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Host Response to Sendai Virus in Mice Lacking Class II Major Histocompatibility Complex Glycoproteins

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The development of Sendai virus-specific cytotoxic T-lymphocyte (CTL) effectors and precursors (CTLp) has been compared for mice that are homozygous (−/−) for a disruption of the *H-2I-A*^{b} class II major histocompatibility complex glycoprotein and for normal (+/+) controls. The generation of CD8$^+$ CTLp was not diminished in the −/− mice, though they failed to make virus-specific immunoglobulin G class antibodies. While the cellularity of the regional lymph nodes was decreased, the inflammatory process assayed by bronchoalveolar lavage (BAL) of the pneumonic lung was not modified, and potent CTL effectors were present in BAL populations recovered from both groups at day 10 after infection. There was little effect on virus clearance. Production of interleukin-2 by both freshly isolated BAL inflammatory cells and cultured lymph node cells was greatly diminished, though the −/− mice still made substantial levels of gamma interferon. However, treating the mice with a single dose of a monoclonal antibody to this cytokine, at least some of which is made by CD8$^+$ T cells, did not decrease CTLp frequencies. As found previously with CD4-depleted *H-2*^{b} mice, the development of Sendai virus-specific CD8$^+$ T-cell-mediated immunity is not compromised by the absence of a concurrent class II major histocompatibility complex-restricted response.

The idea that CD4$^+$ helper T (Th) cells are required to promote the virus-specific CD8$^+$ cytotoxic T-lymphocyte (CTL) response is based on many observations that primed CD8$^+$ CTL precursors (CTLp) are not driven to proliferate and to develop CTL effector function under bulk culture conditions unless the Th set, or compensatory growth factors (particularly interleukin-2 [IL-2]), are also present (36). This reflects the substantial cytokine production characteristic of CD4$^+$ Th cells (33). However, it is now apparent that effector CD8$^+$ lymphocytes also make most lymphokines (26, 28), though generally at a lower level.

The CD8$^+$ CTL recognize viral peptide presented in the context of class I major histocompatibility complex (MHC) molecules (*H-2KDL* in the mouse), while the CD4$^+$ set is targeted to peptide+class II MHC glycoprotein (*H-2I*) (5, 41). The first in vivo analysis (2) of Th cells for virus-specific CD8$^+$ CTL used this divergence in MHC restriction phenotype: *H-2I*^{d} *D*^{d} T cells were filtered through irradiated (950 rad) *H-2I*^{b} *D*^{d} mice to remove Th precursors reactive to the allogeneic *H-2I*^{b} class II MHC glycoprotein and then stimulated with vaccinia virus in a further set of irradiated *H-2I*^{b} *D*^{d} recipients. The absence of a class II MHC-restricted T-cell response (in the context of *H-2I*^{d}) did not in any way compromise the development of the *H-2D*^{d}-restricted primary virus-specific CD8$^+$ CTL response. The experiment contradicted ideas prevalent at that time and, because of the limitations of the then current (in 1978) technology, was not taken further.

The issue was reopened when monoclonal antibodies (MAbs) to T-cell subsets became readily available (29, 32, 34). Essentially the same result, an absence of a requirement for CD4$^+$ Th cells to promote the CD8$^+$ CTL response, was found following in vivo excision of CD4$^+$ T cells in many (but not all) virus systems (reviewed in reference 11). This protocol showed clearly that Sendai virus-specific CTL and CTLp are readily generated in CD4-depleted C57BL/6J (B6; *H-2*^{b}) mice, though the response is somewhat diminished in CBA/J (*H-2*^{k}) mice treated in the same way (12, 19). The caveat is that the "immunosurgery" may not have eliminated all of the CD4$^+$ T cells, perhaps because some have down-regulated cell surface expression of the molecule. Mice that are homozygous (−/−) for disruption of the CD4 gene generate CD4$^-$ CD8$^-$ lymphocytes that can act as helpers (31).

