A Role for CD4+NK1.1+ T Lymphocytes as Major Histocompatibility Complex Class II Independent Helper Cells in the Generation of CD8+ Effector Function against Intracellular Infection

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Summary

Major histocompatibility complex (MHC) class II (Aβ) knockout mice were vaccinated with ts-4, an attenuated mutant strain of *Toxoplasma gondii*, which in normal animals induces strong T cell immunity mediated by interferon γ (IFN-γ). After challenge with the lethal parasite strain RH, the knockout mice displayed decreased resistance consistent with absence of $CD4^+$ effectors. Nevertheless, these animals generated $CD8^+$ lymphocyte effectors capable of mediating partial protection through IFN-γ secretion. Morever, in vivo neutralization experiments indicated that the development of resistance in knockout mice depends on $CD4^+$ cells as well as interleukin 2 (IL-2). The identity of the IL-2–producing protective cell population was further characterized as $CD4^+$, $NK1.1^+$ by in vitro depletion studies and reverse transcriptase–PCR analysis of fluorescence-activated cell sorter (FACS)–purified $CD4^+NK1.1^+$ T lymphocytes. These results demonstrate that in the absence of conventional MHC class II–restricted $CD4^+$ T lymphocytes, CD8 priming persists and mediates partial protective immunity to *T. gondii*. Moreover, the data argue that $CD4^+$, $NK1.1^+$ cells, previously implicated in the initiation of T helper cell 2 (Th2) responses through their production of IL-4, can also play a role as alternative IL-2–secreting helper cells in Th1-mediated host resistance to infection.

Infection with the intracellular protozoan *Toxoplasma gondii* is characterized by an acute proliferative stage, during which infective tachyzoites invade and replicate within a wide variety of host cells, and a chronic slow growing phase consisting of parasite encystment within tissues of the brain and muscle (1, 2). Although infection is usually innocuous, in immunocompromised hosts encysted parasites can reactivate, leading to uncontrolled tachyzoite proliferation, tissue damage, and encephalitis, which in some cases leads to host death (3, 4).

To investigate control of *T. gondii* infection, we have employed a vaccine model in which mice are immunized with the attenuated parasite mutant ts-4 (5). When these animals are subsequently challenged with the highly virulent strain RH, vaccinated mice are completely protected, whereas nonvaccinated animals die within 10–14 d (6–8). Vaccine-induced control of infection in normal mice has been shown to be dependent on three major elements: the cytokine IFN-γ, $CD8^+$ T lymphocytes, and $CD4^+$ lymphocytes (6, 7). $CD8^+$ cells are likely to be the major, and $CD4^+$ T cells a secondary, source of IFN-γ, as determined by cell transfer and in vivo mAb neutralization studies (6, 9). However, CD4 cells play a pivotal role during induction of immunity, since administration of anti-CD4 mAbs during ts-4 vaccination prevents induction of a protective immune response (6).

In the experiments reported here, we examined the ability of MHC class II–negative mice, which have a targeted mutation in the Aβ gene and therefore lack class II-restricted $CD4^+$ T lymphocytes (10–12), to generate protective immunity to infection. We found that despite the absence of classic $CD4^+$ T cells in these animals, $CD8^+$ lymphocyte priming nevertheless continued to occur in response to ts-4 immunization, resulting in partial resistance to RH challenge. Priming of $CD8^+$ T cells for effector function was found to require an early IL-2–dependent helper activity of a population of cells phenotypically defined as $CD4^+NK1.1^+$. They therefore closely resemble a subpopulation of MHC class II–independent $CD4^+NK1.1^+$ cells that is selected during thymic differentiation by the class I–related molecule CD1 (13, 14) and produces several cytokines, in particular IL-4, in response to anti-CD3 stimulation (15, 16). Our findings demonstrate a role for $NK1.1^+$ T lymphocytes in resistance to infection in Aβ knockout animals and sug-

gest that in addition to IL-4 production, IL-2 production may also be a major functional activity of this cell population.

Materials and Methods

Mice and Parasites. Aβ knockout animals (sixth back-cross generation to C57BL/6), which express no surface MHC class II molecules, were generated as previously described (11) and supplied by GenPharm International (Mountain View, CA). Control C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and were housed under sterile conditions, and cages were changed under a sterile hepa-filtered environment at the National Institutes of Health animal facility. Animals were routinely screened for infection with all common adventitious pathogens. Mice (6–8 wk of age) were immunized by biweekly intraperitoneal injection of 2×10^4, 2×10^5, and 2×10^5 ts-4 tachyzoites. 2 wk after the final inoculation, resistance was evaluated by subcutaneous injection of 2,000 tachyzoites of the virulent RH strain. The latter parasites, as well as the attenuated mutant ts-4 (5), were maintained by weekly passsage at 37°C and 34°C, respectively, on monolayers of human foreskin fibroblasts.

Ab Reagents. Flow cytometric analyses employed the following mAbs (obtained from PharMingen, San Diego, CA): PE-conjugated 145-2C11 (anti-CD3); FITC-conjugated RM4-5 (anti-CD4); FITC-conjugated 53-6.7 (anti-CD8); PE-conjugated H57-597 (anti-αβTCR); PE-conjugated GL3 (anti-γδTCR); PE-conjugated PK136 (anti-NK1.1); FITC-conjugated RA3-6B2 (anti-CD45R). In addition, mAb M5/114 (anti–class II) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). In vivo depletion studies employed mAb XMG1.2 (anti–IFN-γ), GK1.5 (anti-CD4), 2.43 (anti-CD8), PK136 (anti-NK1.1), S4B6 (anti–IL-2), and PC61 5.3 (anti–IL-2 receptor). The mAbs were partially purified from mouse ascites by precipitation in 40% ammonium sulfate. For in vitro depletion, hybridoma supernatants containing the mAbs RL174.2 (anti-CD4), SW3A4 (anti-NK1.1), RA3-3A1 (anti-B220), or J11D (anti–B cell marker) were used.

