β2-Microglobulin–dependent NK1.1$^+$ T Cells Are Not Essential for T Helper Cell 2 Immune Responses

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Summary

A number of investigations have established the critical role of interleukin 4 (IL-4) in mediating the development of T helper (Th)2 effector cells in vitro and in vivo. Despite intensive study, the origin of the IL-4 required for Th2 priming and differentiation remains unclear. Natural killer (NK)1.1$^+$ α/β T cell receptor$^+$ T (NT) cells, a unique lineage of cells capable of producing large amounts of IL-4 after activation in vivo, are important candidates for directing Th2 priming. These cells are selected by the nonpolymorphic major histocompatibility complex (MHC) class I molecule, CD1, and are deficient in β2-microglobulin (β2m)-null mice. We used β2m-deficient mice on both BALB/c and C57BL/6 backgrounds to examine their capacity to mount Th2 immune responses after challenge with a number of well-characterized antigens administered by a variety of routes. As assessed by immunization with protein antigen, infection with *Leishmania major*, embolization with eggs of *Schistosoma mansoni*, intestinal infection with *Nippostrongylus brasiliensis*, or induction of airway hyperreactivity to aerosolized antigen, β2m-deficient mice developed functional type 2 immune responses that were not substantially different than those in wild-type mice. Production of IL-4 and the generation of immunoglobulin E (IgE) and eosinophil responses were preserved as assessed by a variety of assays. Collectively, these results present a comprehensive analysis of type 2 immune responses in β2m-deficient mice, and indicate that β2m-dependent NT cells are not required for Th2 development in vivo.

Differentiation of CD4$^+$ T cell subsets can be modulated by a number of variables, including route of antigen administration (1), antigen dose (2, 3), activation of distinct costimulatory pathways (4), interactions with distinct populations of APC (5), use of altered ligands (6), underlying genetic propensities (7), and changes in the cytokine milieu during the period of T cell priming (8–11). Of these, the latter is particularly dominant, as demonstrated by the profound effects on T helper cell (Th)[1] development in vitro or in vivo by exogenous cytokines or anticytokine antibodies and in mice with disruption of distinct cytokine genes.

Of major importance are the cytokines IL-12 and IFN-γ for Th1 development and IL-4 for Th2 development. The critical role for IL-12 and IFN-γ in Th1 differentiation has been suggested by studies with neutralizing antibodies (12–14) and in mice with disruption of the IL-12 p40 (15) or IFN-γ (16) genes in which Th1 responses are substantially abrogated in response to pathogens that otherwise induce type 1 immune responses by the host. Macrophages, and perhaps dendritic cells, are generally regarded to be the primary sources of IL-12 in vitro and in vivo (17), whereas NK cells may be the predominant source of IFN-γ produced early in the immune response (18). These findings have supported a model whereby cells of the innate immune system generate these cytokines upon their interaction with various pathogens, thus directing the development of Th1 responses by cells of the adaptive immune system (19).

A critical role for IL-4 in Th2 development has also been demonstrated through use of exogenous IL-4 or neutralizing antibodies (8–10), and in mice with gene disruption of IL-4 (20) or the IL-4R–dependent transcription factor,

[1] *Abbreviations used in this paper:* Ach, acetylcholine chloride; β2m, β2-microglobulin; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NT, natural T cells; R_L, lung resistance; Th, T helper cell.

STAT6 (21, 22). Despite these data, the cells that contribute the IL-4 required during antigen priming for the development of Th2 cells have not been identified. Studies using passive transfer of $CD4^+$ T cells into mice with disruption of the IL-4 gene suggested that $CD4^+$ T cells alone were sufficient for the generation of IgE production upon subsequent antigen challenge (23). Thus, although mast cells and basophils (24, 25), and perhaps eosinophils (26), can produce IL-4, these cell types did not seem to be required in vivo.

An unusual $CD4^+$ T cell that expresses the phenotype $CD4^+$ $NK1.1^+$ α/β TCR^+ was recently shown to produce essentially all of the IL-4 generated over the initial 30–120 min in response to intravenous injection of anti-CD3 mAb in vivo (27). This population is distinguished by expression of a skewed TCR repertoire, characterized by an invariant TCR α chain, Vα14Jα281, together with a β chain biased toward use of Vβ8, Vβ7, or Vβ2 (28). In contrast to conventional $CD4^+$ α/β TCR^+ cells that are selected by MHC class II/peptide complexes expressed on thymic epithelial cells, $NK1.1^+$ T cells or natural T (NT) cells are selected by the nonpolymorphic class I molecule, CD1, expressed on immature $CD4^+CD8^+$ thymocytes (29, 30). Disruption of the β2-microglobulin (β2m) gene impairs surface expression of CD1 (31, 32) and NT cells are physically and functionally absent from such mice as assessed by flow cytometric analysis and by the lack of IL-4 production after injection of anti-CD3 in vivo (32, 33). The absence of IgE production by such mice after injection of anti-IgD, a powerful stimulus for Th2 development in vivo, suggested the hypothesis that NT cells might represent a critical cell population required as a source of IL-4 in directing the differentiation of Th2 cells (33). To evaluate the role of NT cells in Th2 differentiation in vivo, we used β2m-deficient mice to assess $CD4^+$ subset development in a variety of well-characterized Th2 challenges, which included responses to soluble subcutaneous antigen, intestinal helminth infection, induction of airway hyperreactivity, eggs of *Schistosoma mansoni*, and the intracellular protozoan, *Leishmania major*.

