively transferring CFSE<sup>high</sup>-labeled BCG-expanded CD4+CD25<sup>hi</sup> cells and CFSE<sup>low</sup>-labeled control CD4+CD25<sup>hi</sup> (natural Tregs) cells to mice 72 h after the recipients had received BCG vaccine and treatment either with anti-NK1.1 or isotype control Ab. Eighteen hours after adoptive transfer of CD4+CD25<sup>hi</sup> cells, CFSE<sup>high</sup> and CFSE<sup>low</sup> cells were quantified by flow cytometry, and the percent lysis of expanded CD4+CD25<sup>hi</sup> cells was calculated. In isotype control Ab-treated mice, 59 ± 7% and 33 ± 6% of expanded CD4+CD25<sup>hi</sup> cells were lysed in the spleen and PLNs, respectively. NK1.1+ cell depletion reduced these values to 5 ± 5% and 3.8 ± 3.8%, respectively (p = 0.002 and p < 0.001, respectively, Fig. 4A), suggesting that NK1.1+ cells lyse substantial numbers of expanded CD4+CD25<sup>hi</sup> cells in vivo. In contrast, NK1.1+ cells did not lyse natural CD4+CD25<sup>hi</sup> natural Tregs in both spleen and PLNs. A representative flow cytometry result is shown in Fig. 4B.

NK1.1+ cells and IL-22 enhance the protective efficacy of BCG vaccination

The results above show that BCG vaccination elicits IL-22 production by NK1.1+ cells (Fig. 1C) and that depletion of NK1.1+ cells at the time of BCG vaccination enhances expansion of immunosuppressive Tregs and increases bacillary replication in the lungs of mice challenged with M. tuberculosis H37Rv (Fig. 3E). To determine if IL-22 could substitute for the effects of NK1.1+ cells on CD4+ cell responses, we treated BCG-vaccinated mice with anti-NK1.1, isotype control Ab, or anti-NK1.1 plus recombinant IL-22. After 60 d, mice were challenged with M. tuber-
culosis H37Rv by aerosol. Sixty days postinfection, we measured the number of Tregs in lungs. Similar to the findings in Fig. 3A, depletion of NK1.1+ cells at the time of BCG vaccination and challenge with M. tuberculosis H37Rv markedly increased the number of Tregs in lungs compared with isotype control Ab-treated mice (9.2 ± 1.7 × 10^5 versus 2.2 ± 1.0 × 10^5 cells per lung, p = 0.04, Fig. 5A). In contrast, IL-22 abrogated the effects of anti-NK1.1, with the number of Tregs falling from 9.2 ± 1.7 × 10^5 to 1.0 ± 0.2 × 10^5 cells per lung (p = 0.01, Fig. 5A). A representative flow cytometry result is shown in Fig. 5B.

We next evaluated the effect of NK1.1+ cell depletion and IL-22 on mycobacterial Ag-induced IFN-γ production by lung cells. Thirty days after M. tuberculosis H37Rv infection of BCG-vaccinated mice, lung cells were isolated and stimulated with gamma-irradiated M. tuberculosis H37Rv, Ag85a, and Candida Ags. Depletion of NK1.1+ cells at the time of BCG vaccination inhibited IFN-γ production by lung cells in response to gamma-irradiated M. tuberculosis H37Rv by 80%, and this was reversed by recombinant IL-22 (Fig. 5C). Similar findings were noted in the response to Ag85, except that IL-22 increased IFN-γ production to a greater extent (Fig. 5D). No IFN-γ was detected when cells were stimulated with Candida (data not shown).

NK1.1+ cell depletion at the time of vaccination reduced the protective efficacy of BCG against challenge with M. tuberculosis

