Emergence of NK1.1⁺ Cells as Effectors of IFN-γ Dependent Immunity to Toxoplasma gondii in MHC Class I-deficient Mice

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Summary

CD8⁺ T lymphocytes have been reported to play a major role in the protective immune response against acute infection with Toxoplasma gondii. In order to further assess the role of CD8⁺ cells in resistance against this protozoan we examined the ability of β2m-deficient mice, which fail to express MHC class I molecules and peripheral CD8⁺ lymphocytes, to survive tachyzoite challenge following vaccination with an attenuated parasite mutant. Surprisingly, vaccination of β2m-deficient mice induced strong resistance to lethal challenge, with >50% surviving beyond 3 months. Vaccinated β2m-deficient mice, but not control heterozygotes, showed a five- to six-fold expansion in spleen cell number and ~40% of the splenocytes were found to express the NK markers NK1.1 and asialo GM1. Spleen cells from the vaccinated β2m-deficient animals failed to kill either infected host cells or the NK target YAC-1. However, high levels of IFN-γ were secreted when the cells were cultured in vitro with soluble T. gondii lysate, and this response was abolished by NK1.1⁺ but not CD4⁺ and CD8⁺ lymphocyte depletion, implicating the NK1.1⁺ population as the major source of IFN-γ. More importantly, vaccine-induced immunity in β2m-deficient mice was completely abrogated by in vivo administration of antibody to NK1.1, asialo GM1, or IFN-γ. Together, the data suggest that in class I-deficient mice vaccinated against T. gondii, the absence of CD8⁺ effector cells is compensated for by the emergence of a population of NK1.1⁺ and asialo GM1⁺ cells which lack cytolytic activity, and that the protective action of these cells against the parasite is attributable to IFN-γ production. The induction of this novel NK population may provide an approach for controlling opportunistic infections in immunocompromised hosts.
In order to further define the role of CD8+ T lymphocytes in immunity to Toxoplasma, we examined the response of MHC class I-deficient mice following vaccination with the attenuated T. gondii mutant ts-4. These animals were constructed by targeted disruption of the gene encoding the β2 microglobulin (β2m) subunit of the class I molecule (10). As a result, the peripheral CD8+ compartment fails to develop (11) and the mice fail to display normal resistance to infection with Trichomonas cruzi (12), Mycobacterium tuberculosis (13) and certain viruses (14). As described below, β2m-deficient mice unexpectedly were found to develop high levels of protective immunity to T. gondii following vaccination. This resistance was associated with a massive parasite-induced expansion of a splenocyte population expressing NK1.1 and asialo GM1 (ASGM1),1 phenotypic markers characteristic of NK cells. The latter population, while producing IFN-γ in response to parasite Ag, was unable to mediate lysis of either T. gondii-infected host cells or conventional NK targets, and thus appears to be functionally unique. The appearance of these cells in CD8+ deficient animals suggests that they could be induced as alternative effectors of parasite immunity in immunodeficient hosts.

Materials and Methods

Mice and Parasites. Chimeric mice homozygous (−/−) for the disrupted β2m gene were derived from (129 × B6)F2 founder stock as described (10). Animals used in this study were from the fifth backcross generation. Control animals heterozygous for the disrupted β2m gene (+/−) were obtained by crossing −/− mice and certain viruses (14). As described below, β2m-deficient mice unexpectedly were found to develop high levels of protective immunity to T. gondii following vaccination. This resistance was associated with a massive parasite-induced expansion of a splenocyte population expressing NK1.1 and asialo GM1 (ASGM1),1 phenotypic markers characteristic of NK cells. The latter population, while producing IFN-γ in response to parasite Ag, was unable to mediate lysis of either T. gondii-infected host cells or conventional NK targets, and thus appears to be functionally unique. The appearance of these cells in CD8+ deficient animals suggests that they could be induced as alternative effectors of parasite immunity in immunodeficient hosts.

