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The Cytotoxic T-Lymphocyte Response to Sendai Virus Is Unimpaired in the Absence of Gamma Interferon

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Sendai virus is eliminated from the respiratory tract of gamma interferon (IFN-γ) −/− BALB/c mice with normal kinetics. The level of virus-specific cytotoxic T-lymphocyte (CTL) activity in the cell population recovered by bronchoalveolar lavage is unimpaired, the prevalence of interleukin-4 (IL-4)-producing cells is increased, and the titers of virus-specific immunoglobulins IgG1 and IgG2b are higher in the IFN-γ −/− mice. The emergence of this T-helper 2 response profile in both lymphoid tissue and the pneumonic lung has no obvious deleterious consequences. Virus clearance is slightly delayed following depletion of the $CD4^+$ subset, with the effect being similar in magnitude for IFN-γ −/− and +/+ mice. However, the generation of CTL precursors (CTLp) is diminished in the IFN-γ −/− (but not +/+) mice in the absence of concurrent $CD4^+$ T help. Apparently the clonal expansion of the CTLp population can be promoted either by a cytokine (perhaps IL-2) produced by the IFN-γ −/− $CD4^+$ T cells or by IFN-γ made by other cell types in the +/+ mice.

The gamma interferon (IFN-γ) response is a prominent feature of most virus infections (7, 27). The biological effects of this cytokine can be diminished by in vivo treatment with an appropriate neutralizing monoclonal antibody (mAb) or eliminated completely by using mice homozygous for disruption of the IFN-γ or IFN-γ receptor gene (−/− mice) (6, 25, 27). Somewhat surprisingly, the lack of IFN-γ throughout the course of the infectious process may not obviously modify the overall severity of most virus diseases. Virus clearance tends to occur with normal (or slightly delayed) kinetics, though early, antigen nonspecific resistance mechanisms mediated via nitric oxide synthase may be diminished (13) and $CD4^+$ T cells may switch to more of a T-helper 2 (Th2) response profile (11, 25). The functional deletion of IFN-γ has also been found to have comparatively little effect on the development of cytotoxic T-lymphocyte (CTL) effectors specific for influenza virus, the poxviruses, and lymphocytic choriomeningitis virus (11, 18, 41).

The spectrum of IFN-γ production following intranasal (i.n.) infection with the murine type 1 parainfluenza virus, Sendai virus, has been compared previously (16) for normal C57BL/6J (+/+) and congenic major histocompatibility complex (MHC) class II (*H-2IA^b*) −/− mice (12). The conclusion was that most of the IFN-γ detected following in vitro restimulation of Sendai virus-primed spleen and lymph node populations is normally produced by $CD4^+$ T cells (16). However, analysis of freshly isolated bronchoalveolar lavage (BAL) cells by the single-cell enzyme-linked immunosorbent assay (ELISA) spot (ELISPOT) technique indicated that the numbers of IFN-γ-producing inflammatory cells in the lungs of the $CD4^+$ T-cell-deficient MHC class II −/− mice were equivalent to those in the +/+ controls and that $CD8^+$ cells recovered from the mediastinal lymph nodes (MLN) of these mice produced substantial amounts of IFN-γ following in vitro restimulation (16). Treating these MHC class II −/− mice with MAb XMG1.2 to IFN-γ (4) had no obvious effect on the Sendai virus-specific $CD8^+$ T-cell response (16).

Despite the fact that the in vivo administration of MAbs to IFN-γ can profoundly modify the pathogenesis of parasitic infections (21), this neutralization approach has the limitation that there may be no reduction in the numbers of IFN-γ-producing cells detectable by secondary stimulation in culture or by direct ELISPOT analysis of BAL populations from mice with viral pneumonia (16, 32). This finding raises the possibility that IFN-γ may still be functioning by short-range secretion following the cognate, T-cell receptor-mediated binding of virus-immune T cells (24) to antigen-presenting stimulator or target cells. Direct interaction with Sendai virus-infected respiratory epithelium is known to be essential for the clearance of this virus by $CD8^+$ effectors (14). The present experiments thus focus on the characteristics of the $CD8^+$ T-cell response in IFN-γ −/− BALB/c mice with Sendai virus pneumonia.