A third approach to the CD4-CD8 help problem has recently been provided by manipulated *H-2*^{b} mice that are −/− for the *H-2I-A*^{b} gene (6, 17). These class II MHC −/− animals have small numbers of CD4$^+$ T cells that do not seem able to provide Th function for B-cell responses. The CD8$^+$ CTL response to an influenza A virus was slightly delayed (4), in accord with previous experiments with CD4-depleted +/+ mice (1). A more quantitative analysis using limiting dilution analysis (LDA) approaches has shown substantial diminution in the generation of influenza virus-specific CTLp (40a). We describe here the characteristics of the host response to Sendai virus in these class II MHC −/− mice.

MATERIALS AND METHODS

Mice. The founding stock for the class II MHC −/− *H-2*^{b} B6 mice was supplied by GenPharm International, Mountain View, Calif., and the mice are bred under license at St. Jude Children's Research Hospital. The conventional B6 +/+ controls were purchased from the Jackson Laboratory, Bar Harbor, Maine. All animals are maintained, other than for infection with Sendai virus, under specific-pathogen-free conditions.

Virus infection and T-cell depletion. Anesthetized mice were infected intranasally (i.n.) with 200 50% egg infective doses (EID_{50}) of the Ender's strain of Sendai virus (19). The virus was titrated in embryonated hen eggs, using a standard protocol. In the first experiment, the host response was compared for −/− mice and +/+ controls that had or had not been depleted in vivo by treatment with the GK1.5 MAb to CD4.

Sampling, culture, and cytotoxicity. Mice were anesthetized, bled from the axilla, and then lavaged three times via the trachea to obtain the bronchoalveolar lavage (BAL) population (1, 19). Experiments were generally done with pooled cells from three to seven mice per time point. Except when they were used for the single-cell ELISPOT assays to detect cytokine or immunoglobulin (Ig)-producing cells (21, 22, 38, 39), the BAL cells were adsorbed on plastic to remove most of

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TABLE 1. Quantitation of virus-specific $CD8^+$ CTLp

Expt	Days after infection[a]	Source of cells	Reciprocal of CTLp frequencies[b]		
			+/+	$CD4^{-}$[c]	−/−
1	5	MLN	21,700	104,800	195,600[d]
	7	MLN	9,100	2,340[d]	4,320
		BAL	1,590	3,700	<10[6d]
	10	MLN	9,120	3,890[d]	1,530[d]
		Spleen	19,100	3,680[d]	10,310[d]
		BAL	207	198	>26[d]
	13	MLN	8,200	1,530[d]	458[d]
		Spleen	3,640	3,520	1,870
		BAL	117	60	>26
2	7	MLN	40,316		43,523
	10	MLN	15,294		926[d]
		Spleen	7,527		70,214[d]
		BAL	184		351
	14	MLN	4,030		1,671
		Spleen	7,773		6,840
	18	MLN	3,396		300[d]
		Spleen	6,276		12,256

[a] Cell counts and phenotypes for experiment 1 are shown in Fig. 1; the infection protocols is described in the legend to Fig. 1.

[b] CTLp frequencies were determined by LDA using Sendai virus-infected MHC class I^+ II^- MC57G cells. The frequencies in normal MLN or spleen are <500,000, and the values for microcultures assayed (after well split) on uninfected MC57G targets are of the same order. The LDAs on the BAL populations were done with nonadherent cells.

[c] Some of the +/+ mice were depleted of $CD4^+$ T cells by MAb treatment (see Fig. 1).

[d] Significantly different ($P < 0.005$ or <) from the values for the +/+ mice.

the macrophages. Single-cell suspensions of mediastinal lymph node (MLN) and spleen were not subject to this depletion step. The protocol for LDA to detect CTLp, which has been described previously (18), involves 7-day culture in microwells with irradiated, virus-infected stimulators and the addition of 10 U of recombinant IL-2 per ml. Microcultures causing three times the standard deviation in specific ^{51}Cr release for a 6-h cytotoxicity assay with virus-infected MHC class I^+ II^- MC57G target cells were scored as positive. The addition of recombinant IL-2 optimizes this in vitro system, but the frequency of the CTLp detected depends on the extent of clonal expansion in vivo. Bulk cultures also followed a well-established protocol, again with 10 U of added recombinant IL-2 per ml, and CTL activity was expressed for both cultured lymphocytes and freshly isolated BAL cells as specific ^{51}Cr release from virus-infected MC57G targets.

