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Role of NK1.1$^+$ T Cells in a T_H2 Response and in Immunoglobulin E Production

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Immune responses dominated by interleukin-4 (IL-4)–producing T helper type 2 (T_H2) cells or by interferon γ (IFN-γ)–producing T helper type 1 (T_H1) cells express distinctive protection against infection with different pathogens. Interleukin-4 promotes the differentiation of naïve CD4$^+$ T cells into IL-4 producers and suppresses their development into IFN-γ producers. CD1-specific splenic CD4$^+$NK1.1$^+$ T cells, a numerically minor population, produced IL-4 promptly on in vivo stimulation. This T cell population was essential for the induction of IL-4–producing cells and for switching to immunoglobulin E, an IL-4–dependent event, in response to injection of antibodies to immunoglobulin D.

Interleukin-4 is a major determinant of the differentiation of naïve T cells into IL-4–producing cells and has the capacity to suppress their differentiation into IFN-γ–producing cells (*1*). A potential source of IL-4 that could affect the priming of naïve CD4$^+$ T cells is a set of CD4$^+$NK1.1$^+$ splenic T cells (*2*). These cells produced IL-4 within 30 to 90 min of in vivo challenge with antibody to CD3 or with staphylococcal enterotoxin B. They appear to be related to a population of thymic NK1.1$^+$ T cells that are also prompt cytokine producers (*3–9*). NK1.1$^+$ thymocytes are known to express a limited set of αβ T cell receptors (TCRαβ) specific for the major histocompatibility complex (MHC) class I–like molecule CD1 (*3, 4, 7–10*). Thymic NK1.1$^+$ T cells were markedly diminished in number in β_2-microglobulin knockout ($\beta_2M^{-/-}$) mice (*3, 11*). This is in keeping with the association of β_2M with CD1 (*12*).

Splenic CD4$^+$NK1.1$^+$ T cells were also markedly diminished in $\beta_2M^{-/-}$ mice (Fig. 1A), constituting only 0.1% of spleen cells in contrast to a frequency of 1.0% in C57BL/6 mice (*13*). In CD8$^{-/-}$ C57BL/6 congenic mice, the frequency of CD4$^+$NK1.1$^+$ T cells was similar to that in C57BL/6 mice. MHC class II$^{-/-}$ (Aβ$^{-/-}$) mice expressed almost the same number of CD4$^+$NK1.1$^+$ splenic T cells as were expressed by C57BL/6 mice, although CD4$^+$ T cells constituted only 4 to 5% of their spleen cells. CD4$^{-/-}$ mice did not express any CD4$^+$NK1.1$^+$ cells but did express NK1.1$^+$ T cells, as determined by staining with antibodies to CD3 and NK1.1 (*14*).

As anticipated from their deficiency of CD4$^+$NK1.1$^+$ cells, $\beta_2M^{-/-}$ mice made minimal or no IL-4 90 min after injection of antibody specific for CD3, whereas C57BL/6, Aβ$^{-/-}$, CD8$^{-/-}$, and CD4$^{-/-}$ mice all produced IL-4 (Fig. 1B). In contrast, IL-2 and IFN-γ were produced in response to antibody to CD3 by spleen cells from C57BL/6 mice and from each strain of the knockout mice, including $\beta_2M^{-/-}$ mice. This indicates that CD4$^+$NK1.1$^+$ T cells are not uniquely responsible for the production of IL-2 and IFN-γ. Antibody to CD3 also failed to stimulate $\beta_2M^{-/-}$ mice to produce messenger RNA (mRNA) for IL-13, a cytokine closely related to IL-4, whereas each of the other strains produced IL-13 mRNA (*14*).