Materials and Methods

Mice. Female 6–8-wk-old C57BL/6, BALB/c, and β2m $-/-$ mice backcrossed 10 generations to C57BL/6 or BALB/c were obtained from The Jackson Laboratory (Bar Harbor, ME).

Immunization with KLH. KLH (Calbiochem-Novabiochem, La Jolla, CA) was dispersed into a 1:1 emulsion of PBS with CFA (Sigma Chemical Co., St. Louis, MO). Mice were immunized with 50 μl (75 μg KLH) in each hind footpad. Popliteal lymph nodes were removed after 7–11 d, disrupted into single cell suspensions, and duplicate aliquots of 10^6 cells were cultured in 200 μl complete Iscove's media (10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5.5×10^{-5} M 2-mercaptoethanol) in microtiter wells of 96-well round-bottomed plates in the presence or absence of 100 μg/ml KLH. Supernatants were collected after 48 h for cytokine ELISA (below).

L. major infection. *L. major* (WHOM/IR/$-$/173) was passaged serially in BALB/c mice to maintain virulence. Parasites were harvested from infected animals and cultured in vitro at 26°C in M-199 medium (plus 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) for no longer than 2 mo before experimental infection. Late stationary-phase cultures were enriched for metacyclic promastigotes by incubation with peanut agglutinin–conjugated agarose beads (Sigma Chemical Co.) as described (34). Mice were inoculated with 5×10^5 metacyclic promastigotes in each hind footpad and disease progression was monitored weekly by measuring the footpads with a metric caliper. Designated mice received 5 mg of mAb 11B11 (rat IgG1 anti–murine IL-4) intraperitoneally at the time of infection. After 6–8 wk, the popliteal lymph nodes draining the lesion were collected for cytokine determinations, the footpads and spleens were harvested for parasite burdens, and serum was collected for quantitation of total IgE.

For the quantitation of parasites, footpads were washed in ethanol, rinsed in HBSS, and homogenized in 3 ml M-199 medium. Spleens were homogenized in 3 ml M-199 medium. Aliquots were serially diluted in flat-bottomed 96-well microtiter plates. Plates were sealed with parafilm and incubated at 26°C for 1 wk. Motile promastigotes were detected using inverted microscopy.

S. mansoni Egg-induced Pulmonary Granuloma. Eggs of *S. mansoni* were extracted from livers of infected mice (Biomedical Research Institute, Rockville, MD) and enriched for mature eggs as described (35). Mice were presensitized by intraperitoneal injection of 5,000 eggs. 4 wk later, mice were challenged with intravenous injection of 5,000 eggs to induce synchronous granuloma formation after entrapment in the lungs. Animals were killed for analysis 6 or 10 d after egg injection during the peak period of the immune granulomatous response (36).

For quantitation of granuloma formation, the left lung was inflated with Bouin-Hollande fixative before processing. The size of the granulomas was determined microscopically from 4-μm-thick sections stained using Litt's modification of the Dominici stain (37). The diameters of each granuloma that contained a single egg were measured using an ocular micrometer and the volumes of the granuloma were calculated assuming a spherical shape. The numbers of eosinophils were numerically evaluated in the same sections.

Induction of Airway Hyperreactivity. Airway hyperreactivity was induced and quantitated as described (38). Briefly, groups of BALB/c or β2m $-/-$ BALB/c mice were immunized subcutaneously with 25 μg turkey OVA (Sigma Chemical Co.) and alum weekly for 4 wk and subsequently exposed to three aerosolizations of 50 mg/ml OVA in PBS nebulized by compressed air and administered through a nose-only chamber over 20 min at 2-d intervals. 1 d after the final aerosol, mice were anesthetized and maintained inside a plethysmograph on rodent ventilators (Harvard Apparatus Co., Inc., S. Natick, MA) on 100% oxygen under conditions such that physiologic arterial blood gas parameters other than elevated Pa_{O2} were maintained. Lung resistance (R_L) was determined by continuously quantitating the quotient $\Delta Pt/\Delta V$ (where ΔPt = change in tracheal pressure and ΔV = change in flow) at 70% tidal volume. After establishing a stable baseline for R_L (<5% variation over 3 min), acetylcholine chloride (Ach; Sigma Chemical Co.) was administered intravenously over 1 s in increasing doses. The provocative concentration of Ach (micrograms per gram) that caused a 200% increase in R_L was calculated from linear interpolation of appropriate dose-response curves as described (39).

Immediately after collection of the physiologic parameters, mice were killed and single-cell suspensions of lung cells prepared. Briefly, the lungs were completely blanched with cold

PBS, removed, and minced into fine fragments before dispersing into PBS using a syringe plunger and passage over a 0.75-μm nylon mesh filter. Cells were washed twice, counted, and adjusted to 10^7 cells/ml in RPMI 1640 with 5% fetal bovine serum and antibiotics for cytokine ELISPOT assays (below) and enumeration of eosinophils using phloxine B staining (Unopette; Becton Dickinson and Co., San Jose, CA). Serum was prepared for the determination of total IgE by ELISA (below).

Nippostrongylus brasiliensis Infection. Infective *N. brasiliensis* third-stage larvae were isolated from the feces of experimentally infected rats after 7 d of charcoal and peat moss/fecal culture by direct pipetting after migration into the liquid PBS media. Worms were washed repeatedly in saline, counted, and injected into designated groups of C57BL/6 or β2m −/− C57BL/6 mice subcutaneously at the base of the tail using 500 organisms/mouse in 0.2 ml PBS. Mice were killed after 12 d and the numbers of adult worms were determined by direct visualization after opening the intestines with an enterotome. Immunocompetent mice expulse adult worms in a CD4-dependent manner after 10 d (40). Single-cell suspensions of lung cells were prepared as described above (*Induction of Airway Hyperreactivity*) for cytokine ELISPOT assays, and blood was collected for determination of eosinophil numbers and total serum IgE levels.

