Bovine NK Cells Can Produce Gamma Interferon in Response to the Secreted Mycobacterial Proteins ESAT-6 and MPP14 but Not in Response to MPB70

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Bovine NK cells have recently been characterized and the present study describes the interaction between NK cells, antigen-presenting cells, and secreted mycobacterial proteins. Gamma interferon (IFN-γ) production by NK cells was seen in approximately 30% of noninfected calves in response to the Mycobacterium tuberculosis complex-specific protein ESAT-6, MPP14 from Mycobacterium avium subsp. paratuberculosis, and purified protein derivative (PPD) from M. tuberculosis. In contrast, no response was induced by MPB70, which is another M. tuberculosis complex-specific secreted antigen. The production of IFN-γ by NK cells in whole blood in response to ESAT-6 and MPP14 was demonstrated using intracellular staining together with surface labeling for the NK cell-specific receptor, NKp46, or CD3. Furthermore, the depletion of NK cells from peripheral blood mononuclear cells completely abolished the IFN-γ production. The response was mediated through stimulation of adherent cells and was largely independent of contact between adherent cells and the NK cells. Neutralization of interleukin-12 only partly inhibited IFN-γ production, showing that other cytokines were also involved. The demonstration of NK cell-mediated IFN-γ production in young cattle provides an explanation for the nonspecific IFN-γ response frequently encountered in young cattle when using the IFN-γ test in diagnosis of mycobacterial infections.

Mycobacterial infections are widespread in ruminants and considered of major significance. The most important of these is bovine tuberculosis caused by Mycobacterium bovis, which has large economic implications and is a threat to human health in many parts of the developing world. Another widespread mycobacterial pathogen is Mycobacterium avium subsp. paratuberculosis. This bacterium causes paratuberculosis in ruminants and has been linked to Crohn’s disease in humans (5). Although conclusive evidence for the role of M. avium subsp. paratuberculosis in Crohn’s disease is lacking, the increased public focus on food-borne pathogens has given a renewed interest in this bacterium.

Both M. avium subsp. paratuberculosis and M. bovis are intracellular bacteria that survive and replicate inside host cells and they elicit immune responses with similar characteristics. The outcome of the infection is dependent on a complex interplay between the invading bacteria and the immune responses of the host. Protective immunity is dependent on activation of the cellular immune response with the production of the Th1 cytokine gamma interferon (IFN-γ), which is essential for resistance to mycobacteria (8, 16). The steering of the immune response onto a Th1 pathway is dependent on other cytokines produced by the innate immune system. A key cytokine in this context is interleukin-12 (IL-12), which is essential for the development of a protective Th1 response. The importance of this cytokine has been demonstrated in humans, where disruption of IL-12 or IL-12 receptors gives an increased susceptibility to mycobacterial infections (1, 8, 10, 11). IL-12 also acts in synergy with IL-18 and several studies have demonstrated that monocytes/macrophages infected with mycobacteria secreted IL-12 and IL-18 (15, 17). There may however be a distinct difference in the ability of the antigen-presenting cells to induce IL-12 and IL-18 production (18). Hope et al. have shown that bovine dendritic cells infected with virulent M. bovis and M. bovis BCG produced IL-12, tumor necrosis factor alpha, and little IL-10, whereas macrophages produced tumor necrosis factor alpha, IL-10, and little IL-12 (22).

IL-12 and IL-18 also have a synergistic effect on IFN-γ production from NK cells, which are large granular lymphocytes belonging to the innate immune system (17). Several studies have shown that NK cells respond to mycobacterial infections but they are not believed to be essential for protection (24). Nevertheless, NK cells are likely to play a role in immune modulation and initiation of a Th1 pathway. The findings of Vankayalapati et al. supported a role for NK cells as a link between the innate and adapted immune response when they demonstrated that human NK cells regulated CD8+ T-cell effector function in response to Mycobacterium tuberculosis (42). A direct effect of mycobacteria on NK cells was also recently described by Esin et al. (14). They demonstrated that NK cells responded to extracellular BCG with proliferation, IFN-γ production, and cytotoxicity. Similar results has been reported in the bovine model where NK-like cells from naive calves produced IFN-γ in response to M. bovis BCG-infected dendritic cells (21).

