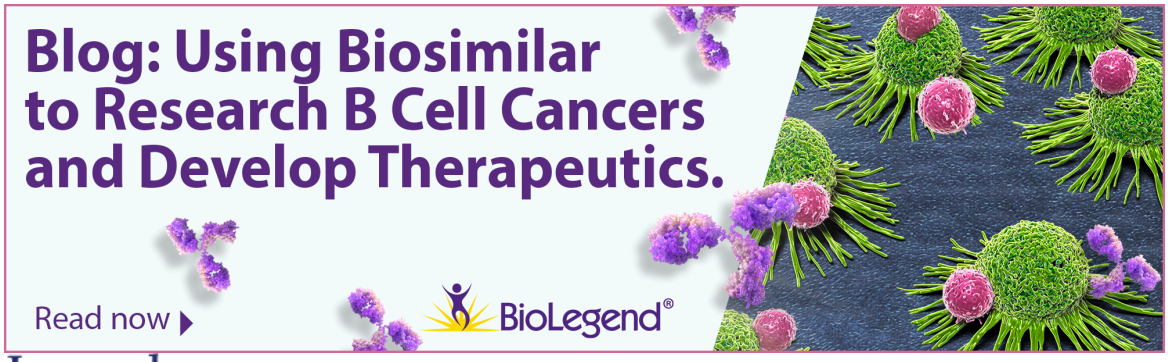

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Cellular Mechanisms Involved in Protection Against Influenza Virus Infection in Transgenic Mice Expressing a TCR Receptor Specific for Class II Hemagglutinin Peptide in $CD4^+$ and $CD8^+$ T Cells[1]

Adrian Bot,* Sofia Casares,* Simona Bot,* Harald von Boehmer,† and Constantin Bona[2]*

Mice transgenic for a TCR that recognizes $peptide_{110-120}$ of hemagglutinin of PR8 influenza virus in the context of MHC class II I-E^d molecules express the transgenes in both $CD4^+$ and $CD8^+$ T cells. We have found that these TCR-hemagglutinin (TCR-HA) transgenic mice display a significantly increased resistance to the primary infection with PR8 virus compared with the wild-type mice. The TCR-HA transgenic mice mounted significant MHC type II and enhanced MHC type I-restricted cytotoxicity as well as increased cytokine responses in both spleen and lungs after infection with PR8 virus. In contrast, the primary humoral response against PR8 virus was not significantly different from that of the wild-type mice. In vivo depletion and adoptive cell transfer experiments demonstrated that both $CD4^+$ and $CD8^+$ TCR-HA^+ T cell subsets were required for the complete clearance of pulmonary virus following infection with a dose that is 100% lethal in wild-type mice. Whereas $CD4^+$ TCR-HA^+ T cells were necessary for effective activation and local recruitment of $CD8^+$ T cells, $CD8^+$ TCR-HA^+ T cells showed a Th1-biased pattern and MHC type II-restricted cytotoxicity. However, in the absence of in vivo expression of MHC type I molecules on the infected cells, the protection conferred by the TCR-HA^+ T cells was impaired, indicating that the enhanced MHC class I-restricted cytotoxicity due to TCR-HA^+ $CD4^+$ Th cells was a critical element for clearance of the pulmonary virus by the transgenic mice. ***The Journal of Immunology,*** **1998, 160: 4500–4507.**

In the thymus, the engagement of TCR$\alpha\beta$ by MHC class I molecules leads to differentiation of the $CD3^{low}$ $CD4^+CD8^+$ thymocytes into $CD8^+CD4^-$ T cells, whereas the interaction with MHC class II molecules leads to differentiation into $CD4^+CD8^-$ T cells. This positive selection process, which depends not only on MHC molecules but also to some extent on peptides presented by thymic epithelial cells (1, 2), ensures that T cells leaving the thymus recognize the Ags in association with self-MHC molecules.

Based on studies conducted in wild-type or TCR$\alpha\beta$ transgenic (Tg)[3] mice, it has been assumed that the full maturation of a T cell clone requires coengagement of MHC molecules by TCR and coreceptors, namely CD8 in the case of T cells recognizing the peptide in association with class I Ag and CD4 in the case of T cells recognizing the peptide in association with class II molecules (3, 4). However, exceptions from this paradigm were reported previously (5, 6). One of the exceptions is illustrated by TCR-HA Tg mice (7) expressing a TCR specific for $peptide_{110-119}$ of PR8 influenza virus hemagglutinin (HA), presented in the context of I-E^d class II molecules (8). In this mouse strain, the TCR transgene is expressed in both mature $CD4^+$ and $CD8^+$ T cell subsets (7). This indicates that TCR restriction imposed by MHC molecules does not always dictate the CD4/CD8 phenotype of mature T cells. Furthermore, recent evidence showing a significant shift of MHC class II-restricted T cells to the CD8 lineage in the absence of CD4 coreceptors is consistent with a quantitative instructional model for T-lineage differentiation (9).