Flow Cytometric Analysis and Sorting. Splenocyte populations were analyzed on a flow cytometer (Epics model 753; Coulter Corp., Hialeah, FL) as described in detail elsewhere (17). In brief, red cells were lysed in hypotonic media, and the remaining cells were stained with mAbs in the presence of saturating amounts of unlabeled 2.4G2 (anti-Fc receptor; PharMingen), washed, and then analyzed in the presence of propidium iodide to exclude dead cells. To obtain highly purified populations of $CD4^+$ $NK1.1^+$ cells, splenocytes from Aβ knockout mice injected 7 d previously with 2×10^4 ts-4 tachyzoites were passed over an anti–mouse Ig column according to the manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN), resulting in an enriched (>80%) T cell population. Cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-NK1.1. Cell sorting was performed on a FACStar sorter (Beckton-Dickinson, Mountain View, CA) with gates set to collect double-positive cells (~0.4% of the total population). Immediately following sorting, cells were pelleted, resuspended in RNazol (Tel-Test, Friendswood, TX), and frozen in dry ice plus methanol to be processed later for reverse transcriptase (RT)[1]–PCR analysis of mRNA.

In Vivo Cell and Lymphokine Depletion Experiments. To assess the function of $CD4^+$ and $CD8^+$ lymphocytes as well as IFN-γ in resistance to RH challenge, mice were biweekly injected intraperitoneally with 0.5 mg RL174.2, 0.25 mg 2.43, 2 mg XMG1.2, and 2 mg control mAb GL113. Treatment commenced 3 d before challenge infection and was continued for the duration of the experiment (30 d). The role of CD4 lymphocytes and IL-2 during vaccination was determined by biweekly injection of RL174.2 (0.5 mg) or a combination of S4B6 and PC61 4.5 (2 mg each). The mAbs were injected 3 d before the first ts-4 injection and throughout the vaccination regimen. Ab injection was halted 7 d after the final ts-4 injection, and 2 wk later, the mice were challenged with RH strain parasites.

In Vitro Cell Depletion and $CD8^+$ Cell Purification. To eliminate $CD4^+$ and $NK1.1^+$ cells from in vitro assays, splenocytes were treated with two rounds of mAbs and rabbit complement (Accurate Chemical and Scientific Corporation, Westbury, NY) as previously described (17). To obtain purified populations of $CD8^+$ lymphocytes, spleen cells were treated with two rounds of mAb cocktail plus complement. The mAb cocktail consisted of mAb RL172.4, SW3A4, RA3-3A1, and J11D (18). After this treatment, cells were passed over an anti–mouse Ig column according to the instructions of the manufacturer (R & D Systems, Inc.). The resulting population of cells was typically >90% $CD8^+$ as determined by flow cytometric analysis.

Lymphokine Assays. Responder $CD8^+$ populations, obtained by treatment of splenocytes with mAbs and complement, were added to wells of a 96-well plate (2×10^5/well). Splenic macrophages were obtained by incubating 10^8 normal spleen cells on a 100 × 15-mm petri dish (Falcon number 3803; Becton Dickinson Labware, Lincoln Park, NJ) for 1 h at 37°C, removing nonadherent cells by washing with warm media, and then adding a 1:1 ratio of 15 kR-irradiated ts-4. Infection was allowed to proceed overnight, and then adherent cells were recovered by vigorous pipetting and washed five times to remove free parasites (confirmed by microscopic examination). Cells were irradiated (2,000 rads) and added to responders at 2×10^5 cells/well. Presence of IFN-γ in 72-h culture supernatants of *T. gondii* antigen-stimulated spleen cells was ascertained by a two-site ELISA (17) using as a standard recombinant murine IFN-γ (Cetus Corp., Emeryville, CA). IL-2 release in supernatants stimulated for 24 h was quantitated with a CTLL proliferation assay (6).

RT-PCR Detection of Cytokine mRNA. To detect cytokine mRNA, an RT-PCR procedure was employed as described in detail elsewhere (19). In brief, RNA was isolated from spleen cells by resuspending in RNAzol B (Tel-Test) and recovering the aqueous phase after addition of chloroform. RNA was precipitated with alcohol, washed, and resuspended in RNase-free H_20. 1 μg of RNA was reverse transcribed with Moloney murine leukemia virus RT (GIBCO BRL, Gaithersburg, MD), deoxynucleotide triphosphates (dNTPs), and random hexamer primers. The reaction mixture was then used for semiquantitative amplification of cytokine mRNA with Taq DNA polymerase (Promega, Madison, WI) and specific cytokine sense and antisense primers. The numbers of amplification cycles were as follows: 23 (hypoxanthine phosphoribosyl transferase [HPRT]), 29 (IL-4), and 35 (IL-2). The RT-PCR products were subsequently analyzed by Southern blotting followed by probing with internal cytokine gene–specific primers and an enhanced chemiluminescent detection system (Amersham International, Buckinghamshire, England). The results were subject to semiquantitative analysis by scanning densitometry followed by normalization to the HPRT standard as described elsewhere (20–22).

[1] *Abbreviations used in this paper:* dNTP, deoxynucleotide triphosphate; HPRT, hypoxanthine phosphoribosyl transferase; RT, reverse transcriptase.