The ability of NK cells to produce large amounts of IFN-γ...
Paratuberculosis and bovine tuberculosis were used in the present study. ESAT-6, MPB70, and PPD and incubated at 37°C in 5% CO2 in air for 24 h. One/H9253 Science Division, Stormont, Belfast, and Peter Andersen, Statens Serum Insti-

**MATERIALS AND METHODS**

**Antigens.** A 14-kDa secreted antigen (MPP14) was purified from *M. avium* subsp. *paratuberculosis* as described previously (33). Purified protein derivative (PPD) from *M. tuberculosis* was obtained from the National Veterinary Institute, Oslo, Norway. Recombinant ESAT-6 produced in *Escherichia coli* and *Lactococcus lactis*, respectively, were kindly provided by John Pollock, Veterinary Science Division, Stormont, Belfast, and Peter Andersen, Statens Serum Insti-

**Intracellular staining for IFN-γ**. We incubated 5 ml heparinized whole blood with PPD, MPP14, ESAT-6, or recombinant bovine IL-2 (rBL-2) (100 units/ml) and rBL-12 (5 units/ml) (20) for 24 h. Nonstimulated control cultures and positive control cultures stimulated with 50 μg/ml phorbol 12-myristate 13-

**Statistics.** The differences in responses to the various antigens were evaluated using analysis of variance (ANOVA) and Dunnett's test using the unpaired *t*-test. The significance level was set at 5%. The results were expressed as means ± standard deviation (SD).

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RESULTS

**IFN-γ production in response to secreted mycobacterial proteins in young cattle.** Young cattle in an area free from bovine tuberculosis and paratuberculosis were tested for IFN-γ production in response to ESAT-6, MPB70, and PPD. Clinically healthy cattle (n = 54) from three different herds were tested four times for IFN-γ production after stimulation of whole blood with PPD, ESAT-6, and MPB70 for 24 h. The mean response in each animal is shown as dots, while the means for each antigen stimulation are shown as lines. Corrected OD is expressed as [(mean of antigen-stimulated wells – mean control wells)/(test positive control – test negative control)].

![Graph showing IFN-γ production between 2 and 18 months of age](http://iai.asm.org/)

**Antigen-induced IFN-γ production in NK cell cultures was dependent on adherent cells.** In an attempt to define the mechanism of IFN-γ production by NK cells, rbiL-2 activated NK cell cultures were stimulated with MPP14 and ESAT-6. No IFN-γ was detected, indicating that the antigens alone were unable to stimulate NK cells directly (data not shown). We also found a large individual variation in the number of IFN-γ-producing NK cells in nonstimulated samples ranging from 0.1 to 3.0% while no spontaneous IFN-γ production in CD3⁺ cells was detected. The two different methods, depletion of NK cells from PBMC and intracellular staining, conclusively established NK cells as a source of IFN-γ after stimulation with ESAT-6 and MPP14.

NK cells produce IFN-γ in response to mycobacterial antigens. To test the hypothesis that NK cells were responsible for the IFN-γ production, PBMC from responding animals were depleted of NK cells using a monoclonal antibody directed against NKP46, followed by secondary antibodies coupled to magnetic beads. The removal of NK cells was monitored using flow cytometry and routinely more than 95% of the NK cells were removed. PBMC and PBMC depleted of NK cells were stimulated with MPP14, ESAT-6, and PPD, and the supernatant was assayed for IFN-γ production after 24 h of stimulation. The samples were stimulated in triplicate, and the assay was repeated three times using PBMC from six different animals, and two representative examples are shown in (Fig. 3). While the amount of IFN-γ produced varied from animal to animal, the IFN-γ responses were always eliminated after depletion of NK cells. This demonstrates that the NK cells produced IFN-γ, or alternatively, were necessary for IFN-γ production from other cell types.