MATERIALS AND METHODS

Mice. The IFN-γ −/− mice (6) were supplied as heterozygotes (+/−) backcrossed to the BALB/c background by Peggy Woods (Genentech Inc., South San Francisco, Calif.) and then bred at St. Jude Children's Research Hospital to give lines of IFN-γ −/− and +/+ BALB/c mice. We found that there was no difference in the response profiles for the +/+ mice and conventional BALB/c mice purchased from the Jackson Laboratory (Bar Harbor, Maine), and so (for reasons of economy) the +/+ controls derived from the original +/− stock were not maintained in the long term. All mice were held and bred in the Animal Resource Center at St. Jude Children's Research Hospital under specific-pathogen-free conditions until used at 8 to 10 weeks of age. The status of the IFN-γ gene was monitored by PCR of mouse tail genomic DNA, using a protocol provided by Genentech. Briefly, mouse cDNA was isolated and amplified by PCR using two pairs of primers: 5′TCAGCGCAGGGGGCGCCCGGTTCTTT3′ and 5′ATCGACAAGACCGGCTTCCAC3′ for the *neo* gene; and 5′AGAAGTAAGTGGAAGGGCCCAGAAG3′ and 5′AGGGAAACTGGGAGAGGAGAAATAT3′ for the IFN-γ gene. The PCR cycling parameters with a DNA Thermal Cycler (Perkin-Elmer Corp., Norcross, Ga.) were an initial cycle at 94°C for 6 min to denature the cDNA, then 55°C for 2 min for annealing, followed by 72°C for 1 min for extension, for a total of 30 to 40 cycles.

Infection and sampling. Mice were infected i.n. with 200 50% egg infective doses (EID_{50}) of the Enders strain of Sendai virus (15, 28) under Avertin (2,2,2-tribromoethanol) anesthesia (1). The mice were anesthetized again at the time of sampling (1) and bled from the axilla. The inflammatory cell population was recovered by BAL, and the cervical lymph nodes (CLN) and MLN were removed and dissociated into single-cell suspensions for functional analysis and immunophenotyping (15, 22, 23). The lungs were frozen, and virus titers were determined later in supernatants from lung homogenates by endpoint titration in embryonated hen eggs (15).

Antibody treatment and immunophenotyping. Groups of IFN-γ −/− and +/+ mice were depleted of $CD4^+$ cells by intraperitoneal inoculation with 0.5 ml of

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TABLE 1. Virus titers in the lung[a]

Day	Mean virus titer ($\log_{10}$ EID_{50}) ± SEM			
	+/+		−/−	
	Intact	$CD4^-$	Intact	$CD4^-$
6	6.6 ± 0.1	ND	5.9 ± 0.4	7.2 ± 0.3
8	4.4 ± 0.8	ND	3.8 ± 1.0	5.8 ± 0.6
10	0	2.1 ± 0.5	0	0.6 ± 0.3
14	0	0.3 ± 0.2	0	0

[a] The mice were infected i.n. with 200 EID_{50} of Sendai virus. The results are titers determined in 100 μl of lung homogenate for groups of three to seven mice. There were no statistical differences ($P > 0.05$) observed between the virus titers for any of the groups analyzed, as determined by Student's *t* test. ND, not determined.

a 1:5 dilution of mouse ascitic fluid containing MAb GK1.5, to the CD4 α chain, on days −1, +1, +3, +6, and +9 relative to infection with the virus (1, 15). The distribution of the various T-cell populations was monitored by two-color flow cytometric analysis in a FACScan (Becton Dickinson, Mountain View, Calif.) following staining (38) with a panel of phycoerythrin- and fluorescein isothiocyanate (FITC)-conjugated MAbs purchased from PharMingen (San Diego, Calif.). MAb RM4-5, which does not compete with GK1.5, was used to stain for CD4.