If the IL-4 that is rapidly produced by CD4$^+$NK1.1$^+$ T cells is important in the commitment to the T_H2 pathway of T cell development, $\beta_2M^{-/-}$ mice would be deficient in immunoglobulin E (IgE) production, as IL-4, a T_H2 product, is the major determinant of IgE production in mice (*15, 16*). Immunoglobulin E is markedly induced in several mouse strains by injection of the polyclonal stimulant goat antibody to mouse IgD (anti-IgD) (*17*). Treatment with antibody to IL-4 (anti–IL-4) completely inhibits such production of IgE (*15*). We have used this model to examine the IgE-producing potential of $\beta_2M^{-/-}$ mice. Normal C57BL/6 mice, Aβ$^{-/-}$ mice, and CD8$^{-/-}$ mice each developed substantial and comparable IgE responses to anti-IgD (Fig. 1C). In contrast, $\beta_2M^{-/-}$ mice made minimal or no IgE in response to an in vivo challenge with anti-IgD. CD4$^{-/-}$ mice also made diminished amounts of IgE in response to anti-IgD, although these mice made normal amounts of IL-4 90 min after injection with antibody to CD3.

Not only did $\beta_2M^{-/-}$ mice fail to produce IgE in response to injection of anti-IgD, but their spleen cells failed to secrete IL-4 spontaneously 5 days after injection of anti-IgD and showed only modest IL-4 production when stimulated in vitro with anti-IgD (Fig. 2). In contrast, C57BL/6 spleen cells produced IL-4 5 days after injection with anti-IgD, and that production was enhanced by culture with anti-IgD, presumably because of the recognition of anti-IgD peptides bound to MHC class II molecules. $\beta_2M^{-/-}$ mice can be primed in vivo to

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produce IL-4 if IL-4 is introduced shortly after injection of anti-IgD. Thus, injection of IL-4, in the form of complexes of IL-4 and anti–IL-4 (*18*), allows $\beta_2M^{-/-}$ mice to produce IL-4 5 days after injection of anti-IgD; this production is further enhanced if their spleen cells are cultured with anti-IgD. These results indicate that the failure of $\beta_2M^{-/-}$ cells to produce IL-4 is not caused by an innate defect in their capacity to develop into IL-4-producers, as long as a source of IL-4 is available at the time of priming.

If the absence of $CD4^+NK1.1^+$ T cells in $\beta_2M^{-/-}$ mice and the consequent failure to produce IL-4 at the time of priming are responsible for the failure of naïve T cells to become IL-4 producers, transfer of cell populations containing $CD4^+NK1.1^+$ cells and CD1–expressing antigen-presenting cells or B cells should enable $\beta_2M^{-/-}$ mice to produce IL-4 and to secrete IgE. $\beta_2M^{-/-}$ mice that were sublethally irradiated and that received 12×10^6 C57BL/6 $CD8^-HSA^{lo}$ (mature) thymocytes (*19*) and 40×10^6 C57BL/6 T cell–depleted spleen cells (ΔT spleen cells) produced substantial amounts of IgE in response to anti-IgD (Fig. 3A). In contrast, $\beta_2M^{-/-}$ mice that received such ΔT spleen cells either with mature thymocytes from $\beta_2M^{-/-}$ mice or without any thymocytes produced modest amounts of IgE. $NK1.1^+$ thymocytes enriched from the C57BL/6 mature thymocyte population (Fig. 3C) produced an even more vigorous IgE response, whereas depletion of $NK1.1^+$ thymocytes in the mature thymocyte population reduced the IgE response to the amounts induced by ΔT spleen cells alone. The transfer of C57BL/6 ($CD1^+$) ΔT spleen cells was also important; irradiated $\beta_2M^{-/-}$ mice that received C57BL/6 $NK1.1^+$ thymocytes with $\beta_2M^{-/-}$ ($CD1^-$) ΔT spleen cells produced one-fifth as much IgE in response to challenge with anti-IgD as $\beta_2M^{-/-}$ mice that received C57BL/6 $NK1.1^+$ thymocytes and C57BL/6 ΔT spleen cells (Fig. 3B).