Cytolytic T Cell Response. The ability of spleen cells from vaccinated mice to lyse infected targets was determined as described (23). Effector CTL populations were generated by culturing immune splenocytes for 7 d in the presence of irradiated (15 kR) ts-4 tachyzoites at a cell-to-parasite ratio of 7.5:1. Bone marrow macrophages cultured 5–7 d in L cell media served as targets of CTL lysis. Cells were infected with ts-4 and then 18 h later were harvested, washed, and labeled for 60 min at 37°C with ^{51}Cr-labeled sodium chromate. After removal of unincorporated radioisotope, the macrophages were added to effector cells, and the assay was allowed to proceed for 4–5 h at 37°C. Supernatants were collected with a supernatant-harvesting press (Skatron Instruments, Lier, Norway), and radioactivity was compared with that obtained with media alone and in the presence of 5% Triton X-100. The SEM of each sample, assayed in triplicate, was consistently <10%.

Results

Phenotypic Analysis of Splenocyte Populations in Normal and Aβ Knockout Mice before and after ts-4 Immunization. In initial experiments, flow cytometric analysis was performed on normal and ts-4–vaccinated animals to ascertain whether there were any abnormalities in the spleen cell populations of the class II knockout mice. Splenocytes from nonimmune and immune Aβ knockout animals showed a normal cell distribution except that, as expected, virtually no cells expressed MHC class II molecules, and there were correspondingly low levels of CD4$^+$ T lymphocytes (Table 1). Importantly, NK1.1$^+$ cells in Aβ knockout mice remained at 3–4% of the splenocyte population after ts-4 immunization. These animals therefore differ from CD8-negative MHC class I knockout animals, which respond to ts-4 vaccination with a large increase in NK1.1$^+$ cells (17).

Aβ Knockout Mice Develop CD8-dependent Immunity to RH Challenge. To assess the ability of class II–negative animals to develop protective immunity to *T. gondii*, mice were vaccinated with ts-4, and survival was monitored after challenge with the virulent RH strain. As shown in Fig. 1 *A*, ts-4–vaccinated wild-type mice were completely protected from a lethal inoculum of RH. In contrast, vaccinated Aβ knockout animals developed only partial resistance to RH challenge (Fig. 1 *A*). The degree of protection in these mice was somewhat variable from experiment to experiment (e.g., compare Figs. 1, *A* and *B* with 3 *A*); nevertheless, in all cases, knockout animals succumbed by 60 d after challenge.

We and others have previously shown that the protective response in normal mice is dependent mainly on IFN-γ produced by CD8$^+$ T lymphocytes and, to a lesser extent, CD4$^+$ T lymphocytes (6, 7, 17). To determine the basis for protection in the class II–negative mice, animals were vaccinated and then treated with depleting mAbs at the time of challenge infection. As in the case of normal animals, ts-4–induced immunity is IFN-γ dependent, since knockout animals treated with mAbs to the latter cytokine died at the same time as, or slightly before, nonvaccinated mice (Fig. 1 *B*). In control experiments, administration of anti–IFN-γ mAb to uninfected mice caused no deaths over the same time period, indicating that mortality was induced by inability to control *T. gondii* infection rather than the activation of an endogenous pathogen harbored by the animals. We also observed a slightly accelerated time to death in IFN-γ–depleted vaccinated mice relative to that in nonvaccinated and CD8-depleted animals. This observation may reflect neutralization of NK cell–derived IFN-γ, since these cells are also a source of the latter cytokine during *T. gondii* infection, particularly during the initial phase of the disease (21, 24). In addition, anti-CD8 mAbs completely abrogated protection, but anti-CD4 treatment had no significant effect relative to mice given control mAbs (Fig. 1 *B*). Together, these results suggest that IFN-γ produced by immune CD8$^+$ T cells provides protection in the class II–negative animals.

The presence of primed CD8$^+$ effector cells in the absence of class II–restricted helper cells was directly tested in vitro by CTL assays on infected macrophages and cytokine production induced by infected macrophages. In killing assays, the ability to lyse ^{51}Cr-labeled infected targets has previously been shown to be a function of MHC class I–restricted immune CD8$^+$ effector cells induced by ts-4 vaccination (23, 25). As shown in Fig. 2 *A*, lysis was equivalent using effector CTLs from wild-type and knockout mice. In neither case were effectors able to lyse noninfected macrophages. Moreover, when CD8$^+$ T cells from vaccinated animals

Table 1. *Phenotypic Analysis of Splenocyte Populations in Wild-type and Aβ Knockout Mice*

		Cells per spleen (× 10^{-7}) expressing*							
Mouse	Genotype	CD3	CD4	CD8	αβTCR	γδTCR	NK1.1	B220	I-A
Normal	Aβ$^{+/+}$	4.16	2.37	1.79	4.08	0.13	0.47	5.03	4.85
	Aβ$^{-/-}$	4.10	0.54	3.47	3.70	0.26	0.69	8.48	0.22
Immune‡	Aβ$^{+/+}$	8.40	3.91	4.15	7.97	0.69	0.65	13.32	12.48
	Aβ$^{-/-}$	8.32	1.37	6.92	8.32	1.06	1.01	16.38	0.31

*Spleen cells were examined for expression of the indicated surface markers by flow cytometric analysis.
‡Animals were vaccinated three times with the attenuated mutant ts-4, and spleen cells were recovered for analysis 2 wk later.