There is a large body of evidence indicating that virus-specific CTLs are part of the response to influenza infection (10, 11) and are able to mediate virus clearance from infected lungs (12). However, in β_2m-deficient ($\beta_2{}^{-/-}$) mice that lack $CD8^+$ CTLs, $CD4^+$ T cells can successfully clear type A influenza viruses from the lungs of infected animals (13). Furthermore, the clearance of influenza virus by $CD4^+$ T cells did not depend on MHC class II expression on lung epithelial cells (14), although significant numbers of MHC class II-restricted CTLs were recovered from the lungs of influenza virus-infected, $\beta_2{}^{-/-}$ mice (13, 15). These studies underlined the pleiotropic effects of the virus-specific, MHC class II-restricted T cells in the defense against influenza virus: cytokine secretion, help for Ab production, and, in certain circumstances, cytotoxicity. These observations motivated us to study the mechanisms of protective response and recovery from influenza virus infection in TCR-HA Tg mice.

We addressed three major questions. First, we asked whether an expanded T cell population specific for only one of the three immunodominant epitopes of HA recognized in the context of class II molecules mediates significant protection following primary infection with influenza virus. Second, we asked whether the class

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[3] Abbreviations used in this paper: Tg, transgenic; TCR-HA Tg, mice transgenic for T cell receptor-hemagglutinin; HA, hemagglutinin; $\beta_2{}^{-/-}$, β_2-microglobulin-deficient mice; LCMV, lymphocytic choriomeningitis virus; $TCID_{50}$, tissue culture 50% infective dose; pCTL, cytotoxic T lymphocyte precursor; HI, hemagglutination inhibition; LD_{100}, 100% lethal dose.

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II-restricted TCR-HA$^+$ T cells exert their protective effects directly or via other lymphocyte subsets. Particularly, we addressed the question of whether CD8$^+$ or CD4$^+$ TCR-HA$^+$ T cells suffice for protection against pulmonary influenza virus infection. Third, this experimental system can provide information regarding the role of Th cells in priming and recruitment of virus-specific MHC class I- or MHC class II-restricted CTLs following influenza virus infection. Previous studies conducted in other experimental systems led to conflicting results; whereas in LCMV infection, CD4$^+$ T cells were not required for induction of the CTL response (16), they were required for the generation of herpes-virus specific MHC class I-restricted CTLs (17).

We demonstrate that TCR-HA$^+$ T cells confer increased protection to primary infection with influenza virus and that they act in a pleiotropic manner. The virus-specific MHC class II-restricted CTLs alone are not sufficient for clearance of the pulmonary virus. The T cells expressing TCR-HA secrete large amounts of cytokines and induce an enhanced activation and local recruitment of virus-specific MHC class I-restricted CTLs.

Materials and Methods

Mice

Six- to eight-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). TCR-HA Tg mice, prepared as previously described (7), were backcrossed to BALB/c (H-2^d) and maintained by sister-brother breeding in the Mount Sinai animal facility (New York, NY). The presence of TCR transgenes was determined by PCR from tail DNA using two pairs of primers corresponding to insert sequences (7). One pair is composed of the forward primer 5′-AATGAACCTTTATCCTGAAC and the reverse primer 5′-ATTGCCTCCACTCAGAGCAC; the second pair contains the forward primer 5′-TAGGAGAAAGCAATGGAGAC and the reverse primer 5′-GTACCTGGTATAACACTCAG.

Mice deficient for β_2m ($\beta_2^{-/-}$) (18) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Reagents and viruses

$HA_{110-120}$ synthetic peptide corresponding to 110 to 120 amino acid residues (SFERFEIFPKE) of PR8 influenza virus HA was prepared by solid phase synthesis using F-moc technology and purified by reverse phase HPLC using a C_2/C_{18} column (Pharmacia Biotech, Uppsala, Sweden).

Rat anti-murine CD4 (GK1.5, American Type Culture Collection (ATCC), Rockville, MD), anti-murine CD8 (TIB 210, ATCC), and anti-TCR transgene (6.5) (7) were purified from culture supernatants on an anti-rat κ mAb (MAR18.5, ATCC)-Sepharose 4 column. Rat IgG (technical grade) was purchased from Sigma Chemical (St. Louis, MO). For in vivo depletion experiments, mice were injected on days -2, -1, and 0 with 0.5 mg of mAb i.p. and i.v. (2/1, v/v) in saline, according to a schedule adapted from a previously described protocol (19). Mice were challenged on day 0 as described below.