To confirm that the cells producing IFN-γ were indeed NK cells, whole blood from responder animals were incubated with ESAT-6 or MPP14 or left unstimulated. Intracellular staining for IFN-γ together with labeling for surface markers and analysis by flow cytometry identified NK cells as the main IFN-γ-producing cell type while little IFN-γ was produced by CD3⁺ cells (Fig. 4). The experiment was repeated six times, and between 1 and 4% of the NK cells produced IFN-γ after stimulation with ESAT-6 or MPP14. The relatively small number of IFN-γ-producing NK cells is in accordance with results from human studies where only a minor population of NK cells produced large amounts of IFN-γ (7). In comparison, less than 0.3% of CD3⁺ cells produced IFN-γ after stimulation with these antigens. CD3⁺ cells were however the major IFN-γ producers after stimulation with phorbol myristate acetate and calcium ionophore (data not shown). We also found a large individual variation in the number of IFN-γ-producing NK cells in nonstimulated samples ranging from 0.1 to 3.0% while no spontaneous IFN-γ production in CD3⁺ cells was detected. The two different methods, depletion of NK cells from PBMC and intracellular staining, conclusively established NK cells as a source of IFN-γ after stimulation with ESAT-6 and MPP14.

**Antigen-induced IFN-γ production in NK cell cultures was dependent on adherent cells.** In an attempt to define the mechanism of IFN-γ production by NK cells, rbiL-2 activated NK cell cultures were stimulated with MPP14 and ESAT-6. No IFN-γ was detected, indicating that the antigens alone were unable to stimulate NK cells directly (data not shown). We thus hypothesized that the IFN-γ production by NK cells was mediated through stimulation of antigen-presenting cells. Adherent cells from four animals were incubated with MPP14, ESAT-6, MPB70, PPD, and medium alone, and the supernatants were assayed for the ability to induce IFN-γ production in nonautologous NK cell cultures. The assay was repeated four times, and the NK cell cultures were stimulated in triplicate. The results are given as the mean increase in IFN-γ production in response to the various antigens compared to medium alone.

There was a relatively large variation in IFN-γ production between NK cell cultures from different animals. However, in all the animals, supernatants from the MPP14-stimulated adherent cells induced significantly higher IFN-γ production compared to MPB70, while three out of four animals had a significantly higher response to ESAT-6 compared to MPB70 (Table 1). These results indicated that MPP14 and ESAT-6 induced cytokine production in adherent cells that subsequently induced IFN-γ production by NK cells. Since activated macrophages are able to secrete IFN-γ, the supernatants from stimulated adherent cells were assayed for the presence of this cytokine, and no IFN-γ was detected. Furthermore, lipopolysaccharide is a potent stimulator of antigen-presenting cells, and to exclude the influence of possible lipopolysaccharide contamination, polymyxin B was added to some of the samples. Minimal inhibition of IFN-γ production was observed.

A likely mechanism for the IFN-γ production in NK cells in antigen-stimulated whole blood is through cytokines from stimulated monocytes. To see whether bovine NK cells in
blood can produce IFN-γ in response to cytokines, heparinized whole-blood samples were stimulated with rbIL-2 and rbIL-12. IFN-γ production was subsequently measured using intracellular staining and flow cytometry. An average of 10.9% of the NK cells produced IFN-γ, with an individual variation ranging from 4.9 to 20.1% (n = 6) (Fig. 5A). In contrast, the average number of CD3+ cells producing IFN-γ was 1.3%. This showed that a combination of rbIL-2 and rbIL-12 induced IFN-γ production in NK cells in whole-blood (Fig. 5A). Likewise, NK cells in cultures stimulated with rbIL-2 and rbIL-12 produced IFN-γ (Fig. 5B), while low levels (1 to 2 ng/ml) of IFN-γ were detected when rbIL-2 (25 to 200 U) or rbIL-12 (2.5 to 20 U) was used alone.