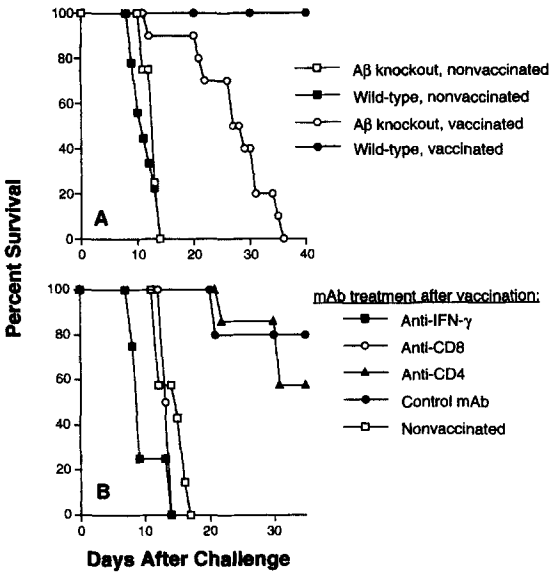

Figure 1. Immunization of Aβ knockout mice with ts-4 results in partial resistance to RH challenge and is dependent on $CD8^+$ T lymphocytes and IFN-γ. In *A*, groups of 6–10 knockout and wild-type mice were vaccinated intraperitoneally with ts-4 tachyzoites and challenged subcutaneously with the virulent strain RH, and their survival was monitored. In *B*, knockout mice (six to eight per group) were vaccinated with ts-4, then treated with the indicated mAb initiated 3 d before RH challenge. See Materials and Methods section for details. The figure shows pooled results from two experiments.

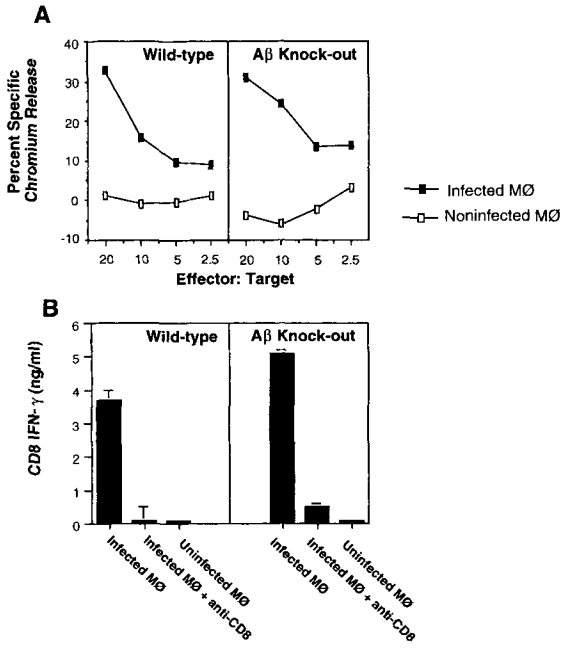

Figure 2. In vitro effector functions of $CD8^+$ cells are unaltered in MHC class II knockout animals. Mice were immunized with ts-4 as in Fig. 1; 2 wk later, spleen cells were isolated, cultured with irradiated tachyzoites for 7 d, and then tested for CTL function by their ability to lyse syngeneic infected target macrophages labeled with ^{51}Cr (*A*). In vitro IFN-γ production was measured by culturing immune $CD8^+$ T lymphocytes (obtained by treatment with a cocktail of anti-NK1.1, anti-CD4, and anti-B cell mAbs and complement, followed by passage over T cell purification columns) with ts-4–infected macrophages with or without blocking levels of anti-CD8 mAb to confirm the source of cytokine as $CD8^+$ cells. After 72 h, supernatants were collected and tested for IFN-γ by a two-site sandwich ELISA as described in detail in Materials and Methods. This experiment was repeated three times with similar results.

were purified and cultured with infected stimulator cells, the levels of IFN-γ released were similar in normal and class II knockout mice (Fig. 2 *B*). Addition of blocking anti-CD8 mAb to the cultures reduced cytokine levels to levels stimulated by uninfected macrophages, confirming that the IFN-γ measured was indeed derived from $CD8^+$ T lymphocytes.

Priming of $CD8^+$ Cells for IFN-γ Production Depends on $CD4^+$, $NK1.1^+$ Cells and IL-2. We were surprised to find that Aβ knockout mice were able to generate effector $CD8^+$ T lymphocytes, as it is generally believed that priming of the latter cell population requires help in the form of IL-2 from MHC class II–restricted $CD4^+$ lymphocytes (6, 26–28), cells that are absent in Aβ knockout animals (10, 11). Nevertheless, a novel population of $CD4^+NK1.1^+$ T cells has been described that differentiates in dependence upon the class I–like molecule CD1 rather than classical MHC class II proteins (13, 14). While the function of these cells during infection is unknown, they have been shown to produce a number of cytokines in response to anti-CD3 stimulation (15, 16).

The above considerations prompted us to investigate the factors involved in priming CD8 cells for IFN-γ production in the absence of class II–restricted CD4 cells. Accordingly, animals were administered mAbs to CD4 and IL-2 during the ts-4 vaccination period, treatment was halted, mice were RH challenged, and survival was noted. When class II knockout animals were given mAbs to IL-2 and its receptor or, more interestingly, CD4 during vaccination, the animals showed a complete loss of resistance to challenge (Fig. 3 *A*). This contrasted with the result when anti-CD4 was given after vaccination, in which case the mAb had no significant effect (see Fig. 1 *B*). As previously shown (6), immune C57BL/6 mice given anti-CD4 but not anti–IL-2 mAbs during vaccination failed to control RH infection, and all mice died between 19 and 21 d after challenge (Fig. 3 *B*).