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Vaccination of β2m-deficient Mice Induces Expansion of NK1.1⁺ Cells. In order to investigate the mechanism by which β2m-deficient mice resist RH challenge, we examined the splenocyte populations from vaccinated and nonvaccinated animals. A dramatic five- to six-fold increase in spleen cell number was observed in ts-4 immunized −/− animals. In contrast, no significant increase in splenocytes was seen in vaccinated +/− or nonvaccinated −/− mice. As expected, flow cytometric analysis revealed only low levels of class I (H-2K b) and CD8 expression in spleen cells from −/− animals (Table 1). In addition, no CD8⁺ lymphocytes were detected in the peritoneum (data not shown), a site where these cells have recently been detected in β2m-negative mice injected with tumor cells (22). Instead, we found a striking increase (from 5 to 39%; Table 1 and Fig. 2) in the percent of splenocytes expressing the NK marker NK1.1 in vaccinated −/− animals. In contrast, only a minor increase in NK1.1⁺ cells (3-7%) was induced by vaccination of +/− animals. The level of CD4⁺ lymphocytes was 10-15% lower in vaccinated β2m-deficient than in nonvaccinated −/− and vaccinated +/− mice, this decrease most probably reflecting the increased percent of NK1.1⁺ cells.

Fig. 2D shows the population of anti-NK1.1 staining spleen cells in vaccinated β2m-negative mice selected for analysis of other surface markers by dual fluorescence. Since there was overlap between positive and negative staining cells, only the brightest 20% of total splenocytes was examined in order

### Table 1. Spleen Cell Composition in Vaccinated and Nonvaccinated −/− and +/− Mice*

| Sample                  | H-2K⁺ | CD4   | CD8     | NK1.1  |
|-------------------------|-------|-------|---------|--------|
| −/− vaccinated          | 6.5 ± 1.5† | 14.1 ± 3.0 | 1.8 ± 0.9 | 39.4 ± 9.3 |
| +/− vaccinated          | 98.2 ± 1.0 | 24.3 ± 1.1 | 16.2 ± 0.9 | 7.3 ± 1.6  |
| −/− nonvaccinated       | 2.0 ± 0.1 | 29.5 ± 2.3 | 1.5 ± 0.8 | 5.1 ± 1.0  |
| +/− nonvaccinated       | 96.3 ± 2.1 | 16.1 ± 3.1 | 8.4 ± 1.1 | 3.3 ± 0.7  |

* Splenocytes were stained with FITC conjugated mAb specific for the indicated markers. See Materials and Methods for details.
† Mean ± SD of individual mice (3-7 per group). Similar results were obtained in 5 independent experiments.
Figure 2. NK1.1 expression by vaccinated +/- and -/- mice. Splenocytes from +/- mice (A and B) and -/- (C and D) animals were stained with a fluoresceinated irrelevant mouse IgG (anti-IA d) (A and C) or anti-NK1.1 (B and D).

To ensure exclusion of NK1.1- cells. In this cell staining experiment, as in the others shown, saturating levels of unlabelled mAb 2.4G2 were included to block nonspecific Fc receptor binding. Cells stained with FITC-labeled NK1.1+ alone showed no PE fluorescence (Fig. 3 A) but virtually all of the NK1.1+ cells expressed ASGM1 (Fig. 3 B). In contrast, minimal staining was detected with mAb specific for Thy1.2 (5%; Fig. 3 C), CD4 (2%; Fig. 3 D), and αβ TCR (3%; Fig. 3 F). However, a small amount of staining (12%; Fig. 3 H) was detected using 5E6, a mAb detecting a marker associated with a subpopulation of NK cells (17). The similar proportion of NK1.1+ cells expressing the latter markers suggested they were produced by the same subpopulation of cells, and indeed 3-color cytometric analysis using mAb to NK1.1, CD3, and γδ TCR confirmed this to be the case (data not shown).

Cytotoxic Activity of T. gondii-induced NK1.1+ Cells. The large increase in NK1.1+ cells was unexpected because it has recently been shown that β2m-deficient mice are defective in NK cell lytic activity (23, 24). We similarly found that poly I:C treatment of nonvaccinated -/- mice resulted in a much smaller enhancement of cytolytic function relative to that stimulated in +/- mice (Fig. 4). Moreover, splenocytes from T. gondii vaccinated class I-deficient mice, although composed of up to 40% NK1.1+ cells, were unable to lyse YAC-1 targets (Fig. 4 B).