Influenza A/PR8/34 (H1N1) and A/HK/68 (H3N2) viruses were grown on allantoic fluid and purified by sucrose gradient ultracentrifugation. Batches of UV-attenuated virus were prepared by 20-min exposure to a UV light source (BBL Microbiology Systems, Albuquerque, NM) under stirring.

Infection and measurement of the pulmonary lung titers

Naive TCR-HA Tg or BALB/c mice were challenged via the aerosol route with doses of 7.5×10^4, 2.5×10^4, 1.5×10^4, or 7.5×10^3 $TCID_{50}$ of PR8 virus/aerosol chamber, according to a previously described technique (20). Other groups of naive BALB/c and TCR-HA Tg mice were infected with doses of 1.5×10^5 or 1.5×10^4 $TCID_{50}$ of HK virus. The survival was followed for 20 days following infection. Virus lung titers of the survivor mice were measured on day 20 following challenge. In subsequent experiments, lungs were harvested on days 3, 7, and 16 from two or three mice in each group, and viral titers were determined as previously described, using a Madine Darby kidney carcinoma cell-chicken RBC hemagglutination assay (20).

Negative selection of CD4$^+$ and CD8$^+$ T cells and adoptive cell transfer

Spleen and lymph node cells (10^8) from Tg mice were treated with 4 ml of hypotonic lysis buffer and then enriched for T cells on a 10-ml Unisorb T&B nylon wool column according to the manufacturer's instructions (Nycomed, Oslo, Norway). The enriched population of T cells (5×10^7) suspended in 1% BSA-PBS was incubated for 15 min with iron beads (BioMag, Perseptive Biosystems, Framingham, MA) coupled to goat anti-rat IgG (H+L) and coated with 100 μg/ml of rat anti-mouse CD4 mAb (GK1.5, ATCC) or 100 μg/ml of a mixture (1/1, w/w) made of two rat anti-mouse CD8 mAbs (TIB105 and TIB210, ATCC). The supernatant containing either CD4 or CD8 negatively depleted T cells was collected, resuspended in Iscove's modified Dulbecco's medium supplemented with 10% FCS, and analyzed for purity by flow cytometry (EPICS Profile II analyzer, Coulter Clone, San Diego, CA) using double staining with anti-CD4 FITC and anti-CD8 biotin conjugates according to standard procedures. FACS analysis showed that >95% of the T cell populations express CD4 or CD8 Ag, respectively.

Unseparated T cells from naive TCR-HA Tg mice were infused via the tail veins in BALB/c or $\beta_2^{-/-}$ mice at 7×10^7 cells/recipient. Recipient mice were infected 30 min later with a dose of 1.5×10^4 $TCID_{50}$ of PR8 virus/aerosol chamber. Alternatively, 5×10^7 CD4$^+$ T cells or 2.5×10^7 CD8$^+$ T cells from TCR-HA Tg mice were adoptively transferred into BALB/c or $\beta_2^{-/-}$ recipient mice that were subsequently infected with PR8 virus. Each recipient group comprised between two and four mice. Seven days after challenge, mice were sacrificed, and spleens and lungs were harvested for cytotoxicity and pulmonary virus titer assessment.

Cytotoxic assay and estimation of pCTL frequency

Nylon wool-enriched T cells from lymph nodes and spleens or negatively selected CD4$^+$ or CD8$^+$ T cells from TCR-HA Tg mice were cultured with PR8 virus-infected or $HA_{110-120}$ peptide-coated and irradiated spleen cells from BALB/c mice in RPMI supplemented with 10% FCS and 50 μM 2-ME. After 3 days of culture, the cytotoxic assay was conducted by incubating various numbers of effector cells with 5×10^3 ^{51}Cr-labeled target cells in 96-well, V-bottom plates. As target cells, we used P815 cells (class I$^+$, class II$^-$) infected with PR8 virus or M12 B lymphoma cells (class I$^+$, class II$^+$) incubated with 10 μg/ml of $HA_{110-120}$ peptide. After a 4-h incubation at 37°C in 5% CO_2, supernatants were harvested, and radioactivity was measured using a gamma counter. The results were expressed as the percent specific lysis, determined in triplicate for each E:T cell ratio as follows: [100 × (actual − spontaneous release)/(maximum − spontaneous release)] − background.