We next examined the ability of $CD8^+$ cells from Aβ knockout and wild-type mice to produce IFN-γ in vitro after mAb treatment during vaccination. Parasite antigen-induced cytokine production from Aβ knockout $CD8^+$ cells was blocked by in vivo administration of anti-CD4 (89% inhibition), anti-NK1.1 (78% inhibition), and anti–IL-2 (93% inhibition) (Fig. 4 *A*). These results suggest that induction of $CD8^+$ effector T lymphocytes in the knockout animals requires, in addition to parasite antigen, cells expressing CD4 and NK1.1, as well as a source of IL-2. In the wild-type situation (Fig. 4 *B*), where CD8 T lymphocyte priming occurs in the presence of class II–restricted CD4 cells, anti-CD4 mAb treatment again abrogated IFN-γ production by $CD8^+$ cells (84% inhibition). In this case,

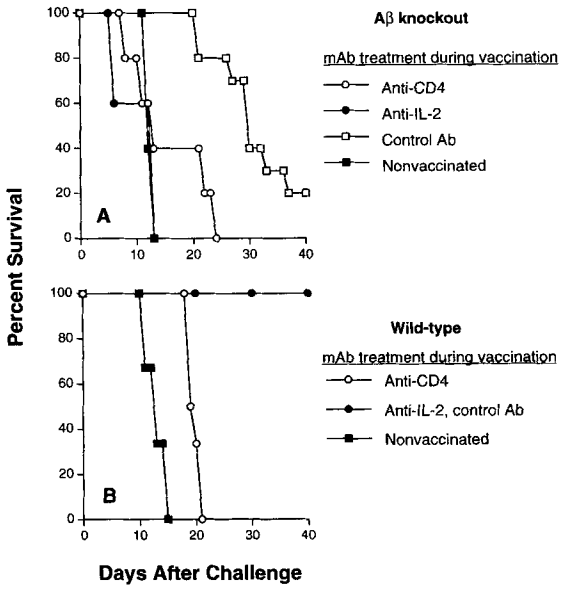

Figure 3. Induction of immunity in Aβ knockout mice requires MHC class II–independent $CD4^+$ cells and IL-2. During ts-4 vaccination, knockout (*A*) and wild-type (*B*) mice were treated with the indicated mAbs. After the final vaccination, mAb treatment was halted, and 2 wk later, animals were challenged with RH strain tachyzoites. At the time of challenge, efficacy of CD4 depletions was confirmed by FACS® analysis (0.1% for both wild-type and knockout vs. 16.3 and 1.9% in control Aβ-treated animals). The data shown were pooled from two independently performed experiments.

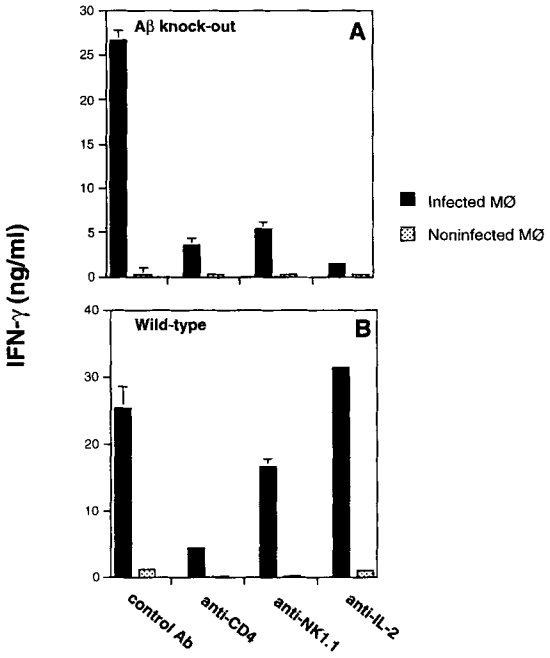

Figure 4. Priming of $CD8^+$ T lymphocytes for IFN-γ production is blocked by treatment with mAbs to CD4, NK1.1, and IL-2 during ts-4 vaccination in MHC class II knockout mice. Aβ knockout (*A*) and wild-type (*B*) animals were vaccinated and simultaneously treated with the indicated mAbs. 2 wk after the final injection, spleen cells were isolated, and $CD8^+$ lymphocytes were purified by treatment with two cycles of anti-CD4, anti-NK1.1, and anti–B cell mAbs plus complement, followed by passage over an anti-Ig T cell purification column. The latter cells (>90% $CD8^+$ as determined by FACS® analysis) were then cultured with irradiated normal and infected macrophages, and IFN-γ release was measured by two-site ELISA. This experiment was performed three times with the same result.

however, anti-NK1.1 treatment had only a partial effect (32% inhibition), and anti–IL-2 administration failed to block CD8 priming for IFN-γ production.

IL-2 Is Produced Early after ts-4 Vaccination. The data in Figs. 3 and 4 suggested that the IL-2 required for $CD8^+$ T lymphocyte priming in Aβ knockout animals was derived from cells expressing the CD4 and NK1.1 markers. Indeed, when either wild-type or knockout animals were injected intraperitoneally with ts-4, IL-2 was produced when splenocytes were restimulated in vitro with irradiated tachyzoites. The response was detectable after 4 d and reached peak levels 7 d after infection (Fig. 5 *A*). Interestingly, when mice were boosted with ts-4 on day 14 and then examined for IL-2 production 3 d later, wild-type mice produced high levels, whereas the class II knockout mice produced little of this cytokine (Fig. 5 *B*). This result suggests that two sources of IL-2 are available, one acting early in an MHC class II–independent fashion, and another (presumably conventional $CD4^+$ T lymphocytes) requiring class II molecules and acting later. Wild-type animals therefore possess both of these activities, and the Aβ knockout mice only have the early class II–independent source.