The ability of splenocytes from vaccinated mice to kill tachyzoite-infected host cells was also assessed. As shown previously (20, 25), vaccination of normal (+/-) animals generated splenic effector cells capable of lysing parasite-infected bone marrow macrophages (Fig. 5 A). A low but significant level of killing (10% specific Cr-release; p <0.05) was detected using restimulated +/- effectors and infected -/- targets (Fig. 5 A). The latter could possibly be mediated by a low level of functionally conformed cell surface class I heavy...
chain expressed by β2m-deficient animals (26), or alternatively by exogenous β2m supplied by the serum in the culture medium. In contrast, neither ts-4 restimulated (Fig. 5 B) nor freshly isolated splenocytes (Fig. 5 D) from −/− animals lysed to a significant degree infected targets of either strain. Taken together, the results in Figs. 5 and 6 show that the parasite-induced NK1.1+ cells in β2m-deficient mice have no detectable lytic activity against either T. gondii-infected targets or the classic NK target YAC-1.

NK1.1+ Cells Induced in β2m-deficient Mice Produce IFN-γ in Response to T. gondii Ag. NK cells are a major source of IFN-γ (27) and T. gondii has recently been shown to trigger in vitro production of this cytokine by splenic NK cells from SCID mice (28). Therefore, we assessed the ability of vaccine-induced NK1.1+ cells to produce IFN-γ in response to the parasite. Nondepleted splenocytes from β2m-deficient and nondeficient animals when stimulated with soluble T. gondii Ag produced 30 and 53 ng/ml of the cytokine, respectively (Table 2), while unstimulated cells synthesized less than 2 ng/ml (data not shown). Depletion of CD4+ and CD8+ lymphocytes, but not NK1.1+ cells, from vaccinated nondeficient animals eliminated the parasite-induced IFN-γ response. In contrast, in vaccinated class I-deficient mice depletion of NK1.1+ cells, but not T cells, abolished T. gondii-stimulated IFN-γ production (Table 2). Similarly, after in vivo NK cell depletion, splenocytes synthesized only low levels of IFN-γ (7 ng/ml) following in vitro stimulation with parasite Ag, and this response was abolished by lysis of T cells prior to culture (Table 2). In addition, removal of CD3+ γδTCR+ cells in the NK1.1+ population by cell sorting did not alter the ability of the remaining cells to secrete IFN-γ in response to parasite Ag (data not shown).

Vaccine-induced Protection in β2m-deficient Mice is Dependent Upon NK1.1+ and ASGMf+ Cells. Since the vaccine-induced NK1.1+ cells produce IFN-γ in vitro in response to T. gondii Ag (Fig. 1 B), we assessed the effects of in vivo depletion of NK cells on the resistance of these mice to challenge infection. While administration of anti-NK1.1 mAb or rabbit anti-
As expected, CD8⁺ depletion did not alter the ability of vaccine-induced immunity in B2m-deficient animals (Fig. 6A). ASGM₁ antibodies failed to alter the ability of vaccinated mice to resist challenge infection (Fig. 6B). The results presented here suggest that in the immune system of class I-deficient animals loss of IFN-γ-producing CD8⁺ lymphocytes is compensated for by production of an unconventional effector cell population capable of producing the same cytokine. The production of novel effector cells in response to immunodeficiency has also been observed in B2m-negative mice infected with Sendai virus (31). Such mice clear the virus, albeit with delayed kinetics relative to normal mice, and although CD8⁺ CTL activity is responsible for virus elimination in class I-expressing animals, in the immunodeficient mice clearance is attributable to the appearance of CD4⁺ CTL. Similarly, infection of B2m-negative animals with murine lymphocytic choriomeningitis virus induces splenic CD4⁺ CTL activity (33). However, it is unlikely that CD4⁺ cytotoxicity plays a major role in the protective response of class I-deficient mice infected with T. gondii because virtually no CD4⁺ cells were detected in the protective NK1.1⁺ population, and no cytolytic activity was detected against parasite-infected cells using splenocyte effectors from vaccinated B2m-negative mice. In addition, immunity was completely eliminated by IFN-γ depletion, implicating this cytokine as the major mediator of immunity rather than cell-mediated cytotoxic activity. Nevertheless, since IL-2 induces NK cell proliferation (34), conventional CD4⁺ lymphocytes could play a helper role in the induction and activity of these cells; this possibility is currently being examined.

Depletion of NK1.1⁺ cells in ts-4 vaccinated +/− mice had no effect on resistance to T. gondii, indicating that such cells probably do not play a major role during the effector phase of immunity in these animals. However, NK cell lytic activity has been detected in other experimental models used to study T. gondii (35-37) and it is possible that in these cases, vaccinia-infected B2m-negative mice have greater numbers of spleen cells than do normal heterozygotes, and part of this increase is due to non-B, non-T cells (30). Therefore, it is possible that control of these infections is attributable to the induction of a protective NK1.1⁺ cell population similar to that described here.