Cytokine mRNA Analysis. For the leishmania experiments, popliteal lymph node cell suspensions were used for extraction of total RNA using RNAzol (Biotecx, Houston, TX). RNA was reverse transcribed using random hexamer primers according to the manufacturer's specifications (Promega Corp., Madison, WI) and used in a competitive PCR analysis as previously described (41). Briefly, a multiple cytokine–containing competitor construct was used to equalize the amounts of input cDNA from the different samples by adjusting for competition with the constitutively expressed hypoxanthine-guanine phosphoribosyltransferase (HPRT) product. These adjusted volumes of cDNAs were then used in subsequent reactions with primers for specific cytokines that were also engineered as larger competitive products in the PCR competitor by insertion of an internal irrelevant DNA sequence. Because of their slower mobility in 2.5% agarose gels, the competitor products can be readily separated from the authentic transcripts on the basis of size. The ratio of the competitor to the authentic cytokine transcripts was used to quantitate mRNA production in vivo.

For the schistosoma experiments, one lobe of the right lung was homogenized using a tissue polytron (Omni International, Waterbury, CT) in 1 ml RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and the total RNA isolated according to the manufacturer's specifications. Cytokines were quantitated using a reverse transcription-PCR–based method using primers for IL-4, IL-5, IL-13, and HPRT as an internal control as previously described (36). The amplified cDNAs were analyzed after electrophoresis in agarose and Southern blotting with cytokine-specific probes. The chemiluminescent signals were quantitated using a 600 ZS scanner (600 ZS; Microtek International, Torrance, CA) and the amount of product determined by comparison of the signal intensity with a standard curve generated from simultaneously amplified stepwise dilutions of cDNA containing large amounts of the specific cytokine mRNAs. Fold increase was calculated as the reciprocal of the equivalent dilution of control (noninjected mouse lung) cDNA.

Cytokine ELISA. Antigen-specific cytokine production was determined by culturing 10^6 draining lymph node cells per well in a 96-well round-bottomed plate at 37°C and 5% CO_2 in complete Iscove's media. Designated wells from *L. major* experiments received 100 μg/ml soluble Leishmania antigens, or antigen plus mAb M5/114 (anti-$IA^{b,d}$ and -$IE^{d,k}$, rat IgG2b). After 48 h, IL-4 and IFN-γ levels in the supernatants were determined by ELISA (PharMingen, San Diego, CA).

Cytokine ELISPOT. For the airway hyperreactivity and *Nippostrongylus* experiments, single-cell suspensions of lung cells were distributed in duplicate aliquots of 10^6 cells in RPMI 1640 with 5% FCS and antibiotics to 96-well microtiter plates (Immunolon IV; Dynatech, Chantilly, VA) that had been precoated with either mAb 11b11 (anti–murine IL-4, rat IgG1) or mAb R46A2 (anti–murine IFN-γ, rat IgG1). Serial threefold dilutions of the cells were performed and the plates were incubated undisturbed for 8 h at 37°C. After washing away the cells, biotinylated secondary antibodies against IL-4 (BVD6-24G.2, rat IgG1) or IFN-γ (XMG-1.2, rat IgG1) were added, and, after 1 h, wells were washed and incubated with 100 μl streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Color was developed with 5-bromo-4-chloro-indolyl- phosphate in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma Chemical Co.) suspended in 0.6% agarose (Sea-Plaque; FMC Bioproducts Inc., Rockland, ME). After solidification of the agar, individual blue spots were counted by inverted microscopy.

Quantitation of Serum IgE. Serum from designated animals was collected at the time of death and total IgE was quantitated by a sandwich ELISA using commercial mAb pairs (PharMingen) according to the manufacturer's instructions.

Results

Evaluation of Th2 Responses to a Subcutaneously Administered Protein Antigen. Subcutaneous administration of KLH with adjuvant establishes substantial IL-4 production by lymph node cells draining the site of immunization that can be demonstrated by restimulation of the isolated T cells with KLH in vitro (42). To assess the contribution by NT cells in priming for IL-4 production, groups of BALB/c β2m +/− or β2m −/− littermates were immunized with KLH and the lymph node cells isolated and restimulated in

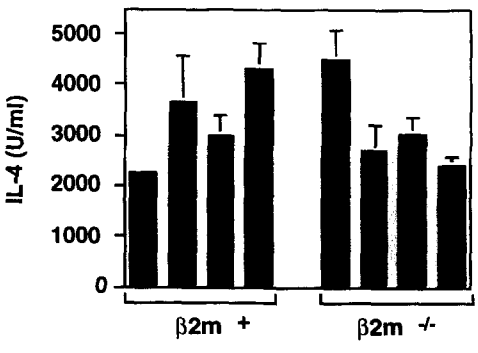

Figure 1. Production of IL-4 after immunization with KLH. Four BALB/c heterozygous (*β2m* +) or deficient (*β2m* −/−) littermates were immunized subcutaneously with KLH in CFA and the draining lymph node cells collected after 11 d and incubated in vitro with 100 μg/ml KLH. After 48 h, supernatants were collected and assayed for IL-4 by ELISA. Cells incubated in the absence of KLH produced <25 U/ml IL-4 under these conditions. Bars represent means and standard deviations of triplicate determinations. Results were comparable in 10 mice.

