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Local IL-4 Expression in the Lung Reduces Pulmonary Influenza-Virus-Specific Secondary Cytotoxic T Cell Responses

Adrian Bot,* Andreas Holz,* Urs Christen,* Tom Wolfe,* Angela Temann,† Richard Flavell,† and Matthias von Herrath*,[1]

*Department of Neuropharmacology, Division of Virology, IMM6, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, California 92037; and †Section of Immunobiology, Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06520

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We studied the effect of lung-specific IL-4 expression on the T cell response during primary and secondary heterologous infection with influenza virus by using transgenic mice that express IL-4 under a lung-specific promoter. Subsequent to primary infection with a type A/H1N1 influenza virus these transgenic mice exhibited similar local recruitment of $CD4^+$ and $CD8^+$ T cells and only slightly decreased virus-specific CTL activity. However, during secondary challenge with a heterologous influenza virus, the local infiltration with virus-specific, MHC class I-restricted $CD8^+$ T cells was significantly decreased compared to that of nontransgenic littermates. The ability of IL-4 transgenic mice to clear the heterologous infection was delayed but not abrogated. This was associated with a faster virus-neutralizing antibody response in IL-4 transgenic mice and with their ability to mount significant Th1 responses even in the presence of increased local IL-4 expression. Our observations demonstrate a negative regulatory effect of IL-4 on memory Tc1/$CD8^+$ T cells, but are also consistent with complementary mechanisms important for virus clearance such as virus-neutralizing antibodies. The reduction of memory CTL in the presence of IL-4 might have consequences for understanding the course of influenza infection in situations where T_H2 immunity is increased.

INTRODUCTION

During immune responses to microbes, antigen-specific class I- and class II-restricted T cells as well as B cells are primed and exert various defensive functions. Early studies defined two subpopulations of functional class II-restricted T helper (Th) cells: Th1 cells that produce IL-2 and IFN-γ and Th2 cells that secrete IL-4 and IL-5 (Mosmann *et al.,* 1986). Accumulating evidence supports the model that Th1 promoting cytokines like IL-12, as well as Th1 cytokines like IFN-γ, exert suppressive effects on the generation and activity of Th2 cells. In contrast, Th2 cytokines like IL-4 inhibit the generation and function of Th1 cells. Thus, during exposure to microbial antigens, the initial pattern of cytokines produced by cells of the innate immune system shape the subsequent adaptive response toward either Th1 or Th2. It is thought that Th1 cells control effective immunity against viruses. In contrast, Th2 cells generally promote responses that are unable to fully protect against infectious challenge. This was previously demonstrated systemically for influenza virus using a model of adoptive transfer with Th1 or Th2 clones (Graham *et al.,* 1994). In these studies, transfer of the virus-specific Th2 clones was followed by impaired responses against influenza virus. Since the Th2 clones produced multiple cytokines, their individual role in the suppression of immunity against influenza virus was not clear.

We sought to investigate the role of IL-4 as a Th2 cytokine with potential regulatory function on class I-restricted immunity. We took advantage of a transgenic model, where the IL-4 cDNA is locally expressed as a transgene under a lung-specific promoter, the rat Clara cell protein 10 promoter (Rankin *et al.,* 1996). The expression of IL-4 is restricted to lungs, specifically to the Clara cells that comprise 50–70% of the epithelial cells in trachea, bronchi, and bronchioles, known to be permissive for infection with influenza virus. The assessment of IL-4 showed levels between 0.15 ng/ml and a few nanograms per milliliter in the broncho-alveolar lavage fluid, compared to less than 60 pg/ml to nondetectable levels in sera. The transgenic mice displayed a certain degree of epithelial hypertrophy, intra-alveolar macrophages, and occasional "islets" of eosinophils in the parenchyma (Rankin *et al.,* 1996) that progressed with age (Jain-Vora *et al.,* 1997).

The immunity against influenza virus consists mainly of B cells producing virus-neutralizing antibodies that bind to hemagglutinin (HA) (Caton *et al.,* 1982), Th cells that recognize dominant class II-restricted epitopes on HA (Haberman *et al.,* 1990), and CTL specific for class I-restricted epitopes on internal proteins, like nucleoprotein (NP) (Townsend and Bodmer, 1989). Whereas the

[1] To whom correspondence and reprint requests should be addressed.

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antibodies and to a certain extent the Th cells recognize epitopes that display increased variation among drift variants and particularly among strains of different subtypes, CTL recognize epitopes conserved among all type A strains (Bastin *et al.,* 1987). We have employed an experimental model where IL-4 transgenic mice and nontransgenic littermates were immunized via the intraperitoneal route with live A/HK/68 (H3N2) influenza virus and challenged with the A/PR/8/34 (H1N1) strain. Since the protective B cell epitopes as well as the dominant Th epitopes are not conserved between these two strains, the protection against infectious challenge depended mostly on memory CTL against internal epitopes. Thus, this experimental system could be used to assess the effect of local expression of IL-4 on memory $CD8^+$ T cells and their role in controlling pulmonary infection and immunopathology.

We assessed the effect of lung-specific expression of IL-4 on the primary and secondary immune response under the conditions mentioned above. We found that local expression of IL-4 exerted negative effects on $CD8^+$ T cells during the secondary response to a heterologous influenza virus. This was not due to a faster clearance of virus by the IL-4 transgenic mice. Unexpectedly, Th1 cytokines were still significantly enhanced in the lungs of the IL-4 transgenic mice infected with influenza virus. Furthermore, *de novo* induction of protective antibodies during infection was more rapid in the IL-4 transgenic mice, explaining why the virus clearance was only delayed but not abrogated.

RESULTS

Primary influenza infection induces similar CTL levels and immunopathology in IL-4 transgenic, IL-4-deficient, or nontransgenic mice