Measuring the CTL response. Levels of MHC class I-restricted CTL effector function were analyzed for BAL cells that were first depleted of macrophages by plastic adherence. The targets used in the ^{51}Cr release assay (1, 14, 15) were virus infected (or peptide pulsed), and uninfected SV-BALB cells (MHC class I^+ MHC class II^- *H-2*^{d}). Virus-specific CTL precursor (CTLp) frequencies were determined for MLN and spleen populations by a well-established limiting-dilution analysis protocol (39) with the SV-BALB targets.

Assaying cytokine and antibody responses. The prevalence of IFN-γ-, interleukin-4 (IL-4)-, and IL-5-producing cells was determined by single-cell ELISPOT analysis (10, 23, 30) for spleen cells restimulated in vitro with concanavalin A (ConA; 2 μg/ml) for 24 h, or for freshly isolated, whole BAL populations, and expressed as spot-forming cells per 10^5 cells. Cytokine levels in culture supernatants were assayed by ELISA following stimulation for 24 to 72 h in vitro with virus-infected, irradiated spleen cells (23, 30). The levels shown in the results are the maximum values recorded. The frequencies of antibody-forming cells (AFCs) producing virus-specific immunoglobulin (Ig) of different isotypes were determined by ELISPOT analysis of freshly isolated MLN and CLN populations, while virus-specific serum Ig levels were measured by ELISA (17, 29).

RESULTS

Virus clearance and the CTL response. The lack of IFN-γ in the −/− mice did not have any obvious inhibitory effect on the elimination of Sendai virus from the respiratory tract. Lung samples from the +/+ and −/− mice had about the same virus titers on days 6 and 8 after infection and were uniformly negative by day 10 (Table 1). Similarly, the slight delay in controlling the infection that may result from depleting the $CD4^+$ T cells (Fig. 1) by in vivo treatment with MAb GK1.5 ($CD4^-$ mice) was not changed by the absence of IFN-γ (Table 1). Potent MHC class I-restricted CTL effectors were readily detected in the BAL populations recovered from all groups of mice at day 10 after infection (Table 2), when most of the virus had been cleared (Table 1). The response then declined over the subsequent 4 days (Table 2).

The numbers of class I MHC-restricted CTLp recovered from the MLN and spleen on day 10 after infection were comparable for the IFN-γ +/+ and −/− mice with intact $CD4^+$ T-cell function (Table 3). Removing the $CD4^+$ T cells from the IFN-γ −/− group consistently decreased the CTLp frequencies in the MLN and spleen, an effect that was not seen with the $CD4^-$ IFN-γ +/+ controls (Table 3). However, even though the CTLp numbers were lower in the $CD4^-$ IFN-γ −/− mice on day 10 (Table 3), the response was still of sufficient magnitude to provide enough $CD8^+$ effector T cells to eliminate Sendai virus from the respiratory tract (Tables 1 and 2). Furthermore, the prevalence of $CD8^+$ T cells in the BAL

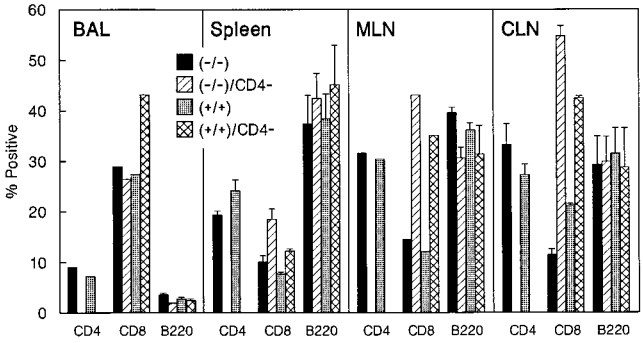

FIG. 1. The phenotypes of $CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells in the lymphoid tissue and BAL were determined at day 10 after i.n. infection with 200 EID_{50} of Sendai virus, by two-color flow cytometry on a FACScan using anti-CD8-phycoerythrin, anti-CD4-FITC, and B220-FITC. Most of the data are for the average of at least two experiments, which gave comparable results. The error bars are shown where at least three determinations were made.