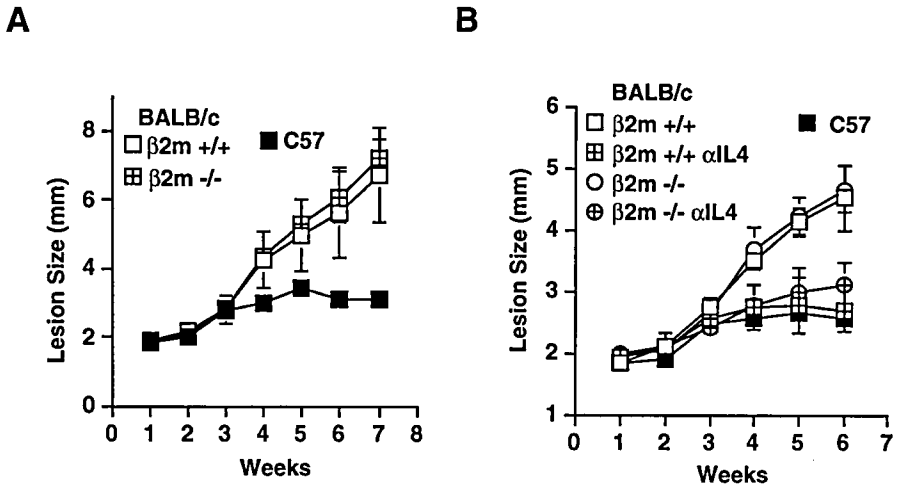

Figure 2. Progression of *L. major* infection in β2m −/− mice. (*A*) The designated mice were inoculated in the hind footpads with *L. major* promastigotes and the size of the footpad lesion quantitated over time using a metric caliper. Symbols represent average sizes of the footpads with standard deviations. The depicted experiment was representative of 10 experiments involving 34 β2m + (both +/+ and +/−) and 23 β2m −/− BALB/c and 18 resistant C57BL/6 mice. (*B*) The designated mice were inoculated in the hind footpads with *L. major* promastigotes with indicated animals also receiving neutralizing IL-4 mAb (αIL-4). Symbols represent average sizes of the footpads with standard deviations. The depicted experiment was representative of three experiments.

vitro in the presence or absence of KLH (Fig. 1). Although variation occurred among animals, no significant differences in IL-4 production could be demonstrated between the two cohorts of mice. Similar results were obtained comparing C57BL/6 β2m +/− to C57BL/6 β2m −/− littermates (data not shown).

Evaluation of Th2 Immune Responses in BALB/c mice Infected with L. major. Although infection with the intramacrophage protozoan, *L. major*, induces strong Th1 responses in most strains of inbred mice, BALB/c mice develop aberrant Th2 responses to infection that underlie the susceptibility of this mouse strain (43). To assess whether this unusual "polymorphism" in Th2 development (7) was dependent on NT cells, BALB/c and β2m −/− BALB/c mice were infected in the hind footpads with infectious promastigotes and the size of the lesions monitored using a metric caliper. Resistant C57 background mice (C57BL/6 or B10.D2) were infected for comparative purposes. As assessed both by the size of the footpad lesions over time (Fig. 2 *A*) and direct quantitation of parasite burdens (data not shown), the absence of the β2m molecule had no effect on the course of the infection in susceptible BALB/c mice. Further, progressive disease in the β2m −/− mice was dependent on early production of IL-4 in vivo, since administration of neutralizing antibody to IL-4 at the time of infection allowed these mice to heal in a manner comparable to wild-type BALB/c mice (Fig. 2 *B*).

When isolated lymph node cells from the infected mice were incubated in vitro with *L. major* antigens, T cells from β2m-deficient and wild-type BALB/c mice produced comparable amounts of IL-4 in the supernatants that was significantly greater than that produced by concomitantly stimulated cells from infected B10.D2 mice (Fig. 3). IL-4 production was MHC class II dependent, consistent with the presence of Th2 cells, since inclusion of mAb against class II abrogated cytokine production. Direct analysis of lymph node IL-4 transcripts using competitive RT-PCR corroborated the restimulation assays: infected BALB/c and

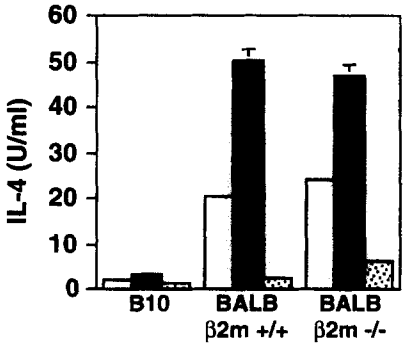

Figure 3. Production of IL-4 by LN cells from mice infected with *L. major*. LN cells draining the infection site were collected from the designated mice and incubated for 48 h with media (*open bar*), or with 100 μg/ml *L. major* antigens without (*closed bar*) or with (*stippled bar*) anti-IA^d mAb. Supernatants were collected and assayed for IL-4 by ELISA. Bars represent mean and standard deviations of triplicate determinations. Comparable results were obtained in six experiments involving 13 animals, including both +/− and −/− β2m littermates.