Cytokine and antibody assays. Levels of secreted cytokine in culture supernatants (or virus-specific antibody in serum) were assayed by enzyme-linked immunosorbent assay (ELISA) as described previously (38, 39). Freshly isolated cells producing secreted protein were quantified as spot-forming cells (SFC) per 10^4 cells for the cytokines (37–39) and antibody-forming cells (AFC) per 10^5 cells for the plasma cells (21, 22), using variants of the ELISPOT technique (24). Cytokine levels were determined as units per milliliter relative to standard curves, and the results shown are for maximum levels detected at 24, 48, or 72 h after in vitro stimulation with irradiated, virus-infected stimulators (38).

Flow cytometry and fluorescence-activated cell sorting (FACS). Lymphocytes were stained with conjugated MAbs (Pharmingen, San Diego, Calif.) and analyzed for cell surface phenotype in two-color mode by using a FACScan or separated by using a FACStar Plus (Becton Dickinson, Palo Alto, Calif.) as described previously (18, 19).

RESULTS

The $CD8^+$ T-cell response. The $CD8^+$ set in the −/− mice has developed in the absence of possible $CD4^+$ help and may thus be functionally skewed to compensate for this defect (10). A contemporary comparison was thus made of the generation of virus-specific $CD8^+$ CTLp in −/− and +/+ mice, some of which were depleted of $CD4^+$ T cells (1, 18) with MAb GK1.5 (Table 1 experiment 1). The cellularity of the regional lymph node (MLN) was less for the CD4-depleted ($CD4^-$) and −/− mice after day 5 (Fig. 1), and given that the prevalence of $B220^+$ lymphocytes did not change (Fig. 2), B-cell recruitment

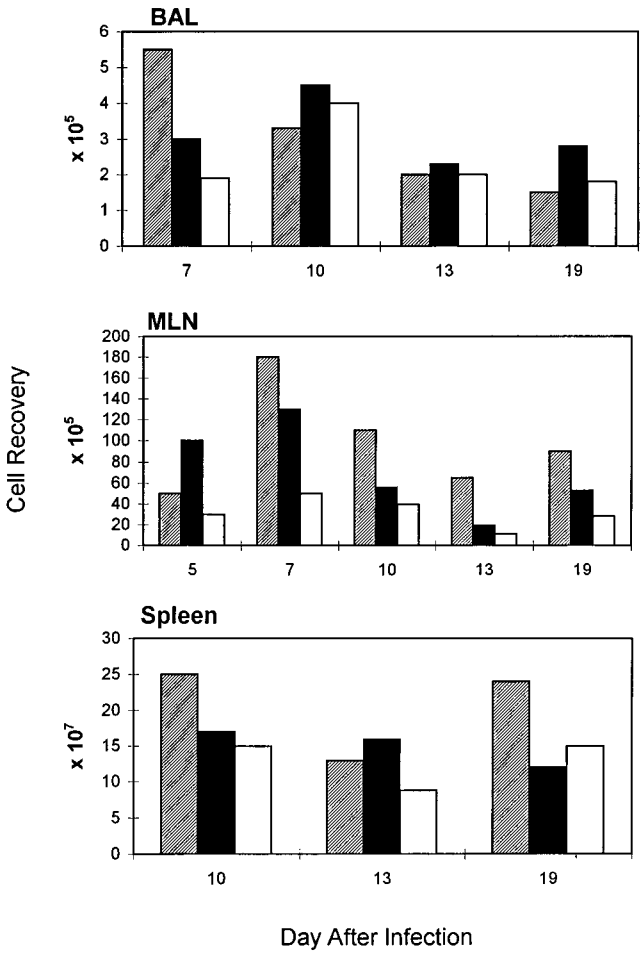

FIG. 1. Cellularity of the BAL, MLN, and spleen for the conventional (hatched bars), CD4-depleted (solid bars), and class II MHC −/− (open bars) groups for mice infected i.n. with 200 EID_{50} of Sendai virus on day 0. This is the experiment shown in Table 1, experiment 1.

was thus reduced. The numbers of cells in the BAL of the $CD4^-$ and −/− mice were lower on day 7, but there was no difference by day 10 (Fig. 1). Subsequent to day 7, $CD8^+$ T-cell recruitment to the BAL was, if anything, enhanced in mice lacking $CD4^+$ T cells (Fig. 1 and 2). As described previously for uninfected −/− mice, small numbers of lymphocytes expressing CD4 were found consistently in the MLN (Fig. 2), though this was not apparent for the +/+ animals that were treated with the MAb to CD4.