was in no way diminished by the lack of IFN-γ or by the absence of the $CD4^+$ subset (Fig. 1), while the extent of $CD8^+$ T-cell activation to the $CD62L^{lo}$ phenotype (38) was not obviously modified for any of the sites analyzed (data not shown). Clearly, IFN-γ is not essential for the promotion of either $CD8^+$ T-cell activation or effector function in mice with Sendai virus pneumonia, but this cytokine becomes more important for the process of CTLp generation when the $CD4^+$ T cells are removed (Table 3).

Cytokine production and the antibody response. Analysis of cytokine production profiles confirmed the expected lack of IFN-γ in the IFN-γ −/− mice (Fig. 2; Table 4). The absence of IFN-γ was associated with substantially increased numbers of IL-4- and IL-5-secreting cells, detected at 10 days after infection by ELISPOT analysis of freshly isolated BAL populations (Fig. 2A) and ConA-stimulated spleen cells (Table 4). The profiles in the $CD4^-$ IFN-γ −/− mice were essentially similar (Fig. 2; Table 4). This skewing to a Th2 response could not be shown by ELISA of culture supernatants from secondarily stimulated spleen cells (Fig. 2B). Apparently the combination

TABLE 2. Primary CTL activity in the BAL[a]

Day	Expt[b]	E:T ratio	% Specific ^{51}Cr release			
			+/+		−/−	
			Intact	$CD4^-$	Intact	$CD4^-$
10	1	10	42 ± 7	48 ± 5	59 ± 11	46 ± 4
	1	5	34 ± 4	29 ± 5	49 ± 6	35 ± 5
	1	2.5	23 ± 5	19 ± 4	40 ± 2	17 ± 3
	2	20	22 ± 3	65 ± 7	82 ± 10	71 ± 7
	2	10	18 ± 3	46 ± 6	76 ± 7	12 ± 2
	2	5	9 ± 3	35 ± 4	60 ± 5	2 ± 2
14	2	20	15 ± 4	18 ± 3	22 ± 5	43 ± 6
	2	10	14 ± 4	13 ± 5	14 ± 5	40 ± 9
	2	1	7 ± 2	11 ± 2	8 ± 4	8 ± 2

[a] Freshly isolated BAL cells were first adsorbed on plastic to remove most of the macrophages and then assayed for cytotoxic effector function at different effector/target (E:T) ratios.

[b] The target cells were ^{51}Cr-labeled SV-BALB fibroblasts that were either infected with Sendai virus (experiment 1) or pulsed with the $NP_{221-236}$ peptide (experiment 2). Uninfected target cells were used as controls for nonspecific cytotoxicity. Nonspecific cytotoxicity ranged from 3 to 10% lysis for all experiments shown.

TABLE 3. Virus-specific CTLp frequencies at day 10 after primary infection[a]

Expt[b]	CD4 depletion	Reciprocal of CTLp frequency			
		MLN		Spleen	
		+/+	−/−	+/+	−/−
1	−	4,804	2,718	1,496	8,300
	+	4,043	13,881[c]	6,419	24,082[c]
2	−	15,272	22,166	27,362	21,382
	+	20,644	48,602[c]	14,076	>10^6[c]
3	−	8,500	12,700	6,200	5,300
	+	3,800	26,600[c]	5,700	18,800[c]
4	−	6,736	1,181	11,217	17,903
	−			9,576	9,436
	−			20,883	5,108
	+	2,418	>10^6[c]	12,797	>10^6[c]
	+			30,259	>10^6[c]
	+			12,817	63,922[c]

[a] MLN and spleen cells from mice infected i.n. with 200 EID_{50} of Sendai virus 10 days previously were cultured for 7 days under limiting-dilution assay conditions with 10 U of recombinant IL-2 per ml (16) and then assayed by adding ^{51}Cr-labeled virus-infected or uninfected SV-BALB fibroblasts. Levels of specific ^{51}Cr release >3 times the standard deviation for uninfected targets were considered positive. All of the MLN results, and the spleen results from experiments 1 to 3, are for pools made from three mice. Individual spleens from three mice were assayed in experiment 4.