FIGURE 4. NK cells lyse expanded Tregs during the response to BCG vaccination in vivo. (A) C57BL/6 mice (five mice per group) were unimmunized or immunized s.c. with 10^6 CFU BCG in 100 µl PBS. After 72 h, CD4+CD25hi cells were isolated from pooled spleen and lymph node cells using the Treg isolation kit. CD4+CD25hi cells from BCG-vaccinated mice and control unvaccinated mice were designated as expanded and natural Tregs, respectively. Expanded and natural Tregs were labeled with 5 µM (CFSE<sup>hi</sup>) and 0.5 µM (CFSE<sup>lo</sup>) of CFSE, respectively, mixed 1:1, and inoculated intravenously (6 × 10^6 cells/mouse) into recipient C57BL/6 mice 72 h after BCG vaccination. Recipient mice were treated with anti-NK1.1 or isotype control Abs 0, 1, and 2 d after vaccination. Eighteen hours after transfer of Tregs, CFSE<sup>hi</sup> and CFSE<sup>lo</sup> populations were detected by flow cytometry of single-cell suspensions from spleens and PNs draining the vaccination site. The percent of in vivo lysis of BCG-expanded Tregs was calculated using the formula given in Materials and Methods. Mean values and SEs of five independent experiments are shown. (B) A representative flow cytometry result of cells from a BCG-vaccinated mouse is shown. CFSE<sup>hi</sup> cells are CD4+CD25hi BCG-expanded Tregs, and CFSE<sup>lo</sup> cells are CD4+CD25hi natural Tregs.

FIGURE 5. NK1.1+ cells and IL-22 enhance the protective efficacy of BCG vaccination. C57BL/6 mice were unimmunized or immunized s.c. with 10^6 CFU BCG. Some BCG-vaccinated mice were treated with anti-NK1.1 or isotype control Ab (0.3 mg per mouse 0, 24 and 48 h after vaccination). Some anti-NK1.1–treated mice received recombinant IL-22 (2 ng) at the same time points. Sixty days after BCG vaccination, mice were challenged with 50–100 CFU M. tuberculosis H37Rv by aerosol. (A) Thirty days postinfection, CD4+CD25Foxp3+ T cells in lungs were measured by flow cytometry. (B) A representative flow cytometry plot of CD4+CD25Foxp3+ T cells is shown. Gating was performed as in Fig. 3B. (C and D) Thirty days postinfection, lung cells were isolated and stimulated with gamma-irradiated M. tuberculosis (C) or Ag85a (D). After 72 h, IFN-γ levels were measured by ELISA. Values shown are those obtained after subtracting IFN-γ levels from wells containing medium alone. (E) Sixty days postinfection, lungs were homogenized and plated on 7H11 agar with THC, and CFUs per lung were counted after 3 wk.
H37Rv, with CFUs 2 mo postinfection increasing 5-fold ($p = 0.02$; Fig. 5E). IL-22 abrogated the effect of NK1.1$^+$ cell depletion on CFUs ($p = 0.007$; Fig. 5E).

**IL-22 expands memory CD4$^+$ cells after BCG vaccination and *M. tuberculosis* H37Rv infection**

To determine whether NK1.1$^+$ and IL-22 regulate antimycobacterial memory CD4$^+$ cell responses, we immunized mice with BCG and treated some of them with anti-NK1.1, isotype control Ab, or anti-NK1.1 plus recombinant IL-22. After 60 d, mice were challenged with *M. tuberculosis* H37Rv by aerosol. Thirty days postinfection, CD4$^+$CD44$^+$CD117$^+$CD127$^+$ cells in the lungs were measured by flow cytometry. CD117 is expressed by human CD4$^+$ cells (10), CD117$^+$ cells have previously been identified as major producers of IFN-$g$ (11), and we found that most CD4$^+$ IFN-$g$-producing cells in BCG-vaccinated mice were CD117$^+$ (R. Dhiman and R. Vankayalapati, unpublished observations). CD44 and CD127 were used to identify cells with a memory and activation phenotype, respectively. BCG vaccination doubled the number of CD4$^+$CD44$^+$CD117$^+$CD127$^+$ cells per lung in *M. tuberculosis* H37Rv-infected mice from $1.6 \pm 0.7 \times 10^5$ to $3.6 \pm 1.1 \times 10^5$ (Fig. 6A). Depletion of NK1.1$^+$ cells at the time of BCG vaccination reduced the number of CD4$^+$CD44$^+$CD117$^+$CD127$^+$ cells per lung to $2.3 \pm 0.4 \times 10^5$, and IL-22 increased this number to $6.7 \pm 1.1 \times 10^5$ cells per lung ($p = 0.03$, Fig. 6A). A representative flow cytometry result is shown in Fig. 6B.