Generation of virus-specific $CD8^+$ CTLp may have been slightly delayed at the onset of the response in the mice lacking $CD4^+$ T cells, as the numbers were lower than for the +/+ MLN taken on day 5, and CTLp were not found in the BAL of the −/− mice on day 7 (Table 1, experiment 1). Previous experiments with conventional B6 mice also showed that CTLp are first found in the MLN on day 5 and the BAL on day 7. There was clearly no decrease in CTLp frequency for all sites assayed on days 10 and 13, while in the BAL and MLN, there seemed to be enrichment of the virus-specific CTLp in the −/− mice (Table 1, experiment 1). In a repeat experiment, comparison of CTLp numbers indicates that the equilibration in CTLp frequencies between MLN and spleen may have been somewhat delayed for the −/− mice sampled on day 10, while the patterns were generally similar to those found in the initial

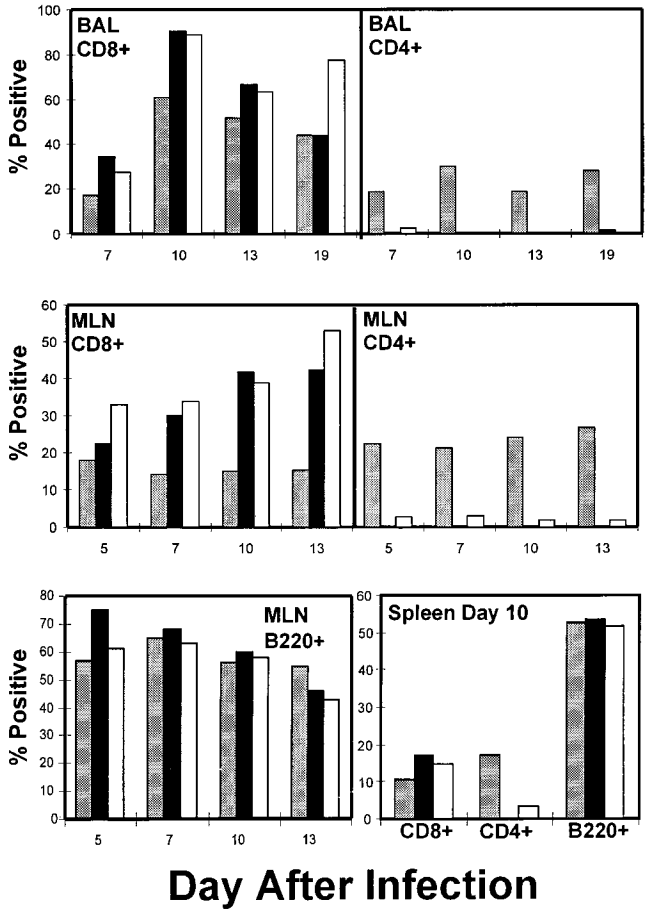

FIG. 2. Phenotypes of lymphocytes recovered from the conventional (hatched bars), CD4-depleted (solid bars), and −/− (open bars) mice for MLN and BAL samples from the experiment described in Fig. 1 and Table 1, experiment 1.

experiment for the later time point (Table 1, experiment 2). Furthermore, the levels of virus-specific CTL activity for freshly isolated day 10 BAL cells (Fig. 3a) and for day 7 MLN and spleen populations that were stimulated in bulk culture (Fig. 3b) were comparable for the +/+ and −/− mice. Thus, as

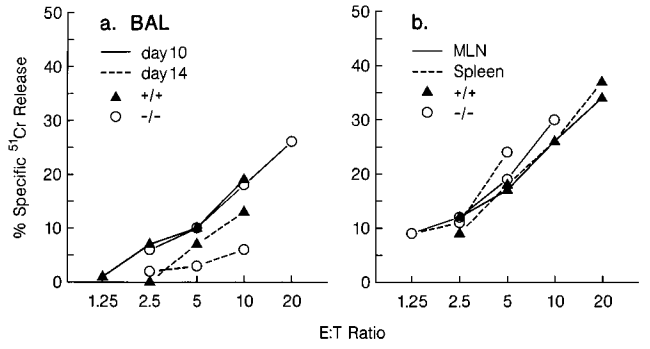

FIG. 3. Level of virus-specific CTL activity for freshly isolated BAL cells recovered at 10 or 14 days after i.n. infections with 200 EID_{50} of Sendai virus (a) or for day 7 spleen or MLN cells that were cultured in vitro for 6 days (b). The results are specific ^{51}Cr release from Sendai virus-infected MHC class I^+ II^- MC57G cells. Assay on normal MC57G targets caused <3% lysis. E:T, effector/target.