$NK1.1^+CD4^+$ T Lymphocytes Produce IL-2 in Response to Parasite Stimulation. To determine the source of early IL-2 produced in the parasite-stimulated cultures of both wild-type and knockout animals, C57BL/6 mice were infected, and 7 d later, spleen cells were isolated and treated with mAbs and complement before in vitro restimulation. Removal of either $CD4^+$ or $NK1.1^+$ cells in this manner eliminated the ability to produce IL-2 in response to tachyzoite stimulation (Fig. 6), indicating that cells expressing the latter markers are a source of IL-2 in these cultures. The observation that either anti-CD4 or anti-NK1.1 completely abolished IL-2 production suggested that a single population of cells coexpressing the latter markers were responsible for cytokine secretion. Indeed, flow cytometric analysis revealed a subpopulation of cells in both knockout and wild-type animals expressing both CD4 and NK1.1 (Fig. 7, *A* and *B*). The latter cells were present at ~0.6 and 0.9% of the total splenocyte population, or 2.4 and 19.9% of $CD4^+$ lymphocytes, in normal and knockout animals, respectively. These percentages were equivalent in both infected and noninfected Aβ knockout mice (Fig. 7 *C*) and wild-type (data not shown) mice. In contrast, we failed to see this small subpopulation when spleen cells from β2-microglobulin–negative mice were examined (Fig. 7 *D*). The latter result was expected on basis of the fact that $NK1.1^+$ T cells require CD1 for their positive selection during thymic maturation (13, 14).

To directly determine whether the double-positive cells produced cytokines after infection, spleen cells from non-

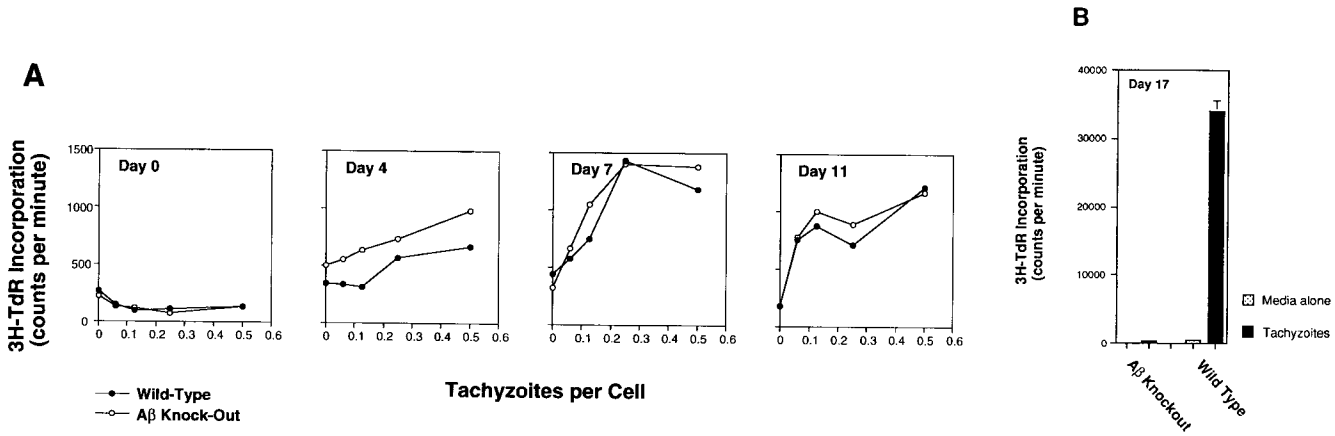

Figure 5. IL-2 is produced by spleen cells early after tachyzoite injection in normal and knockout mice. In *A*, groups of four animals were injected intraperitoneally with 2×10^4 ts-4 tachyzoites, and then at the indicated days after injection, spleens were removed from each group, and cells were cultured in the presence of irradiated tachyzoites for 24 h. IL-2 release was measured by the ability of the supernatants to stimulate proliferation (measured by [^{3}H]TdR incorporation) of IL-2–responsive CTLL cells. In *B*, after 14 d mice were reinjected with ts-4 (2×10^5 tachyzoites), and then 3 d later, spleen cells were removed and parasite-induced IL-2 production was measured.

infected and day 7 *T. gondii*–infected Aβ knockout mice were stained for CD4 and NK1.1; then highly purified double-positive cells were isolated by flow cytometric cell sorting, and gene transcripts were analyzed by RT-PCR. The results shown in Fig. 8 demonstrate that $CD4^+$ $NK1.1^+$, but not $CD4^+NK1.1^-$, T lymphocytes from infected class II knockout animals produce IL-2 mRNA, whereas as little or none was detected when cells from uninfected animals were examined. Interestingly, $CD4^+$ $NK1.1^+$ cells appeared to be downregulated for IL-4 at the same time as being upregulated for IL-2 production as a consequence of *T. gondii* infection, although the significance of this finding awaits a more detailed investigation. Thus, $CD4^+NK1.1^+$ T lymphocytes are induced to produce IL-2 in response to *T. gondii* infection, and the levels made are sufficient to lead to priming of $CD8^+$ T lymphocytes for effector function.