Neutralization of IFN-γ production in NK cells was achieved by adding anti-IL-12 antibodies (Fig. 5B). We subsequently assessed whether IL-12 from antigen-stimulated adherent cells was responsible for the induced IFN-γ production by NK cells. The presence of IL-12 in the supernatants was estimated in an

FIG. 2. IFN-γ responses in 14 young calves during the first 2 months of life. Whole blood was stimulated for 24 h with MPP14, ESAT-6, and MPB70 at a concentration of 2 μg/ml and PPD at a concentration of 10 μg/ml. The responders (n = 7) are animals with an IFN-γ response (corrected OD > 0.3) at at least one time point against any of the purified antigens. Nonresponders (n = 7) never had a corrected OD of >0.3 against any of the antigens. Stimulations were made with (A and B) PPD, (C and D) MPP14, (E and F) ESAT-6, or (G and H) MPB70.
IL-12 assay as described previously (20) and small amounts of IL-12 (<1 U/ml) were detected. Anti-IL-12 was added to NK cell cultures together with supernatant from antigen-stimulated adherent cells to see if a possible effect of IL-12 could be neutralized. The addition of anti-IL-12 antibodies gave a reduction of IFN-γ production varying from 15% to 47%, showing that IL-12 was partly responsible for the observed IFN-γ production from NK cells (Fig. 5C). However, other cytokines were also likely to be involved.

**DISCUSSION**

The present study investigated the interaction between NK cells, adherent cells, and secreted mycobacterial antigens. We found that the mycobacterial antigens ESAT-6 from the *M. tuberculosis* complex and MPP14 from the *M. avium* complex induced IFN-γ production from NK cells. The response was dependent on adherent cells and likely mediated through cytokine production.

We have previously demonstrated innate IFN-γ production in healthy calves in response to MPP14 (34). The recent characterization of bovine NK cells and the generation of a monoclonal antibody raised against the bovine NK cell marker NKp46 (38, 39) facilitated a more detailed study of this innate IFN-γ production. ESAT-6, MPP14, and MPB70 are secreted antigens available to the immune system at an early stage of the infection, and several such proteins can induce strong IFN-γ production from sensitized T cells (2, 25). In the present study we have shown that ESAT-6 and MPP14 were able to induce IFN-γ production from NK cells.

**TABLE 1.** Increase in IFN-γ production in NK cell cultures after stimulation with supernatant from antigen-stimulated adherent cells compared to medium alone

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>MPP14</th>
<th>ESAT-6</th>
<th>PPD</th>
<th>MPB70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 ± 0.1*</td>
<td>2.8 ± 0.7*</td>
<td>4.6 ± 0.8</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>2.5 ± 0.6*</td>
<td>1.9 ± 0.4*</td>
<td>1.8 ± 0.5</td>
<td>0.0 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>5.0 ± 0.5*</td>
<td>4.5 ± 0.5</td>
<td>0.8 ± 0.4</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>4.8 ± 1.1*</td>
<td>3.0 ± 0.8*</td>
<td>3.8 ± 1.1*</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

*P = 0.05, Wilcoxon rank test, compared to MPB70.
NK cells are important in immune regulation and play a role in directing the immune system towards a Th1 pathway in other species (40, 42), however, the significance of NK cells in cattle remains to be established. ESAT-6 and MPP14 may be of importance in the development of a protective Th1 response and are thus good candidates to be evaluated in new vaccines against M. bovis and M. avium subsp. paratuberculosis. The ability of ESAT-6 to initiate a Th1 response has been shown previously in the mouse model, where ESAT-6 delivered as a fusion protein and as a DNA vaccine induced IFN-γ production to an unrelated antigen (29, 30). Mycobacteria are likely to harbor antigens that induce both beneficial and detrimental immune responses. A detailed examination of the effect of various mycobacterial antigens on the innate and the adaptive immune system will facilitate a rational strategy for the design of new vaccines.

The mechanisms for the antigen-induced IFN-γ production from NK cells were not fully explained. However, a definite requirement for antigen-stimulated adherent cells was clearly detected. Most likely, the observed IFN-γ production from NK cells was a result of cytokines secreted from the adherent cells. Despite the low levels of IL-12 present in the samples, neutralization of IL-12 partly inhibited the observed IFN-γ production from NK cells. It is thus likely that other cytokines were involved, and that these cytokines acted in synergy with IL-12. A likely candidate is IL-18. This cytokine is produced by monocytes and macrophages and has a variety of biological functions, including induction of type 1 cytokines and enhancement of NK cell functions (12). The production of IFN-γ from NK cells in response to IL-18 produced by Mycobacterium leprae-stimulated human monocytes was demonstrated by Garcia et al. (17).