HA of influenza virus bears dominant B and Th epitopes, whereas NP carries the major CTL epitopes (Caton *et al.,* 1982; Townsend and Bodmer, 1989). Our hypothesis was that expression of IL-4 in the lungs as a transgene under the control of the lung-specific Clara 10 protein promoter (Rankin *et al.,* 1996) would influence the local T cell responses as well as viral clearance during primary and/or secondary influenza infections. We tested this hypothesis by infecting IL-4 transgenic mice, nontransgenic littermates, and IL-4−/− mice with A/PR/8/34 (H1N1) influenza virus, followed by assessment of the local T cell infiltration. We found that at day 7 after primary infection, lungs of IL-4 transgenic mice displayed significant infiltration with $CD4^+$ and $CD8^+$ T cells, comparable with nontransgenic littermates and IL-4−/− mice (Figs. 1A–1D). Noninfected IL-4 transgenic mice exhibited rare $CD4^+$ and $CD8^+$ cells throughout the lung parenchyma before the age of 3 months. Thus, transgenic expression of IL-4 in the lung did not have significant effects on the local infiltration with $CD4^+$ and $CD8^+$ T cells during the primary response to PR8 influenza virus. The histology generally correlated with the ELISPOT analysis (Figs. 2B and 2D), with the single amendment that the frequency of T cells producing cytokines upon polyclonal stimulation was reduced in the lungs of IL-4 transgenic mice. The primary infection with PR8 was also followed by induction of significant virus-specific, class I-restricted T cell responses in lung (Figs. 2B and 2D) and spleen (Figs. 3A and 3B). No significant Tc1 to Tc2 switch was noted as a consequence of local, constitutive expression of IL-4 (Figs. 2A–2D). The systemic responses of IL-4−/− and IL-4 tg mice were not dramatically altered when compared to nontransgenic littermates, except for an increased frequency of virus-specific IL-4 and IFN-γ producing T cells in the spleens of IL-4 transgenic animals compared to nontransgenic littermates (Figs. 2A and 2C). Furthermore, primary infection of IL-4 tg mice with a partial lethal dose of influenza virus was followed by a survival rate that was not significantly reduced compared to that of littermates (4 of 6 versus 3 of 5; $P > 0.05$). Similar results were obtained with a different H1N1 strain of influenza virus, A/WSN/32 (data not shown). Measurement of virus titers in the nasal wash of IL-4 transgenic mice and littermates at day 5 after primary infection revealed no significant differences in virus levels or percentage of mice that showed detectable infectious virus (IL-4 tg mice $(5.0 \pm 0) \times 10^2$ tissue culture infective dose 50% ($TCID_{50}$)/ml, 100%; versus littermates $(5.0 \pm 2.4) \times 10^2$ $TCID_{50}$/ml, 80%). Together, these data indicate that neither the specific T cell responses nor the overall protective responses are significantly affected by local expression of IL-4 during primary infection with influenza viruses.

Lung $CD8^+$ T cell numbers in response to heterologous secondary influenza infection are lower in IL-4 transgenic mice

Dominant B cell epitopes as well as class II-restricted epitopes on HA recognized by $CD4^+$ Th cells are conserved only among strains of influenza virus from the same subtype. In contrast, the major class I-restricted epitopes localized on NP and recognized by $CD8^+$ CTL are extensively shared among type A strains of influenza virus. Thus, by infecting intraperitoneally immunized mice with a strain of a different subtype, one can test the activation and recruitment of memory $CD8^+$ T cells to the site of infection. We tested the effect of local expression of IL-4 on the activity of memory CTL primed by ip immunization with live A/HK/68 (H3N2) virus. Interestingly, IL-4 transgenic mice immunized with HK virus and infected 1 month later with influenza virus PR8 displayed significantly reduced numbers of $CD8^+$ T cells in the lungs on day 5 postinfection, compared to nontransgenic littermates or C57BL/6 and IL-4−/− mice (Figs. 1E–1H and 4). The density of the $CD8^+$ T cells as revealed by

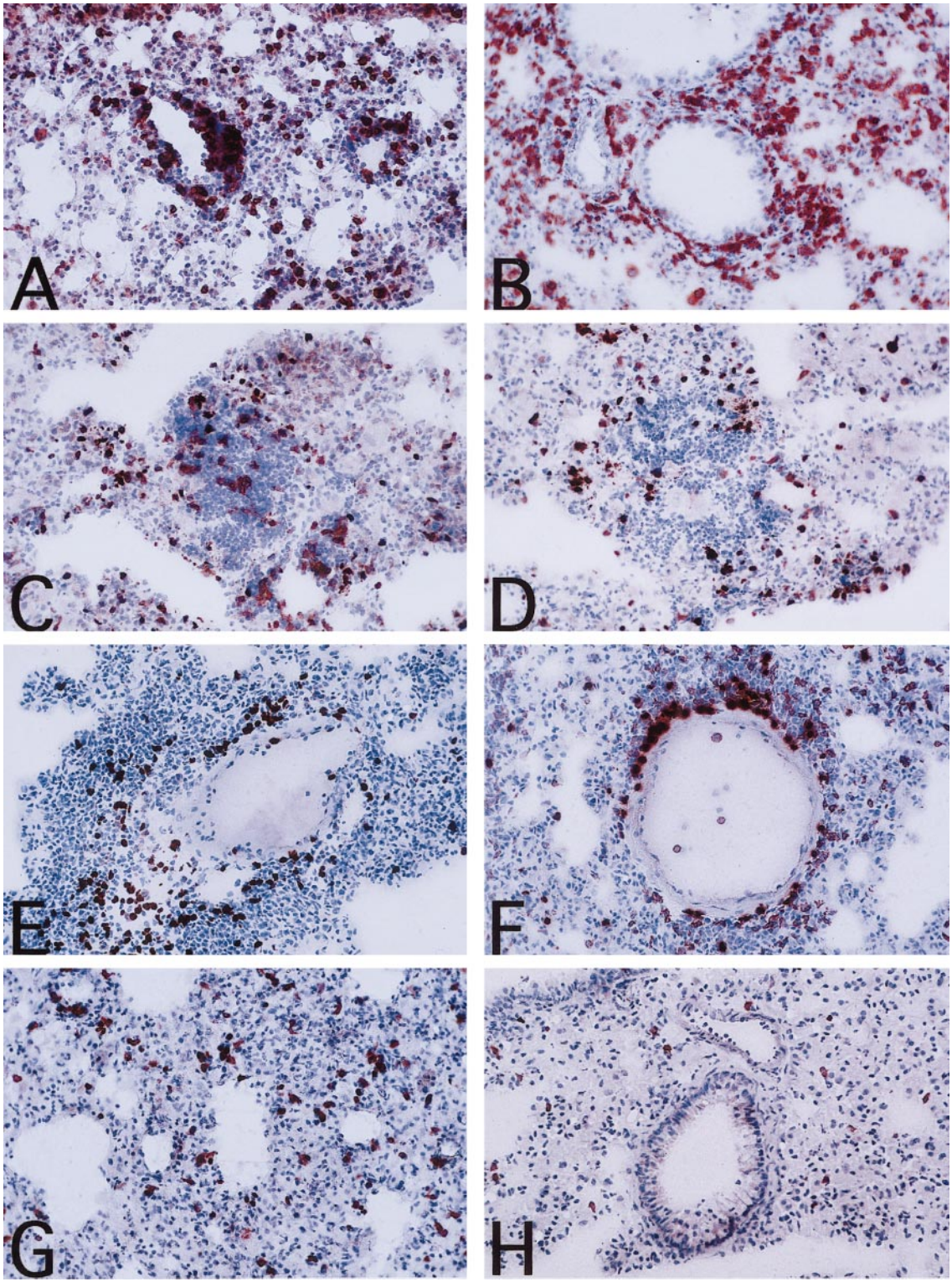

FIG. 1. $CD4^+$ and $CD8^+$ T cells in lungs of IL-4 transgenic and nontransgenic mice during primary or secondary infection with influenza virus. Lungs were harvested on day 7 after primary infection (A–D) or day 5 after secondary infection (E–H) and stained for CD4 or CD8. A, C, E, and G correspond to anti-CD4 staining and B, D, F, and H to anti-CD8 staining. A, B, E, and F correspond to nontransgenic littermates. C, D, G, and H correspond to IL-4 transgenic mice. Representative data for at least three animals/group are shown.