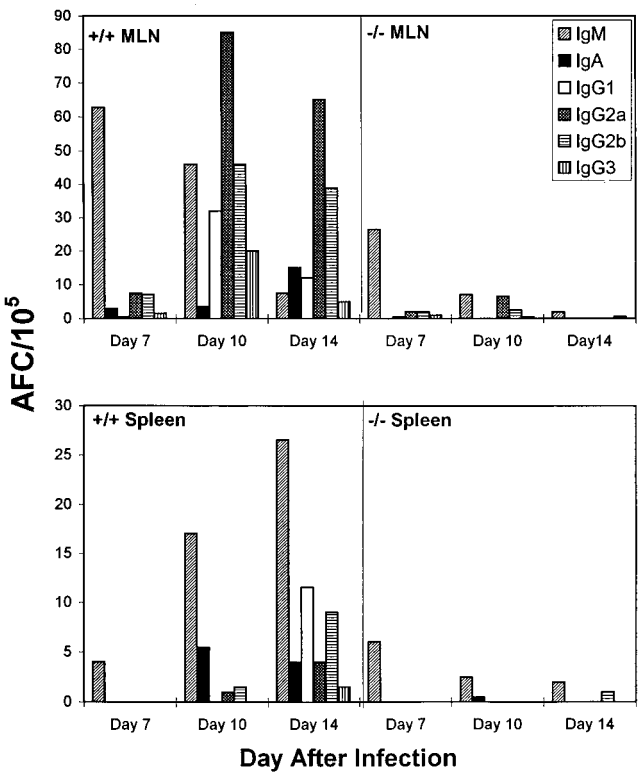

FIG. 4. Virus-specific antibody response determined by ELISPOT analysis with detergent-disrupted virus coated onto the assay plates for the MLN and spleens of +/+ and −/− mice infected i.n. with Sendai virus 7, 10, and 14 days previously. The results are expressed as AFC per 100,000 cells. Comparable samples from normal mice show values of <2 when assayed in this way.

found in an earlier study with $CD4^-$ mice, there was no significant diminution of either effector or precursor CTL development for class II MHC −/− mice infected i.n. with Sendai virus.

Virus clearance and the antibody response. Comparison of virus clearance for homogenized, lightly centrifuged lung samples showed that three of three mice from both the +/+ and −/− groups were positive on day 7, two of three (only at a 10^{-0} dilution) from the +/+ group and three of three (only at a 10^{-1} dilution) from the −/− group were positive on day 10, and no virus was recovered from any of the six mice sampled on day 14. This variant of Sendai virus does not cause productive infection in sites other than respiratory epithelium (19).

The AFC response in the −/− mice was almost exclusively a property of IgM-producing cells and decreased rapidly after day 7 (Fig. 4). The small numbers of IgG2a and IgG2b AFC seen on days 10 and 14 in the MLN and/or spleen may be reflected in the low levels of virus-specific Ig of these isotypes in serum on day 14 (Table 2), but evidence for persistence of this minimal response was not apparent by day 42 (Table 2). Thus, the results are consistent with a complete absence of CD4 help for B cells in the class II MHC −/− mice.