[b] MAb GK1.5 was given intraperitoneally on days −1, +1, +3, +6, and +9 relative to virus infection. Fluorescence-activated cell sorting analysis (1, 38) of the MLN cells indicated that there were <0.5% residual $CD4^+$ T cells at the time of sampling.

[c] The values for the undepleted and depleted mice were not within 95% confidence limits and are thus significantly different (37).

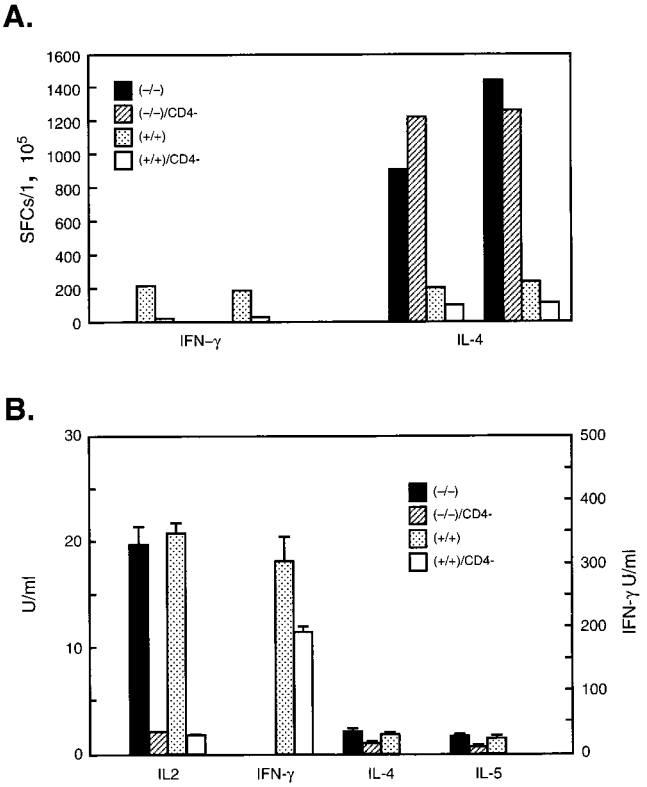

FIG. 2. (A) The numbers of cells producing IFN- or IL-4 (spot-forming cells [SFCs]) are shown for ELISPOT analysis of freshly isolated BAL cells recovered at 10 day after infection. The results are for two separate experiments. (B) The levels of cytokine in tissue culture fluid were determined by ELISA at 24 to 72 h following the in vitro stimulation of pooled spleen cells taken at day 10 after infection with irradiated, virus-infected stimulators (23, 31). The results are the maximum values recorded for the different cytokines and are expressed as mean ± standard error of the mean for at least three experiments.

of short-term ConA stimulation and ELISPOT analysis (Fig. 2; Table 4) is a much more useful method for detecting IL-4 and IL-5, perhaps because these cytokines are rapidly consumed when secreted into a large volume of tissue culture fluid (Fig. 2). The ELISA analysis did, however, establish that most of the IL-2 is being produced by $CD4^+$ T cells (Fig. 2B), while the ELISPOT studies showed clearly that both IL-4 and IL-5 can be made by other cell types ($CD4^-$ [Fig. 2A; Table 4]).