immunohistochemistry was between 10 and 100 times lower in the transgenic mice than in controls (Figs. 5 and 1H versus 1F). The analysis of the frequency of class I-restricted NP-specific T cells by ELISPOT showed significantly lower values in the lungs of IL-4 transgenic mice versus nontransgenic littermates (Fig. 2F). How-

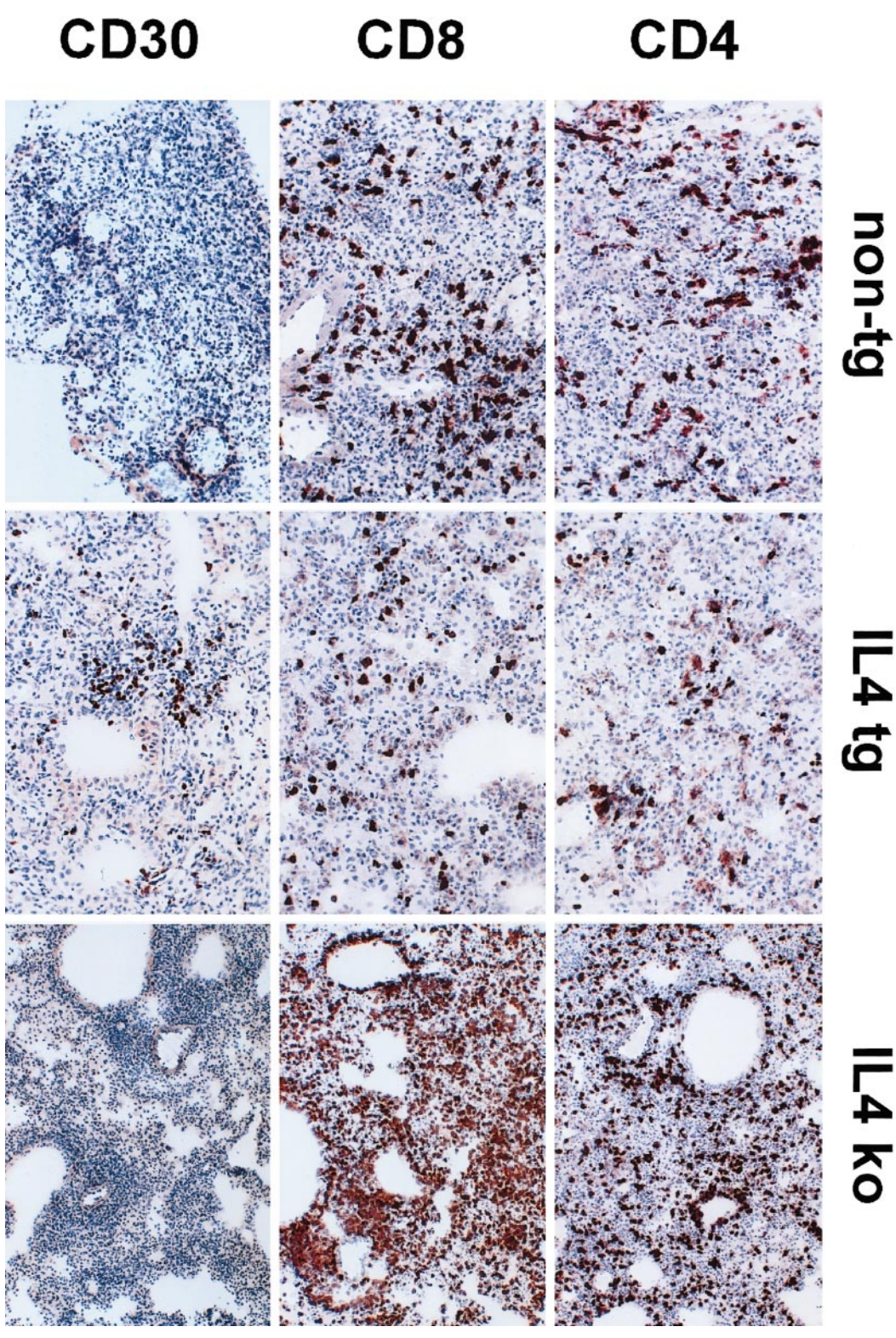

FIG. 4. Expression of CD30 in IL-4 transgenic mice, IL-4−/−, mice and nontransgenic littermates. Histological staining of lungs harvested from HK-immunized mice on day 5 after heterologous challenge with PR8 was performed as described under Materials and Methods, using reagents specific for CD4, CD8, and CD30. Mice and stain under observation are indicated. The data shown are representative for three animals/group.

ever, the difference was only three- to fourfold, which is less than observed by immunohistochemical staining of lung parenchyma. This quantitative discrepancy may be due to two factors: the collagenase digestion cannot discriminate between the parenchymal and BALT-derived cells and second, the lung volume of IL-4 trans-