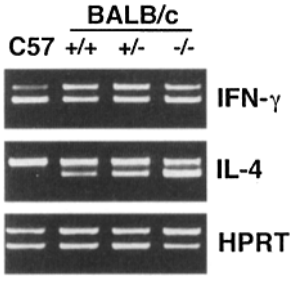

Figure 4. Analysis of LN cell IL-4 mRNA transcript levels in mice infected with *L. major*. LN draining the infected site were collected from the designated mice 6 wk after infection with *L. major* and the total RNA used to prepare cDNA by reverse transcription. Input cDNAs were adjusted until comparable PCR-mediated amplification of the constitutively expressed HPRT gene was achieved as assessed by competition from an exogenous competitor construct that contains a larger PCR product that can be resolved as the upper band after gel electrophoresis from the more rapidly moving wild-type, lower band. Adjusted cDNAs were used as templates for amplification in the presence of the same competitor that also contains IL-4 and IFN-γ pseudogenes. The ratio of the wild-type (*lower*) to competitor (*upper*) amplification products allows a semiquantitative analysis of the relative amounts of cytokine transcripts in lymph node cells. Results were comparable in five experiments.

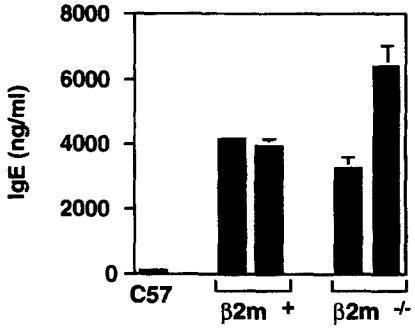

Figure 5. IgE levels in mice infected with *L. major*. Mice in the designated groups had serum prepared 6 wk after infection with *L. major*. Total IgE was quantitated using ELISA. Results represent means and standard deviations of triplicate determinations from individual littermates either BALB/c +/− (β2m +) or −/− β2m. Comparable results were obtained from four infected animals in each group.

β2m +/− or β2m −/− littermate BALB/c mice had substantially greater amounts of IL-4 mRNA than infected C57BL/6 mice (Fig. 4). Finally, serum IgE levels were also comparably elevated in infected BALB/c β2m +/− and β2m −/− littermates (Fig. 5), consistent with enhanced levels of IL-4 in vivo during the course of infection.

Evaluation of Schistosoma Egg-mediated Pulmonary Granulomas. Intravenous injection of eggs of *S. mansoni* into mice presensitized with egg antigens induces prominent granulomatous infiltration around the embolized eggs in the lungs that is associated with strong Th2 immune responses (35). These responses include induction of multiple type 2 cytokines and predominant eosinophilic infiltrates. To examine the role of NT cells in this type 2 granulomatous response, C57BL/6 and β2m −/− C57BL/6 mice that had been presensitized to egg antigens were injected intravenously with 5,000 eggs and the pulmonary response assessed histologically and by evaluation of the immune response.

At 6 and 10 d after intravenous injection of eggs, granuloma volumes did not differ between C57BL/6 wild-type and β2m −/− mice (Fig. 6 *A*). Histologic examination of the tissues revealed no significant differences in the numbers of eosinophils in the granulomas (Fig. 6 *B*). To assess the immune response, lung tissue was used to isolate RNA for quantitation of Th2 cytokines by reverse transcription-PCR. As measured by fold increases from baseline (uninjected) lung values and standardized to the constitutively expressed gene, HPRT, no diminution in the pulmonary Th2 response was evident in these two cohorts of mice (Fig. 7).

Evaluation of Intestinal Immunity against Nematodes. When injected subcutaneously larvae of *N. brasiliensis* migrate to the bloodstream and thence escape into the pulmonary alveolae. They are subsequently coughed up and swallowed, and thus gain access to the small intestine where they mature into the egg-laying adults. Immunocompetent mice expulse the adult worms shortly after the onset of egg laying, and expulsion is usually complete by 10–12 d. Depletion of

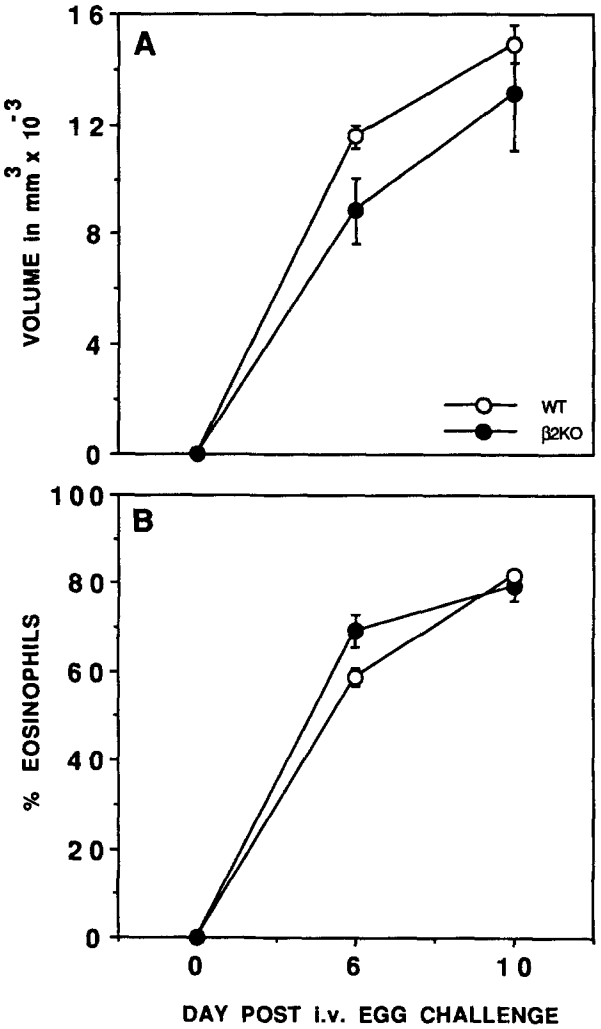

Figure 6. The granulomatous response to eggs of *S. mansoni* in β2m −/− mice. (*A*) Four C57BL/6 or C57BL/6 β2m −/− mice were killed 6 or 10 d after embolization of 5,000 eggs intravenously and the volume of the pulmonary granulomas was assessed microscopically on stained sections. Results represent means and standard errors from the pooled results of four mice in each group. (*B*) The percentage of eosinophils in granuloma cells surrounding single eggs was calculated using the histologic materials examined in *A*. Results represent means and standard errors of the means.