More virus-specific IgG1- and IgG2b-producing cells were found in the MLN (Fig. 3) and the CLN (data not shown) of the IFN-γ −/− mice, and virus-specific serum Ig titers were increased for these two isotypes (Table 5). The IgG2a response that is classically associated with IFN-γ production was also slightly decreased. Removal of the $CD4^+$ T cells greatly diminished virus-specific antibody production for all isotypes, including IgM (Fig. 3; Table 5). Clearly, other cytokines can substitute for IFN-γ in, for example, the promotion of a substantial IgG2a response to Sendai virus. Thus, though the antibody response was to some extent skewed to show a more Th2 profile in the IFN-γ −/− mice, the effect was relatively slight.

DISCUSSION

Previous experiments with MAb-depleted and MHC class II −/− *H-2*^{b} mice established that the participation of $CD4^+$ T cells is not essential for the clearance of either Sendai virus (16) or the HKx31 (H3N2) influenza A virus (39) from the respiratory tract. However, many fewer influenza virus-specific $CD8^+$ CTLp were generated in the MHC class II −/− mice (40), while the CTLp numbers were essentially equivalent for MHC class II −/− and +/+ mice exposed to Sendai virus (16). The other difference was that influenza virus infection of the MHC class II −/− mice seemed to induce a cytokine (including IFN-γ) response much smaller than that found for compa-

TABLE 4. Frequencies of cytokine-producing cells in the spleens of Sendai virus-infected mice[a]

Expt	Mouse	CD4 depletion	Spot-forming cells/100,000 cells		
			IFN-γ	IL-4	IL-5
1	+/+	−	320	10	0
	+/+	+	360	8	0
	−/−	−	0	13	13
	−/−	+	0	90	50
2	+/+	−	339	0	0
	+/+	+	435	0	0
	−/−	−	0	39	97
	−/−	+	0	44	94
3	+/+	−	94	30	0
	+/+	+	103	41	0
	−/−		0	126	60
	−/−	+	0	117	76

[a] Spleen cells taken from mice that were infected with 200 EID_{50} of Sendai virus on day 10 after infection were restimulated with ConA (2 μg/ml) for 24 h before analysis in the cytokine ELISPOT assay (10, 23, 30).

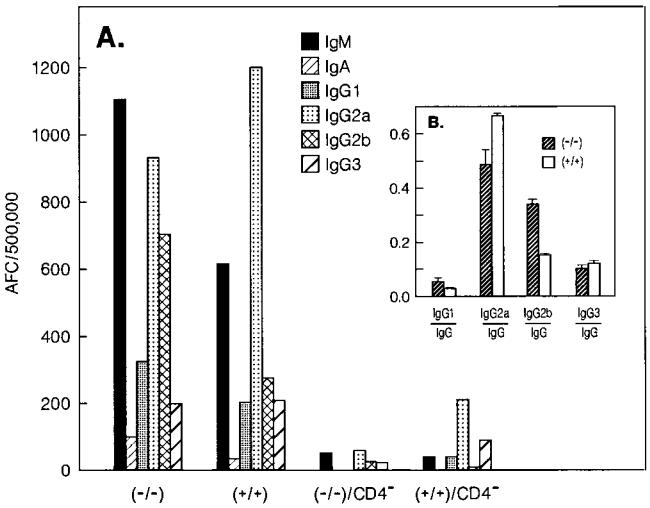

FIG. 3. The prevalence of AFCs was determined by ELISPOT analysis for the MLN at day 10 after infection. (A) Results, expressed as mean ± standard error of the mean, for the intact mice (four separate experiments) and for the CD4-depleted mice (two experiments). (B) Ratios of AFCs for the different virus-specific isotypes.

rable animals exposed to Sendai virus. The present analysis with IFN-γ −/− and +/+ *H-2*^{d} mice indicates that the capacity of other cell types to produce IFN-γ is a significant factor in the promotion of the CD8$^+$ CTLp response in the absence of CD4$^+$ T help. A likely source of the IFN-γ is the CD8$^+$ T cells themselves (16, 18, 19, 24). This need for IFN-γ is not apparent when CD4$^+$ T cells, which make most of the IL-2, are present.