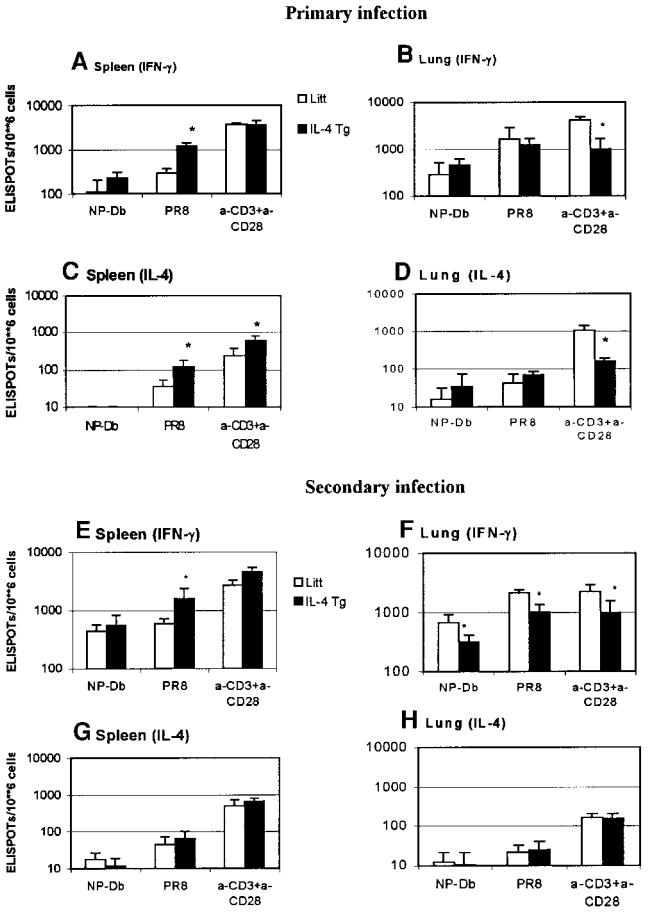

FIG. 2. ELISPOT analysis of T cells from lungs or spleen of IL-4 transgenic and control mice, during primary (A–D) or secondary (E–H) infection with influenza virus. Organs were harvested on day 7 (primary infection) or 5 (secondary challenge) after infection and single-cell suspensions of lymphocytes were prepared as described under Materials and Methods. Cells were stimulated with NP-D^b peptide, PR8 virus, or anti-CD3 + anti-CD28 antibodies, in nitrocellulose wells coated with anti-IFN-γ or anti-IL-4 antibodies. The analysis was developed after 72 h. The results are expressed as frequency (spots/10^6 responder cells) of IFN-γ or IL-4 producing cells (means ± SD, $n = 3$ animals/group).

genic mice was consistently two- to threefold higher than that of nontransgenic littermates, due to IL-4-induced hyperplasia (Rankin *et al.,* 1996; Jain-Vora *et al.,* 1997). No down-regulation of specific $CD8^+$ T cells was noted in the spleen (Fig. 3) and no significant Tc1 to Tc2 switch occurred in the IL-4 transgenic mice (Fig. 2). However, the frequency of $CD4^+$ T cells infiltrating the lungs subsequent to secondary infection with PR8 virus was only slightly and infrequently decreased in the IL-4 transgenic mice (Figs. 1E and 1G). The apparent decrease in the local frequency of virus-specific IFN-γ producing T cells (Fig. 2F) cannot be accounted for solely by a reduction of $CD4^+$ T cells and is probably due to the decreased frequency of $CD8^+$ T cells. Overall, the balance of cytokines produced by the recruited T cells was slightly shifted toward a Th2 profile, mainly due to decreased frequency of virus-specific IFN-γ producing T cells, but not due to an increased frequency of IL-4 producing T cells (Figs. 2F and 2H). Since dominant CTL epitopes like the D^b-restricted NP 366–374 are conserved among influenza virus strains of different subtypes (like H3N2 and H1N1), this result shows a local negative effect of IL-4 on primed $CD8^+$ T cells.

Consistent with the ELISPOT analysis, the measurement of systemic secondary CTL responses in spleens of IL-4 tg mice primed with HK and challenged with PR8 virus revealed no significant differences compared to the group of littermates (Fig. 3C). We further explored the potential mechanism through which IL-4 can locally reduce influenza specific memory CTL. As shown in Fig. 4, up-regulation of the CD30 antigen shown to be a potent inhibitor of CD8 function (Kurts *et al.,* 1999) that is ex-

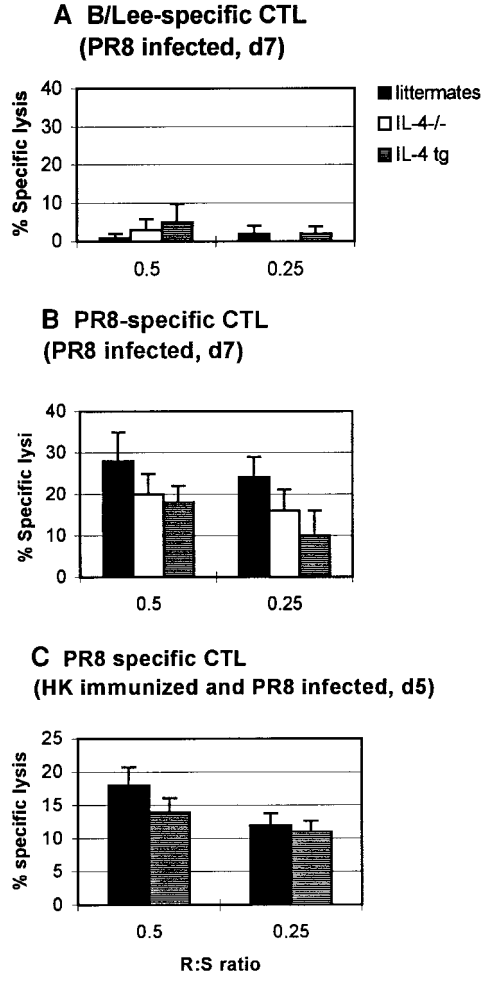

FIG. 3. Induction of cytotoxic cells subsequent to primary (A, B) or secondary (C) infection with influenza virus. Spleens were harvested on day 7 (primary infection) or day 5 (secondary infection) from three mice/group and splenocytes were *in vitro* stimulated with virus-infected splenocytes. Subsequently, standard ^{51}Cr-release assays were carried out. The specific lysis against B/Lee virus (A) or homologous virus (B, C) was measured at different responder/stimulator (R/S) ratios. The results are expressed as means ± SEM of the percentage of specific lysis measured individually.

TABLE 1

Pulmonary Virus Titers during the Response of IL-4 Transgenic Mice after Primary Heterologous Challenge

Mice	Immunization	Infection	Pulmonary virus titers[a] Day 7	Day 14
C57BL/6 ($n = 3$)	None	PR8	$8.7 \pm 5.8 \times 10^4$(3/3)[b]	ND[c]
C57BL/6 IL-4$^{-/-}$ ($n = 5$)	HK	PR8	0[d] (0/3)	ND
Nontransgenic littermates of IL-4 Tg ($n = 4$)	HK	PR8	0 (0/4)	0 (0/6)
IL-4 transgenic ($n = 6$)	HK	PR8	$2.0 \pm 0.9 \times 10^3$ (3/6)	0 (0/5)[e]

[a] Pulmonary virus titers were measured at day 7 after infection and are expressed as means ± SEM of $TCID_{50}$ per 2 ml of lung homogenate.
[b] Number of mice displaying detectable virus levels/total number in the group.
[c] Not done.
[d] No detectable pulmonary virus.
[e] One of six mice died on day 8.

pressed on T_H2 lymphocytes and CTL (Bengtsson *et al.,* 1995; Kurts *et al.,* 1999) was seen only in IL-4 transgenic mice after infection with heterologous virus (Fig. 4, lower middle panel). No significant CD30 staining was noted in the lungs of noninfected transgenic or nontransgenic mice.