$CD4^+$ T cells significantly delays expulsion (40), and administration of IL-4 to infected SCID mice enables these mice to expulse the worms (44). Infection is associated with dominant type 2 cytokine responses in the lungs and intestinal lymphoid tissues. To assess the role of NT cells in the IL-4 response induced by *Nippostrongylus*, C57BL/6 wild-type or β2m −/− mice were inoculated with 500 third-stage larvae and examined for worm expulsion and the type of immune response produced.

When examined 12 d after infection, neither wild-type nor β2m −/− mice had any adult worms in the small or large intestines. Single-cell suspensions of isolated lung cells were used in ELISPOT assays to quantitate the numbers of IL-4–producing cells that had been produced during the mi-

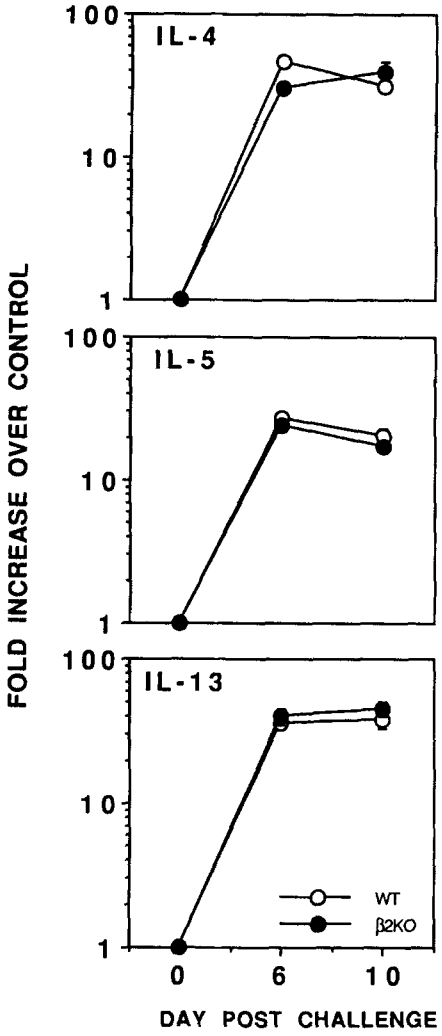

Figure 7. Th2 cytokine responses in the lungs of mice challenged with eggs of *S. mansoni*. Lung homogenates were used to prepare cDNA for quantitative analysis of amounts of IL-4, IL-5, and IL-13 transcripts as expressed as fold increases as compared with uninjected control animals that are given an arbitrary value of 1. Results represent means and standard errors of the means from the lungs of four individual mice in each group.

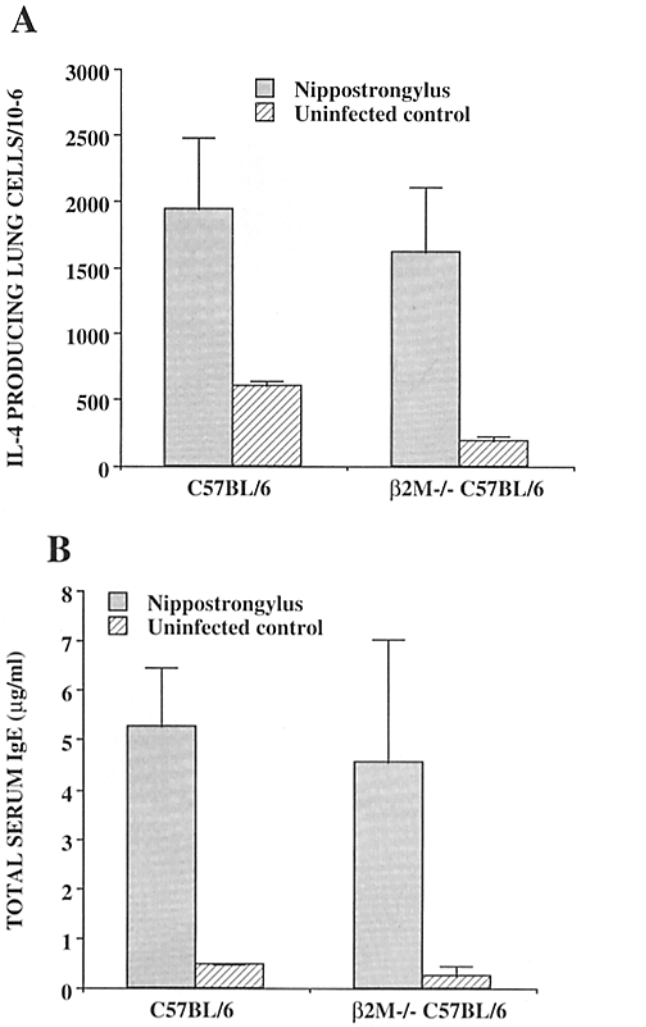

Figure 8. Type 2 immune responses in mice infected with *N. brasiliensis*. (*A*) Wild-type C57BL/6 or β2m −/− C57BL/6 mice were either uninfected or challenged with larvae of *N. brasiliensis*. After 12 d, single-cell suspensions of the whole lung were analyzed for numbers of IL-4–producing cells using the ELISPOT assay. Bars represent means and standard deviations from duplicate determinations from five animals. Results are representative of two experiments. (*B*) Wild-type C57BL/6 or β2m −/− C57BL/6 were either left uninfected or infected with larvae of *N. brasiliensis*. After 12 d, serum was prepared and analyzed for total IgE by ELISA. Results represent means and standard deviations of duplicate determinations from five mice in each group. Results are representative of two experiments.