Our results provide strong evidence that the early in vivo production of IL-4 by $NK1.1^+$ T cells is essential for IgE production in some situations. In the anti-IgD system, IL-4 is required to prime naïve "conventional" $CD4^+$ T cells (presumably specific for goat Ig-derived peptides) to differentiate into T_H2 cells, as shown by their capacity to secrete IL-4 in response to in vitro challenge with anti-IgD 5 days after injection of the antibody. However, the finding that reconstitution of irradiated $\beta_2M^{-/-}$ mice with highly enriched $NK1.1^+$ T cells and ΔT spleen cells from C57BL/6 mice allowed production of IgE suggests that under certain circumstances, the $NK1.1^+$ T cells, or cells that copurify with them, may be able to help B cells directly. This would be consistent with the ability of MHC class $II^{-/-}$ mice to produce IgE in response to anti-IgD, inasmuch as $CD4^+$ cells from class $II^{-/-}$ mice should be unable to recognize goat Ig peptides presented by B cells. However, this finding is at odds with the failure of $CD4^{-/-}$ mice to produce IgE,

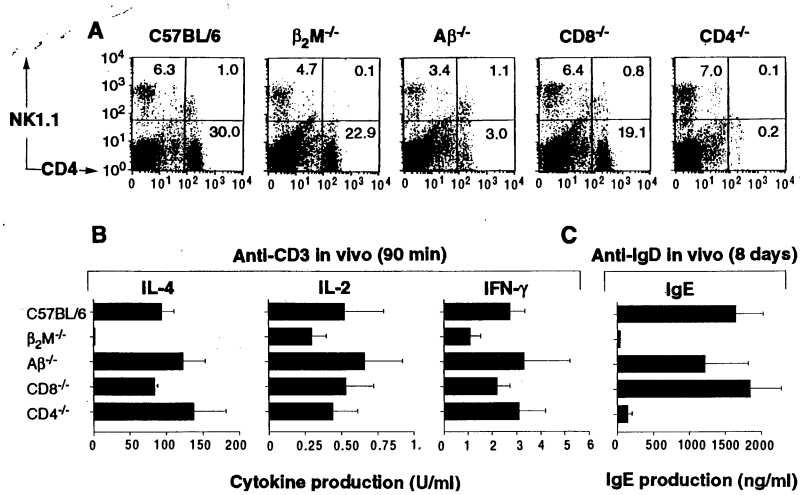

Fig. 1. Function of $NK1.1^+$ T cells in acute production of IL-4 and in priming for IgE production. (**A**) $CD4^+NK1.1^+$ splenic cells in C57BL/6 mice and in homozygous $\beta_2M^{-/-}$, $A\beta^{-/-}$, $CD8^{-/-}$, and $CD4^{-/-}$ mice on a C57BL/6 background. Percent of cells in selected quadrants are indicated. (**B**) Acute cytokine production in response to in vivo treatment with antibody to CD3 (anti-CD3). (**C**) Immunoglobulin E production in response to in vivo treatment with antibody to IgD (anti-IgD). Results are mean +1 SD.

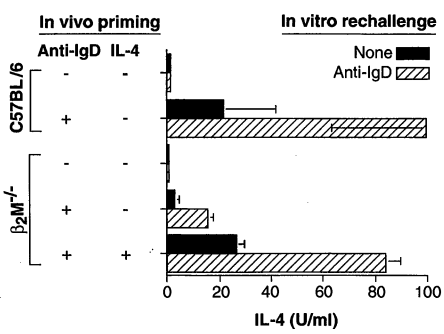

Fig. 2. Priming of $\beta_2M^{-/-}$ mice to produce IL-4 in vivo. Spleen cells (10^6) from C57BL/6 and $\beta_2M^{-/-}$ mice injected subcutaneously with 100 μl of goat antiserum to mouse IgD 5 days earlier were cultured in 48-well plates with or without 5 μg/ml of purified goat antibody to mouse IgD (anti-IgD) for 48 hours. Supernatants were harvested and IL-4 content was measured. Two days after treatment with anti-IgD, one group of the $\beta_2M^{-/-}$ mice was injected with a mixture of pure IL-4 (10 μg per mouse) and antibody to IL-4 (11B11; 30 μg per mouse) as previously described (*18*). Results are geometric means; error bar indicates 1 SEM.