Clearance of secondary influenza infection is delayed but not abrogated in IL-4 transgenic mice

Determining viral titers in lungs and nasal washes assessed the effect of local transgenic expression of IL-4 on the ability of the immune system to clear a secondary heterologous influenza infection. Fifty percent of the IL-4 transgenic mice that were previously primed with HK virus and subsequently infected with PR8 virus displayed one log lower viral titers in lungs at day 7 after infection compared to nonimmunized, nontransgenic mice (Table 1). None of the immunized nontransgenic littermates or IL-4−/− mice displayed detectable viral titers at day 7 when similarly infected. These results show a significantly delayed clearance of influenza virus in IL-4 transgenic mice after secondary infection with a strain of a different subtype ($P < 0.05$ by Fisher's exact test, when we compared the rate of virus detection in IL-4 transgenic mice versus pooled controls). When checked at day 5 for the presence of virus in the nasal cavity, the IL-4 transgenic mice displayed higher virus titers than nontransgenic littermates ($5.0 \pm 3.2 \times 10^3$ $TCID_{50}$/ml vs $2.8 \pm 2.0 \times 10^2$ $TCID_{50}$/ml). By day 14 all survivors had completely cleared the pulmonary virus (Table 1). In this experiment carried out with a 100% infectious dose, 6/6 nontransgenic and 5/6 IL-4 tg mice survived. Thus, the lower number of $CD8^+$ T cells in the lungs of IL-4 transgenic mice was not a result of more rapid clearance of influenza virus, but most probably the cause for the impaired clearance of virus during the memory response. However, when compared to naive mice infected with similar high doses of influenza virus, the IL-4 tg mice previously immunized with HK displayed clear tendencies to eliminate the pulmonary virus (Table 1). This is consistent with compensatory clearance mechanisms such as the *de novo* generation of neutralizing antibodies.

Cytokine levels in lungs of IL-4 transgenic mice

We compared the expression of cytokines relative to mRNA levels in lungs of HK-immunized animals, infected or not with PR8 virus. We used nontransgenic littermates as controls for the IL-4 transgenic mice. The assessment of cytokine mRNAs in the lungs by RNase protection assay confirmed increased levels of IL-4 mRNA in transgenic mice (Fig. 6A), consistent with previous data (Rankin *et al.,* 1996). Relative to the levels of IL-4 mRNA in transgenic mice, the nontransgenic littermates exhibited no significant expression. Surprisingly, local expression of IFN-γ on day 5 after heterologous challenge was not decreased in uninfected or infected IL-4 transgenic mice, compared to nontransgenic littermates. There was

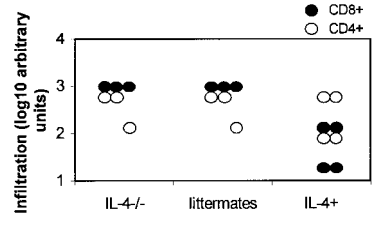

FIG. 5. Comparison of lung infiltration during secondary response to influenza virus between IL-4 transgenic and control mice. Lungs from IL-4 transgenic mice, nontransgenic littermates, and IL-4−/− mice were harvested on day 5 after heterologous infection and stained for CD4 and CD8. For each animal, at least five different fields (objective 20×) were screened for the presence and number of $CD4^+$ and $CD8^+$ T cells. The results were individually plotted in a logarithmic semiquantitative manner as follows: "1" corresponds to 1–10 cells/field; "2" corresponds to $10–10^2$ cells/field, and "3" corresponds to $10^2–10^3$ cells/field.

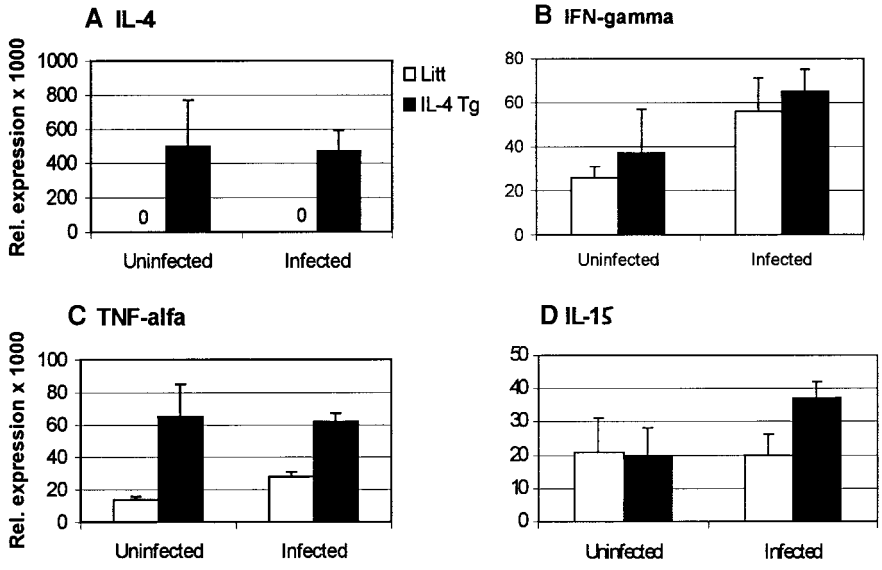

FIG. 6. Expression of mRNA of various cytokines in the lungs of IL-4 transgenic mice and nontransgenic littermates after heterologous infection. RNase protection assays were individually carried out using lung tissue from three mice/group harvested on day 5 from HK-immunized and PR8 infected (second set of bars) or HK-immunized and uninfected mice (first set of bars). The bands corresponding to protected mRNAs were scanned and quantified and the resulting signals were individually normalized to a standard housekeeping message (L32). The results are expressed as means ± SEM of relative expression × 1000. Black bars represent IL-4 transgenic mice ("IL-4 Tg"); white bars represent nontransgenic controls ("Litt").

a clear-cut tendency of IFN-γ up-regulation upon infection in both transgenic and nontransgenic littermates (Fig. 6B). The expression of TNF-α increased upon infection, in the nontransgenic littermates. However, although the basal levels of TNF-α expression were higher in the IL-4 transgenic mice than in nontransgenic littermates, they failed to increase upon infection (Fig. 6C). In contrast, only the IL-4 transgenic mice exhibited significant up-regulation of IL-15 mRNA upon infectious challenge (Fig. 6D). IL-15 is thought to be produced mainly by antigen-presenting or stromal cells and has IL-2-like activity, promoting the proliferation of recently activated T cells (Johnston *et al.,* 1995).