Profiles of cytokine production. ELISPOT analysis of freshly isolated BAL cells on day 10 showed a substantial drop in the prevalence of IL-2 producers for the −/− mice (Table 3, experiment 1). Otherwise, all of the cytokines that were assayed were being made by significant numbers of cells. Restimulation in vitro (Table 3, experiments 2 to 4) confirmed the defect in IL-2 production and showed that FACS-separated $CD8^+$ T cells (Table 3, experiment 3) from both the +/+ and −/− mice

TABLE 2. Virus-specific serum antibody response

Days post-infection[a]	Mice	Reciprocal ELISA titer for indicated antibody isotype						
		Total	IgM	IgA	IgG1	IgG2a	IgG2b	IgG3
14	+/+	5,000	900	0	2,000	3,000	3,500	800
	−/−	200	<100	0	0	200	200	<100
42	+/+	3,500	150	0	1,000	2,000	2,500	200
	−/−	<100	<100	0	0	<100	<100	<100
	+/+	3,000	100	0	1,500	1,500	3,000	350
	−/−	<100	<100	0	0	<100	<100	<100

[a] Days after i.n. infection with 200 EID_{50} of Sendai virus.

could make some gamma interferon (IFN-γ). Furthermore, equivalent amounts of IL-2 and IFN-γ were produced by −/− MLN cells that were obtained on day 10 and restimulated in vitro, with or without prior removal (by FACS) of the few $CD4^+$ T cells (Fig. 2) that are present in these mice (data not shown).

The most prominent cytokine throughout was IFN-γ (Table 3). Treating mice with XMG1.2 MAb to IFN-γ has been shown to modify profiles of Ig class switching and alter cytokine-related susceptibility patterns in parasitic and viral infections (14, 30, 39). However, IFN-γ-producing cells were still present on subsequent culture of MLN cells from Sendai virus-infected −/− mice treated with MAb XMG1.2 (as found previously with influenza virus [39]), and there was no major switch to the production of Th2 cytokines. Much the same was found in this study for both the −/− and +/+ mice that were given anti-IFN-γ and Sendai virus (Table 3, experiment 4). Also, $CD8^+$ CTLp frequencies in the MLN and spleens of treated and untreated −/− mice were comparable at day 9 after infection (Table 4).

DISCUSSION

Though lymphokines that are normally produced in substantial amounts by $CD4^+$ Th cells are essential to drive virus-specific $CD8^+$ memory CTLp to develop CTL effector function under bulk culture conditions, this requirement has not always been apparent for $CD8^+$ T cells stimulated in the microcultures used for LDA (35). Similarly, the primary, in vivo $CD8^+$ CTL response to many viruses, including Sendai virus, does not require concurrent involvement of the $CD4^+$ subset (11, 19). A likely explanation is that the smaller amounts of lymphokine that are produced by $CD8^+$ T cells are sufficient to drive both proliferation and differentiation when lymphocytes and stimulator cells are tightly packed together in the greatly distended (four to five times normal) responding lymph node, or in a round-bottom microculture well, but are diluted in a larger volume of culture fluid.

The other factor is that many cytokines that are made in vivo and could potentially influence $CD8^+$ T-cell differentiation (8, 9) may either be missing from the in vitro cultures or be present in much lower concentrations. Cytokines such as IL-6 are made by epithelial and vascular cells (40), which will be discarded in the processing of lymphoid tissue. Also, it is very likely that regulatory molecules that are not yet discovered may play a part in the development of $CD8^+$ T-cell responses (15). Furthermore, the possible interactive effects between known cytokines are imperfectly understood, especially when we consider that there is no information on levels of locally available cytokines in responding lymphoid tissue. The fact that priming with peptide in Freund's adjuvant induces the development of memory $CD8^+$ CTLp (25) shows clearly that any help that might be required is simply a function of cytokines that contribute to the development of an activated environment (7) in lymphoid tissue and does not need to be virus specific.

The continued presence of IFN-γ^+ cells in the lymph nodes and pneumonic lungs of the Sendai virus-infected −/− mice is not surprising, as IFN-γ is clearly made both by $CD8^+$ T cells and by natural killer cells. We could not show that this cytokine is driving the generation of virus $CD8^+$ CTLp, though it is the only product detected in substantial amounts in the −/− mice. Similarly, IFN-γ −/− mice developed a normal CTL response to influenza virus (16). However, we have not tested for all known lymphokines, some of which, particularly IL-7 (27) or IL-15 (15), could well be involved.