Table 2. Source of IFN-γ in T. gondii Ag-stimulated Splenocytes From Vaccinated Mice*

| Mouse strain | Treatment | IFN-γ (ng/ml) |
|--------------|-----------|---------------|
| +/-          | Nondepleted | 48.4 ± 2.1    |
|              | T cell depleted | 1.7 ± 0.6    |
|              | NK cell depleted | 47.2 ± 2.0   |
| −/−          | Nondepleted | 30.2 ± 3.2    |
|              | T cell depleted | 22.1 ± 2.3   |
|              | NK cell depleted | 2.3 ± 0.1    |
| −/−          | NK cell depleted in vivo | 6.7 ± 0.2    |
|              | NK cell depleted in vitro | 0.8 ± 0.6   |
|              | T cell depleted in vitro | 0.8 ± 0.6    |

* Indicated cell populations were treated with anti-CD4 + anti-CD8 mAb (T cell depletion) and anti-NK1.1 mAb (NK cell depletion) + rabbit complement and then 5 × 10⁶ cells were cultured in vitro with 100 μg/ml soluble T. gondii Ag for 72 h before measuring IFN-γ. See Materials and Methods for details. This experiment is representative of 3 performed.

Discussion

Immunity to T. gondii is dependent upon IFN-γ and, in immunologically intact mice, CD8⁺ lymphocytes appear to be major producers of this cytokine. The results presented in this paper demonstrate that in the absence of CD8⁺ cells, a previously uncharacterized population of effector cells emerge in response to T. gondii vaccination. These cells express NK1.1 and ASGM₁ markers associated with NK cells, and their elimination by in vivo anti-NK1.1 or anti-ASGM₁ Ab treatment ablates immunity to challenge infection. The parasite-induced NK1.1⁺ cells, while failing to lyse either the NK target YAC-1 or T. gondii-infected bone marrow macrophages, release high levels of IFN-γ when cultured with tachyzoite Ag. The production of this cytokine by the NK1.1⁺ population is likely to account for their protective effect since depletion of IFN-γ in vivo abrogates immunity in the CD8⁺-deficient mice.

Our finding that vaccinated class I-deficient mice resist T. gondii challenge contrasts with previously reported experiments demonstrating an inability of these animals to survive infection with Trypanosoma cruzi (12), Mycobacterium tuberculosis (13), and a virulent influenza strain (14). However, B2m-negative mice are able to clear infections with other influenza strains (14, 29) as well as vaccinia and Sendai virus (30, 31). Indeed, while not as striking as the response to T. gondii, vaccinia-infected B2m-negative mice have greater numbers of spleen cells than do normal heterozygotes, and part of this increase is due to non-B, non-T cells (30). Therefore, it is possible that control of these infections is attributable to the induction of a protective NK1.1⁺ cell population similar to that described here.
as with the class I-deficient mice, NK1.1+ cells provide a source of protective IFN-γ. In addition, parasite induction of NK1.1+ cells at initial stages of infection may result in early T cell-independent IFN-γ production, which could in turn drive immunity to the predominant Th1 type of CD4+ response characteristic of T. gondii infection (3).

The results of the present study provide a dramatic demonstration of in vivo induction of IFN-γ producing NK1.1+ cells in response to microbial stimulation. In addition, our findings unequivocally establish that such cells confer strong resistance to challenge with a normally lethal pathogen. Recently, we have shown in an in vitro system that spleen cells and bone marrow derived NK cells from SCID mice produce IFN-γ when cultured with live T. gondii parasites or tachyzoite extract (28). This response, like that induced by the intracellular bacterium Listeria monocytogenes (38, 39), is dependent upon TNF-α and IL-12 released by macrophages after microbial stimulation (40, 41). Preliminary evidence indicates that the NK1.1+ cells induced in vaccinated β2m-negative mice are triggered to produce IFN-γ by a similar accessory cell dependent pathway.

In addition to demonstrating in vivo induction of protective NK1.1+ cells in response to microbial infection, the results of this study provide a major example of redundancy and adaptability in the murine immune system. Furthermore, the ability of a defective host immune system to utilize alternate pathways to produce protective IFN-γ has important clinical implications. Thus, in the case of toxoplasmosis and other opportunistic infections, this property could potentially be exploited to induce T independent resistance in immunocompromised hosts.

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