In conclusion and somewhat unexpectedly, local constitutive expression of IL-4 did not abrogate the expression of IFN-γ. However, the up-regulation of TNF-α subsequent to infection was prevented. Instead, IL-15 increased subsequent to infection only in the IL-4 transgenic mice. Increased basal levels of TNF-α may be due to the recruitment of natural immune cells as previously reported (Jain-Vora *et al.,* 1997). The apparent discrepancy of the ELISPOT analysis with the results of RNase protection assay regarding IFN-γ expression may be due to increased cytokine production on a per cell basis, particularly since $CD4^+$ recruitment was only moderately and inconsistently affected.

Neutralizing antibodies specific for the secondary heterologous influenza strain are increased in IL-4 tg mice

In addition to the effector functions of T cells, neutralizing antibodies that bind to HA and prevent attachment to cellular receptors most probably play a role in influenza virus clearance. Since transgenic expression of IL-4 might increase the ability of B cells to proliferate, differentiate, and produce specific antibodies, we measured the titers of hemagglutination-inhibiting (HI) antibodies specific for the secondary virus (PR8) in mice previously immunized with influenza (HK). Since effective mechanisms of protection should act during the first week subsequent to infection with influenza virus, we studied the HI titers early, on day 5 subsequent to the heterologous challenge. As shown in Fig. 7A, IL-4 tg mice either primed or nonprimed with HK virus mounted significantly higher HI-specific antibodies early during infection against the heterologous strain (PR8), when compared to littermates. The results are consistent with a more rapid onset of humoral responses in the IL-4 tg mice. Most of the IL-4 transgenic mice (5 of 6) displayed early detectable titers of HI antibodies against the virus used for infection, in contrast to 0 of 7 nontransgenic littermates ($P = 0.01$ of Fisher's exact test). Slightly higher IgG ELISA titers were noted in the case of primed versus nonprimed IL-4 transgenic animals (data not shown), but they did not translate into significantly higher protective titers at day 5 (Fig. 7). However, on day 14 after challenge, the HI titers of both IL-4 transgenic and nontransgenic mice were comparable, suggesting a difference in the kinetics but not magnitude of the antibody response (Fig. 7B). Together, these data suggest a compensatory role for virus-neutralizing antibodies in IL-4 transgenic mice during the secondary heterologous infection.

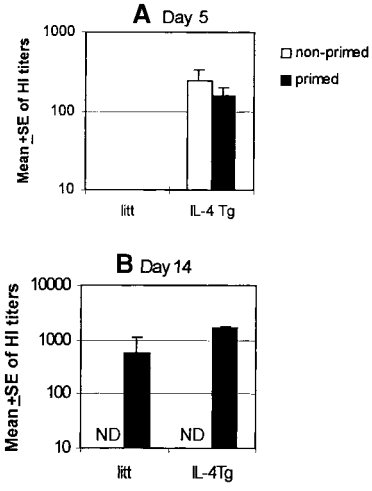

FIG. 7. Induction of virus-neutralizing antibodies in IL-4 transgenic mice. Sera were harvested from IL-4 transgenic or nontransgenic littermates, on day 5 and 14 after primary or secondary infection with influenza virus. The titers of neutralizing antibodies against PR8 virus were individually measured ($n = 3–5$ mice/group) by hemagglutination inhibition and the results are expressed as means ± SE of end-point titers, on a logarithmic scale. The results are representative of two independent experiments. ND, not done.

DISCUSSION

We studied the effect of lung-specific IL-4 expression on the local and systemic response against influenza virus by using a previously described transgenic model (Rankin *et al.,* 1996), in which IL-4 is selectively expressed under the Clara cell protein 10 promoter. During the secondary response against a heterologous strain of influenza virus (PR8) the IL-4 transgenic mice displayed: (i) reduced numbers of $CD8^+$ T in the lungs; (ii) a reduced frequency of MHC class I-restricted IFN-γ producing T cells in the lungs; (iii) delayed but not abrogated clearance of pulmonary virus; and (iv) faster *de novo* induction of PR8-specific neutralizing antibodies, already detectable at day 5 after infection. However, surprisingly, the local expression of IFN-γ was not abrogated in the IL-4 transgenic animals subsequent to primary or secondary infection with influenza virus. This correlated with the lack of switch of the T cell response toward a clear-cut Th2 or Tc2 profile, as shown by both ELISPOT and RNase protection assay. This may be due to quantitative factors, for example, relatively decreased exposure to IL-4 in compartments where priming or expansion of T cells occurs. Alternatively, it may be due to a limited regulatory effect of IL-4 during infection, due to simultaneous activity of other cytokines. The latter hypothesis is more likely, since recent studies involving coexpression of IL-4 and viral antigen by the same vector revealed only quantitative effects on the pool of specific Tc1/CTL but no dramatic Th1 to Th2 or Tc1 to Tc2 shifts (Aung *et al.,* 1999). Thus, a relatively down-regulated class I-restricted T cell response during the recall but not during the primary response suggested that the expansion and/or recruitment of memory CTL directed against conserved epitopes was affected by local IL-4 expression. This was not simply due to a more rapid clearance of pulmonary virus, as shown by virus titration measurements. However, a faster humoral response combined with the robust cytokine production comprising Th1 elements might have compensated for the decreased expansion/recruitment of influenza virus-specific CTL directed against conserved class I epitopes. This correlates with the ability of IL-4 transgenic mice to clear the pulmonary virus. IL-4 may directly or indirectly act on $CD8^+$ T cells at certain stages throughout their life span after they encounter the antigen, by the following mechanisms:

(i) Inhibition of expansion of memory CTL in the interstitial tissue or bronchial-associated lymphoid tissue. This mechanism is supported by previous studies, showing inhibition of IL-2 synthesis by IL-4 (Sad and Mossman, 1995) or down-regulation of antigen presentation (Cua *et al.,* 1996);

(ii) Inhibition of recruitment of specific memory $CD8^+$ T cells in the lung parenchyma subsequent to secondary heterologous infection, due to the alteration of chemokine/chemokine receptor patterns under modified cytokine profiles (Li *et al.,* 1999; Cerwenka *et al.,* 1999);

(iii) Increased decline in the population of memory $CD8^+$ T cells recently restimulated with antigen.

Our findings show that CD30 is up-regulated only in lungs of IL-4 transgenic mice but not in the IL-4-deficient or nontransgenic littermates after secondary influenza infection. Since CD30 expression is a potent inhibitor of CD8 cell function (Kurts *et al.,* 1999) induced in T_H2-type environments (Gilfillan *et al.,* 1998), it could be one of the main factors suppressing CD8 cell activity (Bowen *et al.,* 1996) in the IL-4 transgenic mice.