pCTL frequency estimation was conducted using a method previously described (21). Briefly, single-cell suspensions of splenic responder cells were incubated in six steps of twofold dilution with 2.5×10^5 irradiated syngeneic splenocytes and $HA_{110-120}$ peptide (5 μg/ml) or with PR8-infected stimulator cells. After 5 days in complete RPMI, individual microtiter cultures were assayed by ^{51}Cr release from M12 cells coated with $HA_{110-120}$ peptide. Uncoated M12 cells were used as controls. In parallel experiments we used as targets noninfected and PR8-infected P815 cells, respectively. Those wells exhibiting ^{51}Cr release above background plus 3 SD were considered positive. The percentage of cultures in one dilution step regarded as negative was plotted logarithmically against the number of responder cells per well, and the frequency of CTL precursors was determined by linear regression analysis using the following formula: 1 ÷ number of responder cells/well at the negative well index of 0.37. The number of precursor cells is represented as 1/frequency for purposes of comparison. An identical protocol was used for the estimation of pCTL frequencies in populations of lymphocytes separated from lungs, according to a previously described method (22).

Lymphokine production

Nylon wool-enriched T cells (1×10^5)from spleens or lungs of TCR-Tg or BALB/c mice were harvested on day 3 or 7 after challenge and incubated for 4 days with 2.5×10^5 irradiated BALB/c spleen cells in the presence of IL-2 (1 U/ml; Boehringer Mannheim, Indianapolis, IN) and sucrose-purified UV-attenuated PR8 virus (10 μg/ml). The concentrations of cytokines in supernatants were determined by ELISA, using IFN-γ and IL-4 kits (Biosource International, Camarillo, CA) and were expressed as picograms per milliliter. Values below the background + 2 SD were considered 0.

Estimation of the hemagglutination-inhibition (HI) titers

The measurement of serum HI titers was conducted according to a previously described protocol (23). Briefly, sera were incubated overnight at

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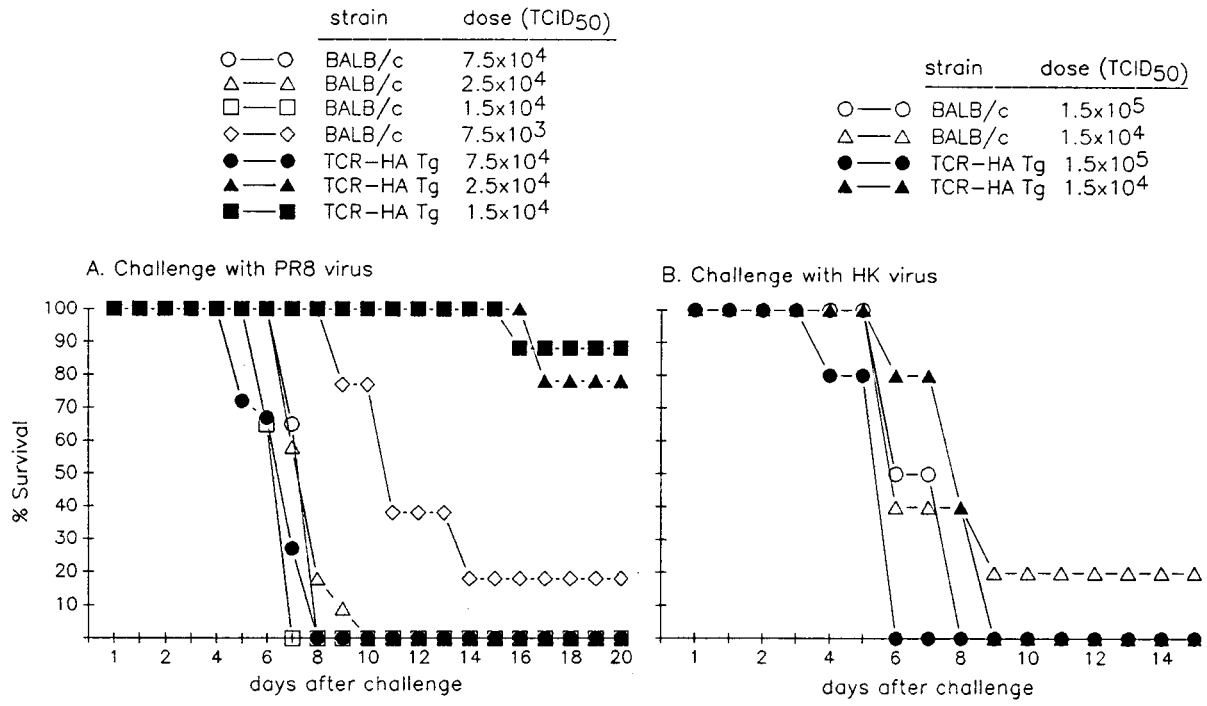

FIGURE 1. Survival of naive BALB/c and TCR-HA Tg mice after aerosol challenge with various doses of PR8 (*A*) or HK (*B*) viruses. Mice were followed until day 20 after infection, and the surviving mice were sacrificed to assess the clearance of the pulmonary virus. At least seven mice in each group were challenged, and the percent survival was plotted against time.