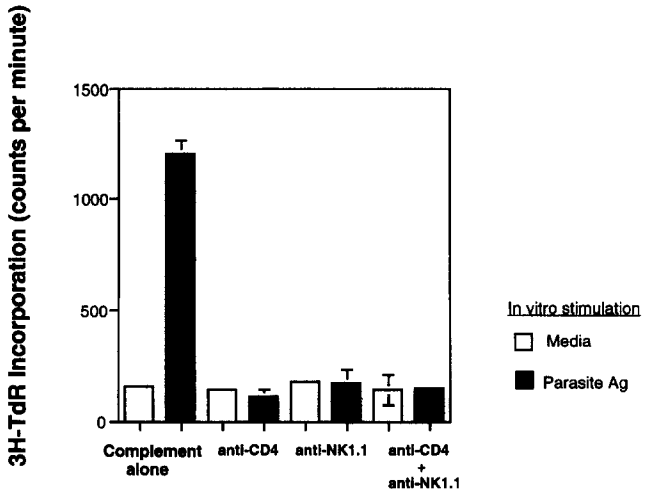

Figure 6. Early IL-2 production is abrogated by removal of either $CD4^+$ or $NK1.1^+$ cells before culture. C57BL/6 mice were injected intraperitoneally with 2×10^5 ts-4 tachyzoites. Spleen cells were isolated 7 d after injection, treated with the indicated mAbs plus complement, and cultured in the presence or absence of irradiated tachyzoites as parasite antigens. Trypan blue exclusion and FACS® analysis at culture initiation confirmed cell viability and depletion specificity. After 24 h, supernatant was collected and tested for IL-2 by a CTL bioassay. See Materials and Methods for experimental details. This experiment was repeated three times with the same result.

Discussion

The results of this study demonstrate that class II knockout mice are able to generate partial immunity to challenge with a virulent *T. gondii* strain after vaccination with an attenuated mutant. The protective response, similar to that occurring in wild-type animals, was dependent on $CD8^+$ T lymphocytes and IFN-γ. Establishment of protective immunity required IL-2 and, interestingly, $CD4^+$ cells during the vaccination period. Similarly, generation of $CD8^+$ cells capable of parasite antigen–specific IFN-γ production in vitro required IL-2, $CD4^+$, and $NK1.1^+$ cells. IL-2 secretion in vitro was detected by use of splenocytes early after ts-4 vaccination, and its production was dependent on cells expressing CD4 and NK1.1 surface molecules. Finally, FACS®-sorted $CD4^+NK1.1^+$ cells from vaccinated animals were directly shown to constitutively express IL-2 mRNA, in contrast with $CD4^+NK1.1^+$ cells from nonimmune mice.

While these results are based on negative depletion data rather than transfer of positively selected cell populations, the data nonetheless lead us to the conclusion that in Aβ knockout mice, $CD4^+NK1.1^+$ T lymphocytes serve as a source of IL-2 that primes $CD8^+$ cells for effector function. As such, our findings provide the first evidence that this novel T lymphocyte population plays an important role in resistance to infection by virtue of its ability to produce IL-2.

Recent studies have shown that the $CD4^+NK1.1^+$ population possesses several unique immunologic properties. First, MHC class II molecules are not used for their selection

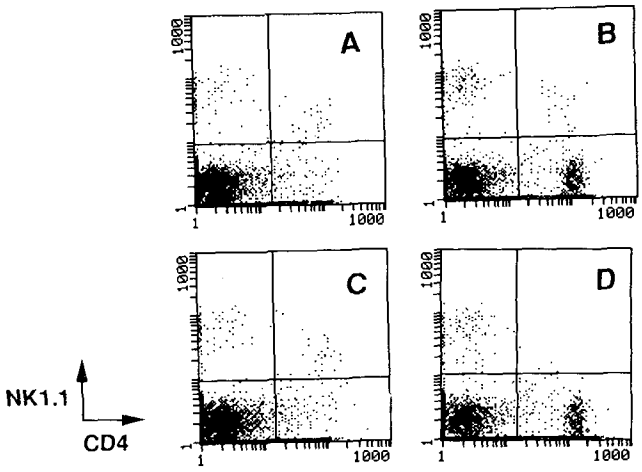

Figure 7. Presence of NK1.1$^+$CD4$^+$ T cells in spleens of wild-type and Aβ knockout mice. Individual spleens (four per group) of MHC class II knockout mice (*A*), wild-type mice (*B*), ts-4-infected knockout mice at 7 d (*C*), and β2-microglobulin knockout mice (*D*) were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-NK1.1 mAb, then examined by flow cytometric analysis. The data shown are from one representative spleen of each mouse strain.

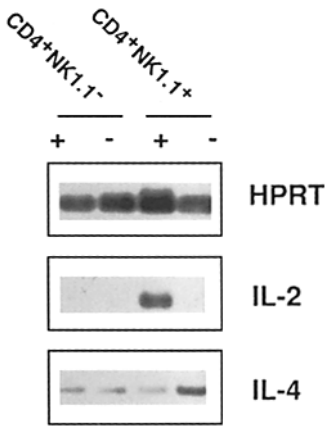

Figure 8. RT-PCR analysis of sorted cells reveals that NK1.1$^+$CD4$^+$ T cells produce IL-2 mRNA after *T. gondii* infection. T lymphocytes from noninfected (−) and 7 d infected (+) MHC class II knockout mice, purified by passage of spleen cells over an anti-Ig column, were stained with FITC-conjugated anti-CD4 mAb and PE-conjugated anti-NK1.1 mAb; then CD4$^+$NK1.1$^+$ and CD4$^+$-NK1.1$^-$ cells were collected by flow cytometric sorting. After RNA purification and reverse transcription to cDNA, mRNA for IL-2, IL-4, and HPRT was assessed by PCR followed by Southern blotting. A semiquantitative analysis of two experiments by scanning densitometry followed by normalization to HPRT revealed the following fold increases or decreases relative to cells from noninfected mice: 9.4, 11.9 (IL-2 from CD4$^+$NK1.1$^+$); 1.3, 1.9 (IL-2 from CD4$^+$NK1.1$^-$); 0.1, 0.5 (IL-4 from CD4$^+$NK1.1$^+$); and 1.2, 1.1 (IL-4 from CD4$^+$NK1.1$^-$). See Materials and Methods for details.