gration of the organisms. No significant effect on IL-4–producing cells could be discerned by the absence of β2m (Fig. 8 *A*). Further, there were no significant differences in blood eosinophilia (wild-type: 13.3 +/− 4% versus β2m −/−: 13.2 +/− 5%, as compared to 1.3% and 1.7% in uninfected control mice of the respective genotypes) nor serum IgE levels (Fig. 8 *B*) in the two groups of infected mice.

Evaluation of Airway Mucosal Immune Responses. Airway hyperreactivity can be established in BALB/c mice by aerosolizing antigen to animals previously sensitized by subcutaneous immunization with the same antigen. Using OVA, hyperreactivity as assessed by increases in airway pressure and resistance after injection of Ach was demonstrated to be dependent on the presence of IL-4 during the initial period of immunization with OVA (38). Neutralizing antibody to IL-4 given during the subcutaneous sensitization period abrogated the subsequent airway response to inhaled OVA, as well as the type 2 cytokine profile in lung cells, but had little effect on pulmonary eosinophilia. To assess the role of NT cells in this airway model of Th2 sensitization, BALB/c or β2m −/− BALB/c mice were immunized subcutaneously with OVA and airway hyperreactivity and immune responses quantitated after multiple aerosol exposures to OVA.

As assessed using incremental increases in Ach to determine the concentration required to increase R_L by 200%,

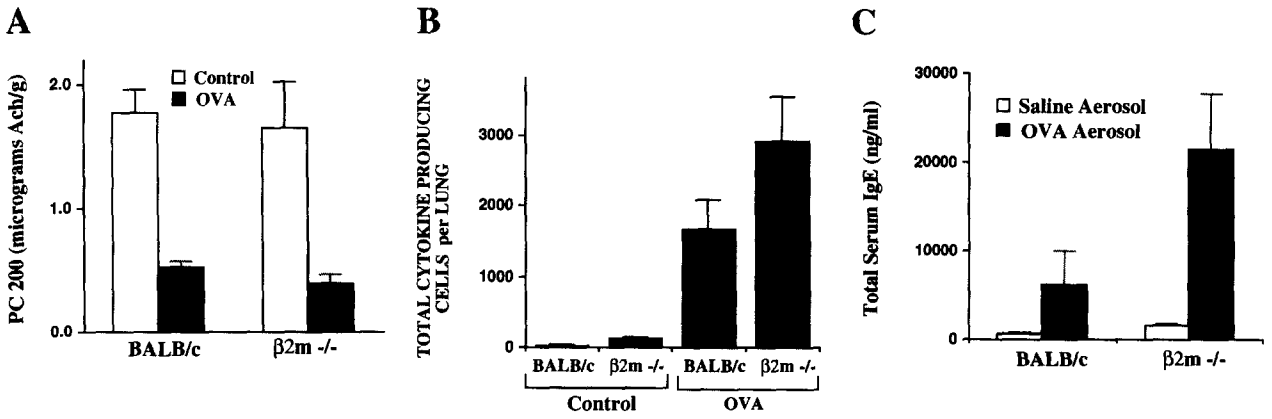

Figure 9. Induction of airway hyperreactivity by aerosolization of antigen to sensitized mice. (*A*) Wild-type BALB/c or β2m −/− BALB/c mice were presensitized with OVA before aerosolization of saline (control) or OVA. After 24 h, animals were anesthetized and placed on rodent ventilators inside a plethysmograph. Incrementally increasing doses of Ach were administered intravenously and the provocative dose that caused a 200% increase in lung resistance was calculated. Bars represent means and standard errors of the means from four individual mice in each group. Results are representative of two experiments. (*B*) Single-cell suspensions from the animals from the groups analyzed in *A* were prepared and assayed for numbers of IL-4–producing cells in an ELISPOT assay. Bars represent means and standard errors of the means from four mice in each group. (*C*) Serum from the animals from the groups analyzed in *A* was prepared and assayed for total IgE by ELISA. Bars represent means and standard errors of the means of duplicate determinations from four mice in each group.

no significant differences in either lung resistance (Fig. 9 *A*) or airway pressure (data not shown) were apparent between β2m −/− mice and wild-type controls. The pulmonary immune response was determined by evaluation of individual IL-4–producing cells by ELISPOT assay of isolated whole lung cells (Fig. 9 *B*) and the serologic response was assayed by quantitating total serum IgE (Fig. 9 *C*). Neither of these assays demonstrated diminished responses in mice without β2m.