37°C with receptor-destroying enzyme (Cholera Filtrate, Sigma Chemical). Twofold dilutions of sera were incubated at room temperature with an appropriate titer of PR8 virus in the presence of human RBCs. The HI titer was read as the end-point dilution of serum that gave inhibition of hemagglutination. The values were expressed as the geometric mean ± SE.

Statistical analysis

Differences in virus lung titers among various groups of mice were analyzed by estimating *p* values of statistical significance determined by the *t* test. Data regarding survival were analyzed using Fisher's exact test.

Results

Striking differences in survival between BALB/c and TCR-HA Tg mice challenged with various doses of PR8 virus

The most faithful criteria to evaluate the protective host response against an infectious agent is to determine the LD_{100}. Studies of survival of naive wild-type and TCR-HA Tg mice challenged with various doses of PR8 virus showed that the TCR-HA Tg mice were more resistant than normal mice (Fig. 1). While the LD_{100} for BALB/c mice was 1.5×10^4 $TCID_{50}$, it was 5 times higher in the case of TCR-HA Tg mice, namely 7.5×10^4 $TCID_{50}$. Further, whereas LD_{50} was higher than 2.5×10^4 $TCID_{50}$ for the Tg mice, it was less than 7.5×10^3 $TCID_{50}$ in the case of BALB/c mice (Fig. 1). TCR-HA Tg mice challenged with doses of 1.5×10^4 or 2.5×10^4 $TCID_{50}$, which are both 100% lethal doses for BALB/c mice, displayed significant survival rates of 90 and 80%, respectively ($p < 0.001$ and 0.004, respectively). Furthermore, the mice that survived until day 20 following challenge displayed no pulmonary virus or weight loss (data not shown). To rule out possible environmental factors responsible for the greater protection of the TCR-HA Tg mice, groups of BALB/c and TCR-HA Tg mice were infected with two different doses of A/HK/68 (H3N2) virus, which lacks the $HA_{110-120}$ epitope of the A/PR8/34 (H1N1) virus. No significant differences in survival subsequent to challenge with HK virus were noted between the BALB/c and TCR-HA Tg mice (Fig. 1). The striking differences in survival rates between normal and TCR-HA Tg mice infected with PR8 virus strongly suggest that T cells expressing the Tg TCR are functional and play a critical role in the protection and recovery from primary influenza virus infection.

Pulmonary virus titers of TCR-HA Tg mice injected with mAbs and challenged with a lethal dose of virus

Recovery from influenza pneumonia is mainly due to the clearance of virus from infected lung cells by CTLs (10), but cytokines and Abs are thought to play a role as well (14). To study the protective mechanisms of TCR-Tg mice, we measured the virus pulmonary titer of BALB/c and TCR-HA Tg mice 3 and 7 days after challenge with a dose of 1.5×10^4 $TCID_{50}$ of PR8 virus. The data depicted in Table I show no significant differences between normal and TCR-HA Tg mice on day 3 after challenge. In sharp contrast,

Table I. *Virus lung titers of BALB/c and TCR-HA Tg mice treated with various Abs previous to challenge with a dose of 1.5×10^4 $TCID_{50}$ of PR8 virus*

Group	Day 3	Day 7	Day 16
BALB/c	4.6 ± 0.2[b]	2.9 ± 0.5	NS[c]
TCR-HA Tg	4.3 ± 0.6	<1.0	<1.0
TCR-HA Tg preinjected with:[a]			
Rat IgG	2.7 ± 0.2	<1.0	ND[d]
Anti-CD4 mAb	3.3 ± 0.4	3.2 ± 0.4	NS
Anti-CD8 mAb	3.4 ± 0.0	2.1 ± 0.4	NS
6.5 mAb	3.1 ± 0.4	3.7 ± 0.5	NS

[a] Mice challenged on day 0 were preinjected at day −2, −1, and 0 with 500 μg of Ab i.v. + i.p. in saline. Two to four mice in each group were sacrificed on day 3, 7, and 16 after challenge, and the pulmonary virus titers were individually estimated.
[b] Results are expressed as mean $\log_{10}$ $TCID_{50}$ ± SD.
[c] NS, no survivors.
[d] ND, not determined.