There are previous studies that pointed toward an important role for IL-4 in the regulation of T cell responses. Disruption of the IL-4 gene in knockout mice is associated with decreased Th2 responses (Kopf *et al.,* 1993). In contrast, low levels of IL-4, together with IL-2, seem to exert a synergistic effect on the expansion of virus-specific CTL (Bachmann *et al.,* 1995). On the other hand, *in vitro* exposure of influenza virus-specific $CD8^+$ T cells to stimulator cells in the presence of Th2 clones resulted in significant inhibition of CTL activity (Palladino *et al.,* 1991). Further, the adoptive transfer of influenza virus-specific Th2 clones also failed to mediate protective effects (Graham *et al.,* 1994). Last, systemic administration of high doses of IL-4 to mice infected with influenza virus significantly decreased primary and secondary responses to influenza virus, probably by affecting the expansion and local recruitment of $CD8^+$ CTL (Moran *et al.,* 1996). Whereas the IL-4-treated, influenza virus-infected mice displayed a delayed virus clearance, their ability to recover from infection was not abrogated.

Other previous studies showed that systemic exposure to IL-4 was associated with various degrees of inhibition of protective antiviral responses, possibly due to down-regulation of CTL activity (Andrew and Coupar, 1992; Sharma *et al.,* 1996). In contrast to these observations, our experimental model of local IL-4 expression allowed dissection of local (lung) from systemic effects of IL-4 during influenza infection. The results indicating quantitative modifications regarding specific CTL were similar to those obtained by another group, using a different strategy for local IL-4 expression (Aung *et al.,* 1999).

The cytokine as well as the antibody response might have contributed to the clearance of influenza virus by the IL-4 transgenic mice. Previous studies showed that IL-4 does not necessarily preclude the generation of proinflammatory cytokines (Erb *et al.,* 1996; Powrie *et al.,* 1993). These results are concordant with our surprising finding regarding the limited effect of local IL-4 expression on IFN-γ production, subsequent to heterologous challenge. Furthermore, the local expression of IL-15, a cytokine that has activity similar to that of IL-2, was enhanced in the IL-4 transgenic mice compared to non-transgenic mice. These findings have two implications: first, they show that there are exceptions to the rule that strong Th1 responses could not coexist with increased levels of IL-4 and second, they pinpoint a potential mechanism that contributes to the recovery from virus infection, in the context of reduced local CTL activity. Such protective mechanisms leading to influenza virus clearance independent of CTL function were previously suggested (Bot *et al.,* 1998; Scherle *et al.,* 1997; Topham *et al.,* 1996). The mechanisms that mediate such effects remain elusive, but at least two might occur: (i) a direct one that limits the virus replication in infected cells and (ii) an indirect one, exerted by increased titers of protective antibodies that have the ability to clear the pulmonary virus (Graham and Braciale, 1997). This last possibility is directly supported by our data showing significant virus neutralizing titers in IL-4 transgenic mice but not littermates early after infection, when protective mechanisms are likely to have a higher impact on the outcome. This result may be explained by the direct positive effect of IL-4 on local B cells specific for virus HA, consistent with the classically described function of IL-4 as a "B cell growth factor" (reviewed by Paul, 1991).

In summary, transgenic expression of IL-4 exerted negative effects on the local function of memory $CD8^+$ T cells during heterologous challenge with influenza virus. This was associated with up-regulation of CD30 and a delayed but not abrogated clearance of pulmonary virus during secondary infection. Nevertheless, the limited inhibition of Th1-type cytokines together with a faster *de novo* neutralizing antibody response contributed to the ability of IL-4 transgenic mice to control the virus infection. Our findings indicate that influenza infection might take a slightly more prolonged and severe course in situations where IL-4 expression is increased in the lungs.

MATERIALS AND METHODS

Mice

IL-4 transgenic mice (IL-4 tg) that bear the IL-4 gene controlled by the Clara cell protein 10 promoter (Rankin *et al.,* 1996) were bred onto the C57BL/6 background for at least six generations and maintained by sister–brother mating. As controls, we have used littermates that did not bear the transgene, C57BL/6 as well as IL-4 knockout mice (The Jackson Laboratories, Bar Harbor, ME), in certain experiments. The IL-4 transgene was routinely detected by PCR using primers specific for the inserted construct, 5′-CCCCAGCTAGTTGTCATCC-3′ and 5′-TAGTGCTCTTTAGGCTTTCC-3′. The mice were housed under specific pathogen-free conditions at the Animal Facility of The Scripps Research Institute (La Jolla, CA). In most of the experiments, we used mice younger than 10 weeks.

Viruses, immunization, and infection

We used three strains of influenza virus, A/HK/68 (H3N2), A/PR/8/34 (H1N1), and B/Lee/40, that were grown on 10-day-old embryonated chicken eggs, as well as one strain A/WSN/32 (H1N1) grown on MDBK cells. The original viruses were obtained from the Mount Sinai School of Medicine (New York, NY) or kindly provided by Linda Sherman at The Scripps Research Institute. Mice were immunized via the intraperitoneal route with allantoic fluid diluted in 100 μl of sterile PBS containing 1×10^4 $TCID_{50}$ of HK. Primary infection was carried out with 5×10^4 $TCID_{50}$ of PR8 virus by intranasal instillation with a volume of 40 μl of allantoic fluid diluted in sterile PBS after anesthesia with metofane. Secondary infection was carried out with 1×10^6 $TCID_{50}$ of PR8 virus. The secondary challenge was carried out 1 month after immunization. Mice were sacrificed at different intervals after infection and various samples (lungs, spleen) were harvested. In certain cases, nasal washes and blood samples were obtained previous to euthanasia.

Measurement of virus titers

Mice were sacrificed 7 days after the infection, lungs were harvested, homogenized, and resuspended in a total volume of 2 ml, with sterile PBS. Serial 10-fold dilutions of homogenate were incubated in triplicate for 1 h with trypsinized MDCK cells seeded 1 day previously in 96-well flat-bottom plates. Complete RPMI–10% FCS medium was added and the cells were incubated for 48 h at 37°C in a humidified CO_2 incubator. After incubation, the supernatants were harvested and coincubated with washed chicken red blood cells for 1 h at room temperature, in 96-well U-bottom plates. The titers were com-

puted by interpolation according to previously established methods (Isobe *et al.,* 1994), taking into consideration the highest dilution of lung homogenate exhibiting detectable hemagglutination. Data were expressed as $TCID_{50}$/volume corresponding to the lungs (2 ml).