Discussion

A number of characteristics suggest that NT cells might be the source of early IL-4 required for commitment of naive $CD4^+$ T cells to the Th2 phenotype during an immune response. First, cross-linking the antigen receptors on NT cells, in contrast to naive α/β^+ T cells, results in rapid production of large amounts of IL-4, the critical cytokine required for Th2 differentiation (27, 33). Second, NT cells display a restricted TCR repertoire that is selected by the nonpolymorphic MHC class I molecule, CD1 (28–31). Such structural constraints would be consistent with recognition shaped by conserved microbial ligands from pathogenic organisms. Indeed, $CD4^-CD8^-$ human T cells have been isolated that recognize mycolic acids and lipoglycans from mycobacteria in the context of CD1b (45, 46). Although mice express only one of the five human CD1 homologues, presumably due to an ancient translocation event that split chromosome 1 (47), the locus has already been duplicated to form two closely related genes, designated CD1.1 and CD1.2, consistent with evolutionary pressure to maintain expression of these molecules. Third, mice deficient in NT cells, either through disruption of β2m necessary for efficient surface expression of CD1 (29–31) or genetically, as in SJL mice, do not mount typical IL-4 responses and IgE elevations in response to anti-CD3 or anti-IgD antibodies in vivo (33, 48). The common requirement for IL-4 production in vivo in response to diverse type 2–inducing agents would be consistent with an invariant population poised to recognize microbial agents and release IL-4 in sufficient amounts to direct Th2 differentiation of naive T cells.

With these considerations, we examined Th2 responses in β2m-deficient mice on both BALB/c and C57BL/6 backgrounds using a variety of challenges that are documented to induce dominant Th2 immune responses in vivo. Neutralization of IL-4 at the time of inoculation of KLH (42), *L. major* (49), eggs of *S. mansoni* (50, 51), larvae of *N. brasiliensis* (52), and subcutaneous immunization with OVA in airway hyperreactivity (38) has consistently abrogated most type 2 responses, including production of type 2 cytokines and elevation of serum IgE. Similarly, neutralization of IL-4 at the time of administration of anti-CD3 or anti-IgD profoundly impairs type 2 immune responses (52), and this effect is presumably due to blocking the differentiation of naive T cells, since the production of IL-4 by NT cells is unaffected by neutralization of IL-4 (33). Despite the comparable effects of anti-IL-4 in these systems, however, we could discern no significant impairment of Th2 responses in the absence of β2m.

The systems investigated represented a variety of antigens administered by different routes to animals on two genetic backgrounds that have been shown to differ in their capacity to generate type 2 responses to some antigens (7). We have also measured responses at two mucosal surfaces (airway hyperreactivity, intestinal worm expulsion), in various tissues (subcutaneous leishmania lesions, schistosoma egg pulmonary granulomas), and in draining lymph nodes (KLH, leishmania responses). Using a variety of functional and immunologic assays, we could discern no substantial

differences in the responses between wild-type, heterozygous littermates or β2m-null mice. Recent studies investigating *L. major* infection (53) and responses to low-dose antigen (54) in BALB/c mice were also unable to incriminate a role for NT cells in type 2 immune responses. These data suggest that most biologic Th2 responses are β2m independent, and thus distinct from the type 2 response generated after anti-CD3 or anti-IgD.

Several possibilities should be considered before dismissing a potential role for NT cells in Th2 responses, however. Although both flow cytometric and functional studies suggest that the NT population is absent in β2m-null mice (29–33), it is possible that a residual population of CD1-restricted T cells remains. A number of hybridomas generated from the small numbers of $CD4^+$ T cells present in class II^0 and class $II^0/\beta2m^0$ mice were reactive to CD1 and shared the mature/activated phenotype typically found on NT cells (55). Expression of transfected CD1.1 and its human homologue, CD1d, has been achieved in the absence of β2m (56). It is possible that a small residual NT population in β2m-null mice is capable of generating IL-4 under the conditions of most type 2 immune responses, but is incapable of responding to rapid activation after administration of anti-CD3 or anti-IgD antibodies. An alternative strategy to eliminate NT cells using mAb depletion of NK1.1-bearing cells from BALB/c mice congenic for NK1.1 expression also failed to ameliorate susceptibility (Brown, D.R., and S.L. Reiner, unpublished results). It is likely, however, that mice with disruption of the CD1.1 and CD1.2 genes will need to be infected with these types of pathogens to exclude definitively a role for NT cells in Th2 development.

An alternative possibility is that these diverse biologic Th2 responses activate distinct populations of cells as compared with anti-CD3 or anti-IgD. Cells of the mast cell/basophil lineage have been implicated in maintaining IL-4 production after *S. mansoni* (57) and *N. brasiliensis* (58) infections, and eosinophils have been implicated in the early production of IL-4 after intraperitoneal injection of *S. mansoni* eggs (59). Intraperitoneal injection of *N. brasiliensis* induced early IL-4 production from $\gamma\delta^+$ T cells (60). In *L. major* infection, mast cell–deficient mice developed smaller lesions that were augmented after passive transfer of mast cells into the lesion site (61). Despite these observations, the most convincing data in each of these systems implicate $CD4^+$ T cells as the major source of IL-4 in directing Th differentiation. An unusual subpopulation of class II–restricted T cells or a propensity for certain antigens to activate naive $CD4^+$ T cells to favor IL-4–mediated responses over IL-12/IFN-γ–mediated responses remain untested but plausible hypotheses.

Although use of β2m-deficient mice remains imperfect, the available data suggest that these animals are incapable of supporting the differentiation of NT cells in vivo. As demonstrated here, these mice support Th2 development in vivo in a manner indistinguishable from wild-type mice in response to a wide variety of immunologic challenges. Such systems should prove invaluable for further studies attempting to identify the critical early source of IL-4 implicated in Th2 effector cell differentiation and activation. Elucidation of the source of IL-4 remains an important goal for understanding and modulating the development of the immune response, particularly in allergic disorders and intestinal host defense.

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