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Table II. *Estimation of the frequency and the total number of MHC class I restricted, virus-specific pCTLs in BALB/c and TCR-HA Tg mice infected with PR8 virus*

Group[a]	Day	1/Frequency		Total/Organ[b]	
		Spleen	Lung	Spleen	Lung
BALB/c	d3	6.6×10^5	5.5×10^5	10^2	10
	d7	6.3×10^4	9.4×10^3	9.8×10^2	6.4×10^2
TCR-HA Tg pretreated with:					
Nil	d3	1.9×10^5	1.1×10^5	3.0×10^2	6.0×10^2
	d7	1.1×10^4	7.8×10^3	5.5×10^3	3.2×10^3
Anti-CD4 mAb	d3	2.1×10^5	$>1 \times 10^6$	1.1×10^2	—[c]
	d7	2.2×10^5	1.5×10^4	10^2	2.9×10^2
Anti-CD8 mAb	d3	$>1 \times 10^6$	$>1 \times 10^6$	—	—
	d7	$>1 \times 10^6$	$>1 \times 10^6$	—	—
6.5 mAb	d3	4.5×10^5	3.4×10^5	1.2×10^2	26
	d7	3.4×10^4	2.4×10^4	3.1×10^2	4.2×10^2

[a] pCTL frequency estimation was carried out on day 3 and 7 after infection using pooled splenocytes from three animals in each group.
[b] Estimation of the total number of pCTL/organ was carried out taking into consideration the average number of cells separated from spleens or lungs in each group.
[c] —, No significant CTL activity was measured.

while the virus was largely cleared by day 7 from the lungs of TCR-HA Tg mice, only a small reduction of viral titers was observed in wild-type mice (difference between the two groups, $p < 0.025$). None of the surviving Tg mice displayed virus in their lungs on day 16 after challenge.

To determine what T cell subset is critical for the clearance of virus, we studied the effects of the following mAbs injected into TCR-HA Tg mice previous to the infection: 6.5 mAb specific for the Tg TCR, GK1.5 anti-CD4, and TIB210 anti-CD8. Treatment with GK1.5 and TIB210 was previously shown to result in the depletion of $CD4^+$ and $CD8^+$ T subsets, respectively (19) (data not shown). In contrast, FACS analysis of T cells from the peripheral blood of TCR-HA Tg mice before and 3 days after the last inoculation of 6.5 mAb showed a minimal depletion effect (<10% of the TCR-HA^+ T cells; data not shown). This suggests that the 6.5 mAb has a reduced ability to induce complement-dependent deletion, a result concordant with its IgG1 isotype as well as the Th2-activating effect (see Table IV). The data depicted in Table I show that there were no significant differences in virus lung titers on day 3 after the challenge among Tg mice treated with rat IgG or the mAbs mentioned above. In contrast, on day 7, TCR-HA Tg mice injected with anti-CD4, anti-CD8, or anti-TCR Abs displayed significant pulmonary virus titers compared with Tg mice injected with rat IgG, which successfully cleared the virus by day 7 after challenge ($p = 0.005–0.05$). These results suggest that $CD4^+$ together with $CD8^+$ T cells expressing the TCR transgene play an important role in the protection and recovery from influenza virus pneumonia of Tg mice.

Enhanced class I-restricted CTL response in TCR-HA Tg mice challenged with PR8 virus

It is well established that while anti-HA Abs prevent penetration of virus into cells, and anti-neuraminidase Abs prevent virus spreading, specific T cells play an important role in recovery from influenza pneumonia. They can prevent virus replication by synthesis of lymphokines, such as IFN-γ, or by lysing the infected cells (10, 24). Peptides from various influenza gene products, such as nucleoprotein, HA, and M protein, are produced by the endogenous pathway and are expressed on the surface of infected cells in association with MHC class I molecules (25). Thus, we estimated the frequency of virus-specific MHC class I-restricted pCTLs in the spleens and lungs of wild-type and Tg mice following aerosol infection with PR8 influenza virus, using as targets P815 mastocytoma cells, which express MHC class I and MHC class II molecules. The TCR-HA Tg mice displayed significantly higher MHC type I-restricted virus-specific pCTL frequencies in both spleen and lung compared with BALB/c mice (Table II). The total number of pCTLs in the lungs was approximately 60 times higher in TCR-HA Tg mice than in BALB/c mice on day 3 after the challenge. On day 7, the Tg mice still showed approximately 1 log more MHC class I-restricted pCTLs in both spleen and lungs. Interestingly, pretreatment of TCR-HA Tg mice with 6.5 mAb decreased the activation and the recruitment of MHC class I-restricted pCTLs (Table II). As expected, pretreatment with anti-CD8 mAb prevented a CTL response. Inoculation of anti-CD4 mAb led to a significant decrease in MHC class I-restricted pCTL frequencies, below the values found in BALB/c mice (Table II). Together, these data indicate that the MHC class II-restricted TCR-HA^+ T cell subset facilitates an early and enhanced MHC class I-restricted CTL response to the virus. Apparently, the MHC class I-restricted CTL response is dependent on the $CD4^+$ T cell subset.