during thymic ontogeny, but rather this function is provided by the class I–like molecule CD1 (composed of a non–MHC-encoded heavy chain associated with a β_2-microglobulin light chain) (14). Second, they express the surface marker NK1.1, a molecule normally associated with the natural killer lineage (13, 29). Third, while CD4$^+$ NK1.1$^+$ cells express the αβ TCR, Vβ8, 7, and 2 families are over-represented (13, 16), and α chain usage is restricted to Vα14-J281 (30). Finally, T cells coexpressing CD4 and NK1.1 are able to secrete a diverse array of lymphokines, and in particular, early IL-4 production has been shown to be triggered by injection of anti-CD3 mAbs (15, 16). It has been suggested that this novel population of cells may provide the early source of IL-4 necessary to drive immune responses toward a type 2 (high IL-4/low IFN-γ) cytokine production pattern, as occurs in helminth infections (15, 31).

While NK1.1$^+$ T cells have been described as a potent early source of IL-4 (15), our data reveal that during *T. gondii* infection, cells of the same surface phenotype are upregulated for IL-2 production. Although it is possible that the NK1.1$^+$ T lymphocytes detected here are a population distinct from those previously described, this is unlikely, because the *T. gondii*–induced cells also appear to have a skewed repertoire of expressed TCR genes (Denkers, E., unpublished observations). Instead, we favor the hypothesis that these cells initially produce IL-4, but that infection drives them toward a dominant IL-2 response. In this context, it is noteworthy that *T. gondii* is also a potent early inducer of macrophage IL-12, which selectively drives type 1 responses (21, 24, 32). In addition, the parasite contains a superantigen activity that can induce IFN-γ directly from naive CD8 cells (18). Thus, in the case of *T. gondii* infection, cytokines inducing both type 1 and type 2 responses would be expected early on, but the immunomodulatory effects of the former appear to cross-regulate the latter. Support for this view comes from the observation that during early infection with the cyst-forming strain ME49, IL-4 is one of the first cytokines induced but subsequently disappears (33). Moreover, in IFN-γ knockout mice, IL-4 is overproduced during early ts-4 and ME49 infection (Scharton-Kersten, T., manuscript in preparation).

Previous work from our laboratory indicates that mAb depletion of CD4$^+$ cells in wild-type mice abrogates the ability to generate CD8$^+$ immunity (6). The results of the present study show that in the absence of MHC class II–restricted CD4$^+$ lymphocytes, T helper function is provided by nonconventional CD4$^+$ cells through the production of IL-2. However, in normal mice, depletion of IL-2 with mAb had no effect on the ability to generate CD8$^+$ immunity. While it is possible that the levels of IL-2 produced in normal mice are high enough that mAb neutralization is incomplete, this would seem unlikely, given that the mice were given a combination of mAbs to the IL-2 receptor and the cytokine itself. In addition, while it is formally possible that wild-type mice produce neutralizing antibodies to the anti-cytokine mAb, this would seem unlikely, because the other rat mAbs given were highly effective in the wild-type mice used in this study. A more interesting possibility is that an alternative source of priming lymphokine is available in MHC class II–positive mice, most likely produced in dependence on CD4$^+$ T lymphocytes. In this regard, it is noteworthy that IL-15, a cytokine with many of the properties of IL-2, is induced during murine *T. gondii* infection (22).

We previously examined the response of MHC class I–negative mice to ts-4 vaccination and RH challenge (34). In that study, and similar others (35), an overexpansion of protective IFN-γ-producing NK cells, induced as a result of infection, appears to compensate for genetic loss of IFN-γ–producing CD8$^+$ lymphocytes. It was of interest to

note in the present study that lack of MHC class II–restricted $CD4^+$ T lymphocytes, another major source of IFN-γ during *T. gondii* infection (6, 21, 36), is not sufficient to induce overexpansion of NK cells. These data lend support, although somewhat indirect, to the hypothesis that $CD8^+$, but not classical $CD4^+$, T lymphocytes are involved in downregulation of NK cell responses during infection of normal mice (35, 37, 38).

The molecular basis for activation of $CD4^+NK1.1^+$ T lymphocytes is presently not understood, but the class I–like molecule appears to be involved (39). In addition, the restricted repertoire of TCR genes expressed by $CD4^+$ $NK1.1^+$ T cells suggests a limited capability of recognizing antigen (13, 14, 30, 40). Although CD1 is able to present certain peptides, as well as nonconventional lipid Ag, to mouse and human T cells (41–43), the molecule alone appears to be sufficient to activate $NK1.1^+$ T cells (14, 44). Thus, while the CD1 molecule is expressed at low levels in the spleen (14), its controlled upregulation could be the basis for $NK1.1^+$ T cell activation. Indeed, preliminary evidence from our laboratory suggests that *T. gondii* infection induces elevated expression of CD1 in infected mice (Denkers, E., unpublished observations).

The finding that *T. gondii* infection triggers $NK1.1^+$ T lymphocytes during early infection provides important clues regarding the role of these cells and their regulation in microbial pathogenesis. While these cells were initially described as an early IL-4 source involved in type 2 response initiation, our results show that this is not their sole function, that in the absence of class II–restricted $CD4^+$ cells, they are crucial for induction of $CD8^+$ T cell responses. The *Toxoplasma* murine model should provide a valuable system for analyzing both the molecular basis of microbial recognition by $CD4^+NK1.1^+$ T lymphocytes and the events involved in their triggering.

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