The lack of antibodies to the bovine Toll-like receptors (TLRs) and other receptors hindered detailed investigation of the mechanisms for the interaction of ESAT-6 and MPP14 with adherent cells. The interaction of mycobacterial components with innate immune receptors such as TLR-2 and TLR-4 have been studied in murine and human models. Lipoarabinomannan and its precursor lipomannan from the mycobacterial cell wall can interact with TLR and induce production of proinflammatory cytokines (9, 28, 41). Furthermore, lipoproteins from M. tuberculosis induced IL-12 production mediated via TLR (3). In these proteins the lipid moiety was believed to be essential for activation. Activation through TLR by protein ligands has not been extensively investigated. However, Ohashi et al. showed that the human heat shock protein 60 induced tumor necrosis factor alpha and NO2− production through TLR-4 (32). We have here demonstrated that secreted mycobacterial protein antigens also can interact with the innate immune system.

There was a remarkable individual difference in the NK cell mediated IFN-γ responses. The explanation for these individual variations is unclear. Young calves will encounter numerous antigens for the first time and they are likely to have a highly activated immune system. The combination of viral, bacterial, and parasitic agents the naïve animal is exposed to, together with inherent predisposition, may steer the general immune response onto a Th1 or Th2 pathway. This was recently demonstrated by Elias et al., who showed that Schistosoma mansoni infection induced a general Th2 response, and that the S. mansoni-infected mice were less able to elicit a Th1 response than the controls (13). The infected mice had a reduced protective effect of BCG vaccination with a lower IFN-γ production.
A general activation of the immune system towards a Th1 or Th2 pathway in the different calves may explain the individual variations in IFN-γ production from NK cells. This may also be reflected in variations in spontaneous cytokine secretion in peripheral blood as detected in the present study, where non-stimulated NK cells showed large variations in IFN-γ secretion. Such variations have been observed by Walker et al. in patients and healthy humans in Malawi, where the frequency of mycobacterial and parasitic infections was high (44). They also showed that spontaneous cytokine production influenced the induced production. Donors who had spontaneous IFN-γ production by NK cells also showed increased production after stimulation with phorbol myristate acetate and calcium ionophore compared to donors without spontaneous IFN-γ production. This is in accordance with the findings in the present and our previous study, where animals that had innate IFN-γ production in response to MPP14 also had low-level spontaneous IFN-γ production (34).

The IFN-γ test has been used in several countries to diagnose early stages of bovine tuberculosis and the use of specific proteins such as ESAT-6 has clearly increased the specificity of this test (4, 35). However, it is widely recognized that the test cannot be used in young animals due to nonspecific IFN-γ production (6, 27). Our results demonstrated that NK cells are likely to be a source of innate IFN-γ production, and this provides an explanation for the problems encountered in the young calves. This is also in agreement with the finding that young calves had the highest proportion of NK cells in peripheral blood (26).

In a broader screening of IFN-γ production in young cattle, we found that approximately one third of the animals under the age of 18 months produced IFN-γ (19). While MPP14 was detected by Western blotting in the M. avium complex and M. scrofulaceum (33), it is unlikely that as many as one-third of the animals were sensitized to mycobacteria harboring both MPP14 and ESAT-6. Consequently, it is likely that NK cells are responsible for the majority of the nonspecific IFN-γ production observed in young cattle. Methods that inhibit IFN-γ production from NK cells would therefore lead to an improvement of the IFN-γ test for detection of bovine mycobacterial diseases.

In conclusion, we have demonstrated that IFN-γ production from NK cells occurred in response to adherent cells stimulated with the secreted proteins ESAT-6 and MPP14. This will have consequences for the interpretation and use of the IFN-γ test for diagnosis of mycobacterial infections in young cattle.

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Mycobacterial antigens induce IFN-γ from NK cells