MHC class II-restricted CTL response in TCR-HA Tg mice infected with PR8 virus

In pilot experiments, we determined the ability of splenic and lymph node cells from TCR-HA Tg mice to lyse PR8 virus-infected M12 cells that express MHC class II I-E^d molecules subsequent to in vitro stimulation with $HA_{110–120}$ synthetic peptide. Both the splenic and lymph node cells were able to lyse the target cells, and the magnitude of specific lysis paralleled the number of T cells expressing the clonotype marker of the TCR transgene, as defined by 6.5 mAb (data not shown). In additional experiments, $CD4^+$ and $CD8^+$ T cells were negatively selected from lymph node and splenic cell populations and stimulated for 48 h with $HA_{110–120}$ peptide. The data depicted in Figure 2 show that $CD8^+$ T cells efficiently lysed PR8 virus-infected M12 cells. A low, but significant, cytotoxicity was also observed in the case of $CD4^+$ T cells. These results are in agreement with previously reported data (7), demonstrating that the $CD8^+$ T cells from TCR-HA Tg mice display most of the MHC class II-restricted CTL activity in this Tg strain.

The data depicted in Table III show that while PR8-infected BALB/c mice displayed no class II-restricted cytotoxicity, significant numbers of $HA_{110–120}$-specific pCTLs were detected in the

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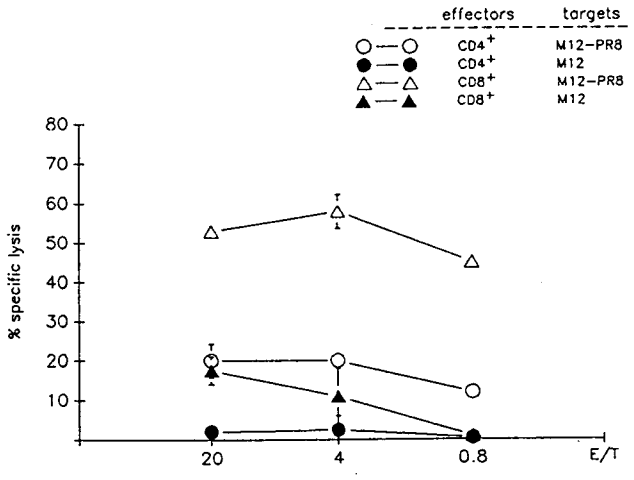

FIGURE 2. CTL activity of $HA_{110-120}$ specific $CD4^+$ and $CD8^+$ T cells. Pooled splenic and lymph node T cells from Tg mice were enriched by nylon wool passage, and $CD4^+$ and $CD8^+$ T cells were obtained by negative selection with magnetic beads coated with anti-CD8 or anti-CD4 mAbs, respectively. T cells were incubated with $HA_{110-120}$ peptide in the presence of haplotype-matched, irradiated splenocytes and were tested 48 h later in a standard 4-h ^{51}Cr release assay against M12 B lymphoma cells infected, or not infected, with PR8 virus. The percent specific lysis was represented as a function of the E:T cell ratio.

spleens and lungs of TCR-HA Tg mice challenged with PR8 virus. Interestingly, although increased numbers of MHC type II-restricted CTLs were present on day 3 in the spleen and lungs of Tg mice, no significant expansion of these cells occurred thereafter. Preinjection of Tg mice with anti-CD8 or 6.5 mAb completely abolished the MHC class II-restricted CTL response. In contrast, depletion of $CD4^+$ T cells led to a delay of pCTL expansion in the spleen, whereas the recruitment of MHC class II-restricted CTLs in the lungs was significantly impaired (Table III). Thus, while most of the in vivo MHC class II-restricted CTL activity of the PR8-infected TCR-HA Tg mice is mediated by $CD8^+$ T cells, $CD4^+$ T cells are critical for the local recruitment of CTLs.