The finding that there are almost normal numbers of IL-4- and IL-5-secreting inflammatory cells in the BAL of the −/− mice is in accord with a previous study (37) showing the per-

TABLE 3. Cytokine profiles in the BAL and MLN[a]

Expt	Source of cells	Mouse strain	Cell enrichment	Anti-IFN-γ[b]	Cytokine level					
					IFN-γ	IL-2	IL-4	IL-5	IL-6	IL-10
1	BAL, day 10	+/+		−	206	180	318	224	141	206
		−/−		−	526	16	280	367	125	166
2	MLN, day 7	+/+		−	45.5	27.1	0	0	0.6	7.1
		−/−		−	56.9	1.8	0	0	0.9	1.6
3	MLN, day 7	+/+		−	50.2	19.1	0	0	0.4	7
		−/−		−	33.4	1.6	0	0	0.4	0
		+/+	$CD8^+$[c]	−	6.2	3	0	0	0	0.8
		−/−	$CD8^+$	−	3.4	0	0	0	0	0
4	MLN, day 10	+/+		−	70.3	12.3	0	0	1.3	2.5
				+[c]	73.6	12.8	0	2.7	1.2	4
		−/−		−	53.6	0	0	0	0	0
				+	29.7	0	0	0	0.8	2

[a] Freshly isolated BAL cells were assayed by the ELISPOT technique; the data are presented as SFC per 10,000 cells. MLN cells were restimulated in vitro, and cytokine levels in culture fluid were determined by ELISA; the data are expressed as units per milliliter.

[b] The mice were treated in vivo with the XMG1.2 MAb to IFN-γ (see Table 4).

[c] Lymphocytes were stained with phycoerythrin-conjugated CD4 cells and fluorescein isothiocyanate-conjugated CD8 cells; the $CD8^+$ cells were enriched by FACS and then cultured.

TABLE 4. CTLp frequencies in mice treated with anti-IFN-γ[a]

Mouse strain	Source of cells	Anti-IFN-γ[b]	% $CD8^+$	Reciprocal of CTLp frequency
+/+	MLN	−	10.4	2,252
		+	9.8	1,169
−/−	MLN	−	42.8	>399
		+	19.3	399
+/+	Spleen	−	5.2	2,337
		+	6.0	2,771
−/−	Spleen	−	14.7	1,692
		+	12.8	798

[a] Pooled samples from three mice were tested at 9 days after i.n. infection with Sendai virus.

[b] Mice were given an intraperitoneal dose of 2 mg of the XMG1.2 MAb to IFN-γ, or phosphate-buffered saline, 1 day prior to infection. None of the LDA values for treated and untreated mice were significantly different.

sistence of this cytokine production profile in the BAL of influenza virus-infected +/+ mice depleted (with MAbs) of the $CD4^+$ and/or $CD8^+$ subsets. Also, it is now clear that $CD8^+$ T cells can be induced to make what are generally regarded as Th2 cytokines (28). We had thought that the IL-4 and IL-5 might be a product of class II MHC-restricted $CD4^-$ $CD8^-$ T cells (1, 37), but this cannot apply to the present results. In the influenza virus model, transcripts for IL-5 (but not IL-4) have been demonstrated in cells that engulf substantial numbers of latex particles and are thus likely to be macrophages (37). Non-T cells have been shown to produce IL-4 following cross-linking of Fc receptors (3).

The present results establish that class II MHC-restricted $CD4^+$ Th cells are necessary to promote the virus-specific antibody response. However, neither the development of the inflammatory process in the BAL of B6 mice infected with Sendai virus nor the differentiation and proliferation of $CD8^+$ effectors requires the concurrent presence of the $CD4^+$ subset. Even so, we did see indications of delayed generation of $CD8^+$ CTLp in CD4-depleted CBA/Ca mice infected with this virus (12). Similarly, the concurrent involvement of $CD4^+$ Th cells was required to promote the primary $CD8^+$ T-cell response to a herpesvirus (23). This could reflect the fact that the $CD8^+$ cells in the particular mouse strains infected with these viruses do not make sufficient lymphokine and/or that cytokine production is not induced in (for example) epithelial cells and stromal cells. Thus, there are exceptions, but absence of class II MHC-restricted $CD4^+$ Th cells is often associated with the generation of a substantially normal $CD8^+$ T-cell response (11). Intercurrent viral infections tend not to be a particular problem for CD4-depleted people infected with human immunodeficiency virus until $CD8^+$ T-cell numbers also start to fall (13).

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