**Discussion**

Murine cells that express NK1.1 include CD3$^-$ NK cells and CD3$^+$ NKT cells, both of which contribute to immunity against cancer, viruses, and bacteria, including *M. tuberculosis*. However, limited information is available on the role of NK1.1$^+$ cells in vaccine-induced protective immune responses. We addressed this question in a mouse model of vaccination with BCG, followed by challenge with virulent *M. tuberculosis* H37Rv. Depletion of NK1.1$^+$ cells at the time of BCG vaccination increased the number of immunosuppressive Tregs, increased the bacillary burden, and reduced Ag-specific T cell responses after challenge with *M. tuberculosis* H37Rv. We also found that IL-22 can reverse the effect of NK1.1$^+$ cell depletion, reducing the number of Tregs, increasing T cell IFN-$g$ production, increasing the number of memory T cells, and reducing the bacillary burden after challenge with *M. tuberculosis*. Our study provides evidence that NK1.1$^+$ cells and IL-22 contribute to the efficacy of vaccination against microbial challenge.

BCG vaccination resulted in infiltration of IFN-$g$– and IL-22–producing NK1.1$^+$ cells into the spleen and PLNs within 24 h after vaccination, paralleling previous findings that intranasal immunization with the *M. tuberculosis* protein Ag85B induced accumulation of activated and cytokine-producing NK cells in nasal-associated lymphoid tissue and cervical lymph nodes (12). BCG vaccination induced expansion of NK cells but not NKT cells (Fig. 1A) and elicited 3-fold more IL-22$^+$ NK cells than NKT cells (Fig. 1C), suggesting that NK cells may play a greater role in BCG-induced responses than NKT cells. We found that NK1.1$^+$ cells lyse *M. tuberculosis* H37Rv-expanded CD4$^+$CD25$^+$ cells in vivo and that depletion of NK1.1$^+$ cells at the time of BCG vaccination induced expansion of immunosuppressive CD4$^+$CD25$^+$ cells after challenge with virulent *M. tuberculosis* H37Rv. These findings confirm our previous work showing that activated human NK cells lyse *M. tuberculosis*-expanded Tregs in vitro (8) and extend this mechanism to a physiologically relevant model of vaccination-induced protection against subsequent infection. It is possible that NKT cells may also destroy Tregs, although there are no published data demonstrating this effect.

Tregs inhibit immune responses to many intracellular pathogens, including *M. tuberculosis*, at least in part by delaying trafficking of effector T cells to the site of infection (13). Prevention of recruitment of Tregs by antagonism of CCR4 enhanced mycobacterial Ag-specific responses to a vaccine expressing Ag85a (14), and depletion of Tregs with anti-CD25 Ab increased T cell responses and improved protection against challenge with herpes simplex virus and *Plasmodium* (15–17). In contrast, treatment with anti-CD25 prior to BCG vaccination led to a stronger T cell response but did not enhance protection against *M. tuberculosis* or *M. bovis* (18). In the current study, depletion of NK1.1$^+$ cells at the time of BCG vaccination increased the number of Tregs, reduced T cell production of IFN-$g$, and increased the bacillary burden after challenge with *M. tuberculosis* H37Rv. These findings suggest that NK1.1$^+$ cells lyse Ag-induced Tregs that normally expand during vaccination and may yield memory Tregs that could further expand during secondary infection (19, 20).

Our in vivo cytotoxicity results strongly suggest that NK1.1$^+$ cells in BCG-vaccinated mice lysed *M. tuberculosis* H37Rv-expanded but not natural Tregs. Although it is theoretically possible that our results could be explained by reduced homing of expanded Tregs to the spleen and lymph nodes in the absence of NK
cells, no published data suggest that depletion of any cell population has a major effect on Treg homing to secondary lymphoid organs. Because BCG vaccination increased the number of IFNgamma- and IL-22-producing NK cells, we speculate that NK cells are activated after BCG vaccination and have an increased capacity to lyse expanded but not natural Tregs, paralleling findings in humans (8). NK cells lyse their targets through interactions between NK cell activating receptors and their ligands. NKG2D is a prominent NK cell activating receptor, and its ligands in human cells include ULBPs (39) and MHC class I-related chain A and B (21, 22). Activated Tregs express higher levels of ULBP-1 than those of natural Tregs, and NK cell lysis of activated Tregs is inhibited by anti–ULBP-1 and anti-NKG2D (8). It is intriguing to speculate that murine expanded Tregs may also have increased expression of the NKG2D ligands RAE-1, H60, and MULT-1 compared with that of natural Tregs and thus be more susceptible to lysis by activated NK cells.