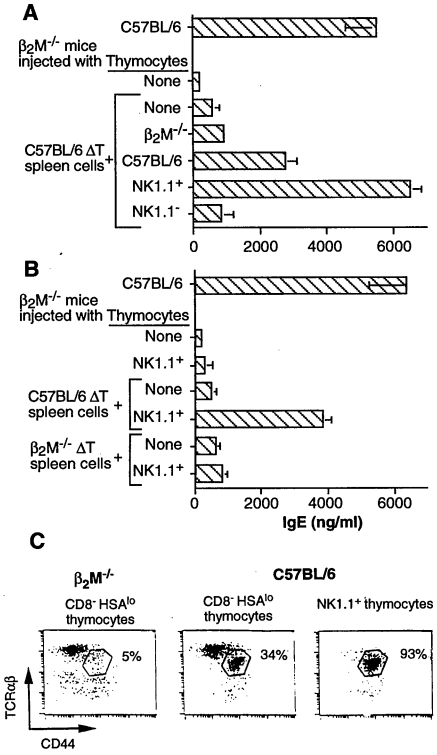

Fig. 3. Transfer of $NK1.1^+$ thymocytes reconstitutes IgE production by $\beta_2M^{-/-}$ mice. (**A**) Immunoglobulin E production by reconstituted $\beta_2M^{-/-}$ mice in response to antibody to IgD (anti-IgD). Irradiated (5 Gy) $\beta_2M^{-/-}$ mice received either nothing or 40×10^6 dense ΔT spleen cells with or without 12×10^6 mature $CD4^+$ thymocytes from C57BL/6 or $\beta_2M^{-/-}$ mice. Other $\beta_2M^{-/-}$ mice received 40×10^6 ΔT spleen cells with 3×10^6 $NK1.1^+$ or 6×10^6 $NK1.1^-$ thymocytes from C57BL/6 mice. (**B**) Transfer of ΔT spleen cells from C57BL/6 mice is critical for induction of IgE production in response to anti-IgD. Irradiated $\beta_2M^{-/-}$ mice received 3×10^6 C57BL/6 $NK1.1^+$ thymocytes with or without 40×10^6 ΔT spleen cells from C57BL/6 or $\beta_2M^{-/-}$ mice. (**C**) Characterization of transferred C57BL/6 and $\beta_2M^{-/-}$ thymocytes. Among adult C57BL/6 $CD4^+CD8^-HSA^{lo}$ thymocytes, almost all of the $CD44^{hi}$ cells expressed NK1.1 (*3, 4, 10*). Results are geometric means; error bar indicates 1 SEM.

despite their capacity to secrete IL-4 in response to antibody to CD3. These results thus leave open the question of whether early cytokine production by NK1.1$^+$ T cells, by itself, is sufficient to cause Ig class switching or whether conventional CD4$^+$ T cells are important for such help. The earlier work of Goroff *et al.* (*20*), indicating that monoclonal C57BL/6 antibodies to BALB/c IgD can elicit IgG1 and IgE responses in BALB/c mice but not in (BALB/c × C57BL/6) F_1 mice, strongly suggests that conventional T cells capable of recognizing peptides derived from anti-IgD of C57BL/6 origin are generally required for these responses.

The specificity of NK1.1$^+$ T cells for CD1 (*4*) and the demonstration that cells genetically capable of expressing CD1 are essential for restoring the capacity of $\beta_2M^{-/-}$ mice to produce IgE indicate that recognition of CD1 may be essential for activating this pathway of priming for IL-4 production. In mice, CD1 is known to be expressed by cortical thymocytes (*21*) and on gastrointestinal epithelium (*22*). In humans, CD1 is also expressed on epidermal Langerhan's cells (*23*), and a distinct CD1 isoform is expressed on a subpopulation of B cells (*24*). It is possible that the stimuli that elicit IgE production, possibly including infection with helminthic parasites and exposure to various allergens, occur either at sites of constitutive CD1 expression, such as the gastrointestinal tract and the skin, or in response to stimuli that cause increases in peripheral expression of CD1. Such CD1 expression could activate IL-4 production by CD4$^+$NK1.1$^+$ T cells, or possibly by a population of γδ T cells (*25*), at the same time as antigen-specific precursors of T_H cells encounter their complementary ligands. This would provide the IL-4 essential for the priming of such precursor cells to develop into IL-4–producing T_H2 cells and for the development of the type of antibody-dominated immune responses that are characterized by high levels of IgE.