Some in vitro experiments indicate that IFN-γ can be a growth-enhancing factor for T lymphocytes (20), though the majority of such studies suggest the contrary (7, 9). The rate of CTLp generation might also be influenced by the capacity of IFN-γ to influence proteasome function (33) and to promote higher levels of viral peptide-plus-class I MHC glycoprotein expression on stimulator cells (36, 42). The involvement of IFN-γ does not, however, seem to be essential for the transition from CTLp to CTL effector. As shown previously for the MHC class II −/− mice infected with influenza virus (38), the smaller pool of Sendai virus-specific CTLp in the CD4-depleted IFN-γ −/− mice was to some extent consumed to give a CTL response that was sufficient to eliminate the virus. Perhaps the terminal differentiation to CTL effector function is driven more by T-cell receptor target interaction in the virus-infected lung than by the cytokines, which are more important for the developing response in lymphoid tissue (8).

The transition to a more Th2-type response in both the intact and CD4-depleted mice is very apparent for these Sendai virus-infected IFN-γ −/− mice. Cloned CD4$^+$ T cells producing IL-4 have been generated from IFN-γ −/− mice that had recovered from infection with an H2N2 influenza A virus (11), but direct evidence for an acute Th1-Th2 switch in lymphoid tissue has been more elusive (32). Use of the short-term (24-h) ConA stimulation protocol combined with ELISPOT analysis has now clarified this situation. Our previous experience with the influenza model was that although mRNA for the various Th1 and Th2 cytokines was detectable at high levels in the regional lymph nodes (3), it was difficult to demonstrate either IL-4 or IL-5 in this site by in vitro culture or by direct ELISPOT analysis of freshly isolated lymphocytes (23, 31, 32). Activated IL-4-producing cells were, however, consistently found in the BAL populations from mice infected with either the HKx31 influenza A virus or Sendai virus (23, 30, 32), and the reasonably high prevalence of IgG1-producing cells in the lymph nodes and spleen indicated that IL-4 was indeed being made (17, 22, 29). Perhaps the amounts of IL-4 delivered via cognate interaction to promote B-cell class switching in vivo are relatively small, and it is only when the T cells are terminally differentiated in the poorly regulated environment of an inflammatory exudate (8) that higher levels are produced.

The greater prominence of IL-4 had no obvious consequences for the generation of CTL effectors in the virus-infected lung, a finding that is in accord with evidence that CD8$^+$ CTLs can be developed from lineages secreting Th1- or Th2-type cytokines (26). The switch to higher levels of IgG1 production and less IgG2a in the lymphoid tissue was to be expected in the absence of IFN-γ (25, 34, 35) and was consistent with the enhanced IL-4-producing cells (Fig. 2; Table 4). However, the increase in IgG2b production was intriguing, and it is clear that not all class switching to IgG2a is IFN-γ dependent. In general, an effective immune response to respiratory viruses is preserved in the absence of IFN-γ (2, 11, 32), though the lack of this cytokine can have dramatic consequences for some bacterial and parasitic infections (5, 21).

TABLE 5. Virus-specific serum Ig levels

Mice	CD4 depletion[b]	Mean Ig titer (1/dilution, 10^3 ± SEM)[a]			
		IgG1	IgG2a	IgG2b	IgG3
+/+	−	8.1 ± 0.0	27.0 ± 0.0	9.0 ± 0.0	4.5 ± 2.9
	+	0.3 ± 0.1	3.2 ± 1.1	0.6 ± 0.4	0.8 ± 0.4
−/−	−	24.3 ± 0.0	15.0 ± 7.3	21.0 ± 7.3	2.7 ± 0.0
	+	0.1 ± 0.04	4.1 ± 0.0	1.4 ± 0.0	1.0 ± 0.4

[a] ELISA titers, determined for three individual mice at day 10 after infection, are expressed as the highest serum dilution giving an absorbance value greater than twice that for corresponding normal serum (22).

[b] The mice were given MAb GK1.5 intraperitoneally and infected with 200 EID_{50} of Sendai virus as described in the footnote to Table 2.

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