Nasal washes were carried out following anesthesia with metofane. Mice were instilled in a drop-wise manner with a total volume of 150 μl of sterile PBS. The nasal fluid was recovered on petri dishes, collected in sterile tubes, and immediately stored at −80°C. The virus titers were measured as described above and the results were expressed as $TCID_{50}$/ml.

Immunohistochemistry

Seven days after primary infection or 5 days after secondary infection the mice were sacrificed following anesthesia with halothane, by bleeding from the axillary artery. The lungs were removed, submerged in resin (TissueTek), and quick frozen on dry ice. Tissue sections were prepared on a cryostat, placed on SuperFrost glass slides, air-dried for 5 min, and fixed in 90% ethanol for 10 min at 4°C. The slides were air-dried again and blocked with 2% FCS/PBS (20 min), followed by biotin and avidin (10 min each) (VectaStain, Vector Laboratories). The sections were washed with PBS and incubated for 1 h at room temperature with anti-CD4, anti-CD8 rat anti-mouse antibodies (PharMingen, San Diego, CA; 1:200 RM4–5 and 1:200 Ly-3.2 + 1:200 Ly-2, respectively), or anti-CD30 hamster anti-mouse antibody (1:100; PharMingen). After being washed, the sections were incubated with biotin-conjugated anti-rat or anti-hamster antibodies (1:200) for 30 min at room temperature. The sections were washed again and incubated with avidin-horseradish peroxidase (Vector ABC kit, Vector Laboratories) and substrate (DAB). The substrate reaction was blocked with saline and the sections were counter-stained for 5 min with hematoxylin (Sigma). After a wash with saline, the sections were mounted and read with a microscope. Controls carried out with secondary antibody showed the lack of background.

Cytotoxicity assays

Mice were sacrificed at day 7 after primary infection or day 5 after secondary infection and spleens were harvested. Single-cell suspensions were obtained by mincing and passing through cell strainers. The red blood cells were lysed with Tris hypotonic buffer and after a wash, the splenocytes were resuspended in complete 10%–FCS RPMI, supplemented with 50 μM 5-mercaptoethanol and 10 U/ml of human rIL-2. Stimulator cells were prepared from spleens harvested from mice of similar genotype, irradiated, and infected for 1 h with PR8 influenza virus (m.o.i. = 5), at 37°C in 1% BSA–DMEM. After an extensive wash, the stimulator and responder cells were coincubated in triplicate at various R/S ratios in 96-well flat-bottom plates, at 37°C and 5% CO_2. After 5 days of incubation, EL-4 target cells (class I^+ class II^-), noninfected or infected with virus (m.o.i. = 5, at 37°C for 1 h, in DMEM–1% BSA) and labeled with ^{51}Cr, were added to the effector cells (5×10^3 target cells/well). Supernatants were harvested after 4 h and radioactivity was measured in a gamma-counter. The results were expressed as specific lysis for particular R/S ratios: [(release − spontaneous release)/(maximum release − spontaneous release)] × 100 − background against noninfected target cells. The results provided by such an experimental set-up were a function of the initial frequency as well as *in vitro* expansion of virus-specific CTL.

RNase protection assay

Lung tissue was harvested on day 5 after secondary infection, immersed in Trizol reagent (TriReagent, Molecular Research Center, Inc.), and homogenized using a tissue mincer. The total RNA was extracted with chloroform followed by isopropanol precipitation and washing with ethanol. After assessment of RNA quantity and quality by spectrophotometry and electrophoresis, we have hybridized the RNA overnight with labeled probes specific for a large array of cytokine messages, provided by a commercial kit (Riboquant, MCK4-3 and MCK4-1; Pharmingen). The RNase protection assay was carried out according to the manufacturer's instructions. The gel was scanned using a STORM 860 Phospho Imaging System (Molecular Dynamics) and the intensity of bands corresponding to various protected mRNAs was quantified using ImageQuant Software (Molecular Dynamics). The results were expressed in arbitrary units relative to the mRNA level of a housekeeping message (L32).

ELISPOT analysis

Nitrocellulose-treated 96-well flat-bottom plates (Millipore) were coated with anti-IFN-γ or anti-IL-4 antibodies, overnight at 4°C (4 μg/ml, in sterile PBS). The wells were blocked with 10% FCS–RPMI for 1 h at 37°C. After being washed with sterile PBS, syngeneic stimulator cells (3×10^5 cells/well) were coincubated with responder cells (threefold dilutions, beginning with 3×10^5 cells/well) in the presence of complete FCS–RPMI medium supplemented with 20 U/ml of human rIL-2 and sucrose purified PR8 virus (4 μg/ml), class I-restricted peptide (NP-D^b 366–374 ASNENMETM), or a mixture of anti-CD3 (2 μg/ml) + anti-CD28 (2 μg/ml) (PharMingen). As control, cells were incubated without antigens but in the presence of IL-2. The stimulator cells were mytomicin-treated splenocytes prepared from animals of matched genotype. No stimulator cells were added in the case of polyclonal stimulation. Some responder cells were prepared by lung digestion with collagenase (Sigma) for 1 h at 37°C, subsequent to tissue fragmentation. The plastic adherent

cells were removed by a 30-min incubation in petri dishes, at 37°C. After 72 h of incubation at 37°C and humidified atmosphere in the presence of 5% CO_2, the cells were washed off with 0.05% Tween–PBS and biotin-conjugated anti-cytokine antibodies were added overnight at 2 μg/ml in 0.1%–FCS 0.05% Tween–PBS. The antibody pairs were purchased from PharMingen. The wells were washed again and incubated with 1:1000 streptavidin:peroxidase (Vector Laboratories) for 1 h at room temperature, followed by substrate reaction. The spots were counted using a stereomicroscope, after the reaction was stopped with tap water and the wells were air-dried. Three to four animals/group were individually studied.

Hemagglutination inhibition assay

The hemagglutination inhibition assay was carried out after treatment of sera with receptor destroying enzyme (RDE/neuraminidase; Sigma, St. Louis, MO) overnight at 37°C, in order to remove the nonspecific hemagglutination-inhibiting activity due to the serum sialoproteins. The RDE was inactivated by incubation with 2.5% sodium citrate at 56°C, for 30 min. The twofold serial dilutions of RDE-treated sera were incubated with 0.5% human erythrocyte saline suspension in the presence of agglutinating titers of influenza virus. The experiment was carried out in triplicate wells. After 45 min of incubation in 96-well round-bottom flexible plates (Falcon) at room temperature, the results were read and expressed as endpoint titers, corresponding to the highest dilution of sera associated with inhibition of hemagglutination.

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