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13. Fluorescence staining was performed at 4°C in 100 μl containing 10^6 spleen cells and fluorescein isothiocyanate–conjugated antibody to mouse CD4 (RM4-5; PharMingen, San Diego, CA) combined with phycoerythrin-conjugated antibody to mouse NK1.1 (PK136; PharMingen) in phosphate-buffered saline containing 0.1% fetal bovine serum and 0.5% NaN_3. Fluorescence analysis was done on a FACScan flow cytometer (Becton Dickinson). For acute cytokine production, we removed spleen cells 90 min after injection of antibody to CD3 (2C11; 1.33 μg per mouse). Cell suspensions were washed twice with Hanks' balanced solution. Spleen cells (5×10^6 per well) were cultured in 24-well plates without additional stimulus for 1 hour. Supernatants were harvested to measure IL-4, IL-2, and IFN-γ content. CT.4S, an IL-4–dependent cell line, and CT.EV, an IL-2–dependent cell line, were used to measure IL-4 and IL-2 content, respectively. We compared responses from serial dilutions of the supernatants with those elicited by known amounts of murine recombinant IL-4 (prepared by C. Watson, NIAID/NIH) and human recombinant IL-2 (Cetus Corp., Emeryville, CA). One unit of IL-4 is equal to ~0.5 pg; 1 U of IL-2, defined as a Cetus unit, is equal to 0.3 ng/ml. Interferon γ was assayed with a specific two-site enzyme-linked immunosorbent assay (ELISA) with known amounts of recombinant IFN-γ (Genzyme Corp., Cambridge, MA) as standards. To induce IgE production, we injected mice intravenously with 200 μg of purified goat antibody to mouse IgD plus 600 μg of normal goat Ig. Sera were obtained 8 days later, and serum IgE content was measured with an ELISA. Homozygous knockout mice were derived from animals in their fourth to eighth generation of backcrosses to C57BL/6 mice.
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TECHNICAL COMMENTS

Use of NMR to Detect Water Within Nonpolar Protein Cavities

The structure of human interleukin-1β (hIL-1β) has a nonpolar "hydrophobic" cavity that, in three independent crystal structures (*1*), appears to be empty. On the basis of nuclear magnetic resonance (NMR) data, however, J. A. Ernst *et al.* (*2*) suggest that the cavity contains disordered solvent. They identify protons on the protein that display nuclear Overhauser enhancement (NOE) cross-peaks with water molecules that are purportedly within the cavity. We question, first, whether the water molecules that display NOEs are in fact in the cavity, and second, whether appropriate controls are available showing that NOEs are not shown to protons that are remote from the cavity and from solvent-exchangeable sites.

The protons identified by Ernst *et al.* (*2*) are within side chains near the cavity and include the methyl protons of Leu^{10}, Leu^{18}, Leu^{26}, Leu^{60}, Leu^{69}, Leu^{80}, Ile^{122}, and Val^{132}; the β-methylene protons of Leu^{10} and Leu^{18}; the γ-methine protons of Leu^{10}, Leu^{18}, and Leu^{60}; and the β-methine and γ-methylene protons of Ile^{122}. The NMR experiment does not provide the actual location of the water proton, only that it is relatively close to the protein proton [stated by Ernst *et al.* (*2*) to be less than about 5 Å].

We examined the structure of hIL-1β, as determined by NMR (*3*), to investigate the environments of the protons listed above.