The best studied murine NKT cells are those bearing an invariant Vα14-Jo18 TCR that recognizes α-galactosylceramide and are restricted by CD1d. Pharmacologic activation of these cells with synthetic α-galactosylceramide improves the outcome of M. tuberculosis infection (23, 24). However, M. tuberculosis and M. bovis BCG do not contain natural Ags for invariant NKT cells, and infection of dendritic cells with M. bovis BCG fails to activate invariant NKT cells (25). There are also no published data demonstrating that NKT cells lyse Tregs. Combined with our current findings that BCG vaccination elicits expansion of NK cells but not NKT cells (Fig. 1A) and of production of IL-22 primarily by NK cells (Fig. 1C), we believe that NK cells are likely to play the dominant role in lysing Tregs, increasing T-cell IFN-gamma production, and increasing protective efficacy against M. tuberculosis challenge after BCG vaccination. However, we cannot formally exclude a role for NKT cells in these processes.

IL-22 is produced by activated T cells (26), particularly Th17 cells (27), and is a critical mediator of early mucosal defense on the lung after challenge with M. tuberculosis infection (36), IL-22 levels are elevated at the site of infection in mouse primates and human NK cells produce IL-22, which is clearly involved in the defenses of NK cells or NK-like cells in secondary lymphoid tissue (31–33), and is a critical mediator of early mucosal defense. We speculate that IL-22 may have an increased expression of the NKG2D ligands RAE-1, H60, and MULT-1 compared with that of natural Tregs and thus be more susceptible to lysis by activated NK cells.

The mechanisms through which IL-22 inhibits Treg expansion and enhances T cell responses remain uncertain. IL-22R is expressed by M. tuberculosis-infected monocyte-derived macrophages (7) but not by T cells (41, 42). Dendritic cells are potent APCs that can be monocyte derived, and different dendritic cell subpopulations favor expansion of Tregs and Ag-reactive Th1 cells (43–45). We speculate that IL-22 may skew dendritic cell subpopulations toward those that favor expansion of Th1 cells and/or elicit migration of these subpopulations toward the site of BCG vaccination or to the lung during aerosol challenge with M. tuberculosis H37Rv.

In summary, using a mouse model of vaccination with BCG, followed by challenge with M. tuberculosis H37Rv, we found that NK1.1+ cells and IL-22 contribute to vaccine-induced protection against microbial challenge by reducing the numbers of immunosuppressive Tregs and enhancing Ag-specific T cell responses. Further delineation of the mechanisms through which NK1.1+ cells destroy Tregs and optimize Th1 responses during BCG vaccination will facilitate development of vaccines against M. tuberculosis and other intracellular pathogens.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental figure legends

**Supplemental Fig. 1.** Anti-NK1.1 depletes mice of NK and NKT cells. Mice were given 0.3 mg of anti-NK1.1 or isotype control Ab by tail vein injection on days 0, 1 and 2, relative to administration of BCG. After 72 h, spleen and peripheral lymph node cells were isolated, and CD3+ and NKP46+ cells were measured by flow cytometry. Four mice were used for each group. A representative flow cytometry result of spleen cells is shown.

**Supplemental Fig. 2.** **A.** FoxP3 expression by CD4+CD25^{hi} and CD4+CD25^{-} cells. C57BL/6 mice were immunized subcutaneously with 10^6 CFU of BCG. After 72 h, CD4^{+}CD25^{hi} cells were isolated from pooled spleen and lymph node cells, using the Treg isolation kit. Purified cells were stained with Abs to CD4 and CD25, and intracellular staining was performed with anti-FoxP3. The experiment was performed three times. A representative flow cytometry result is shown. **B.** Frequency of IL-10-producing CD4^{+}CD25^{hi} and CD4^{+}CD25^{-} cells. The above purified CD4^{+}CD25^{hi} and CD4^{+}CD25^{-} cells were placed on an ELISPOT plate. Following overnight incubation, the frequency of IL-10-producing cells was determined, using ELISPOT kits (Biolegend). Four mice were used.