Increased cytokine production in lymphocytes isolated from spleen or lungs of TCR-HA Tg mice infected with PR8 virus

It is well documented that different subsets of Th cells secrete various types of ILs that mediate distinct helper functions (26) or inhibit virus replication (27). To determine the potential contribution of lymphokines to the recovery of TCR-HA Tg mice from influenza pneumonia, we studied the lymphokine production by splenocytes and lymphocytes separated from lung tissue following the challenge with PR8 influenza virus.

This study was motivated by a preliminary experiment that showed that T cells from nonimmunized TCR-HA Tg mice were able to produce large amounts of IFN-γ and IL-4 after in vitro stimulation with $HA_{110-120}$ peptide or PR8 virus (data not shown).

A dramatic difference in the production of lymphokines was observed between TCR-HA Tg and normal BALB/c mice subsequent to aerosol challenge with a dose of 1.5×10^4 $TCID_{50}$ of PR8 virus (Table IV). First, the lymphocytes from Tg mice secreted large amounts of IFN-γ and IL-4 as early as 3 days after infection, when the cytokine levels of the BALB/c mice were still undetectable. Second, 7 days following infection, the cytokine levels secreted by splenocytes from Tg mice were approximately 1 log higher than those in BALB/c mice. Further, in sharp contrast with BALB/c mice, the lymphocytes isolated from the lungs of Tg mice on day 7 after infection displayed a pronounced Th1 profile (Table IV).

The evaluation of cytokine synthesis by the splenic and lung lymphocytes after virus challenge of Tg mice injected with mAbs showed a major contribution of the class II-restricted TCR-HA^+ T cells. Pretreatment with 6.5 mAb resulted in a dramatically different Th pattern, with a strong Th2 profile dominating the immune response in spleen on day 7 following infection (Table IV). In contrast, injection of rat IgG was not followed by a significant change in the Th profile. These results show that injection of 6.5 mAb is followed by the activation of a subset of splenocytes with the Th2 profile. The in vivo data correlate with previous in vitro experiments that showed a Th2-biased profile of TCR-HA^+ T cells after stimulation with immobilized anti-TCR, anti-Vβ8, or anti-CD3 mAbs (data not shown).

Interestingly, whereas the treatment with anti-CD4 mAb led to a relative decrease in IL-4 production by the splenocytes of Tg mice infected with PR8 virus, the depletion produced by anti-CD8 mAb led to a pronounced Th2 profile in the spleen on both days 3 and 7 after challenge (Table IV). Treatment with anti-TCR, anti-CD4, or anti-CD8 resulted in decreased levels of cytokines secreted by the cells isolated from lung. Together, these results suggest that while the $CD8^+$ TCR-HA^+ T cells differentiate mostly to

Table III. *Estimation of the frequency and the total number of MHC class II-restricted $HA_{110-120}$-specific pCTLs in BALB/c and TCR-HA Tg mice infected with PR8 virus*

Group[a]	Day	1/Frequency		Total/Organ[b]	
		Spleen	Lung	Spleen	Lung
BALB/c	d3	$>1 \times 10^6$	$>1 \times 10^6$	—[c]	—
	d7	$>1 \times 10^6$	$>1 \times 10^6$	—	—
TCR-HA Tg pretreated with:					
Nil	d3	2.0×10^4	5.9×10^4	3.0×10^3	1.0×10^3
	d7	1.8×10^4	1.9×10^4	3.3×10^3	1.5×10^3
Anti-CD4 mAb	d3	6.4×10^4	2.5×10^5	3.5×10^2	15
	d7	1.2×10^4	7.2×10^4	1.8×10^3	60
Anti-CD8 mAb	d3	$>1 \times 10^6$	$>1 \times 10^6$	—	—
	d7	$>1 \times 10^6$	$>1 \times 10^6$	—	—
6.5 mAb	d3	$>1 \times 10^6$	$>1 \times 10^6$	—	—
	d7	$>1 \times 10^6$	$>1 \times 10^6$	—	—

[a] pCTL frequency estimation was carried out on day 3 and 7 after infection using pooled splenocytes from three animals in each group.
[b] Estimation of the total number of pCTL/organ was carried out taking into consideration the average number of cells separated from spleens or lungs in each group.
[c] —, No significant CTL activity was measured.

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Table IV. *Cytokine secretion by splenic and lung lymphocytes harvested from TCR-HA transgenic animals pretreated with various Abs and challenged with PR8 virus*

Group	Cytokines	Day 3 Spleen	Day 3 Lung	Day 7 Spleen	Day 7 Lung
BALB/c mice	IFN-γ	0[b]	0	74 ± 34	46 ± 18
	IL-4	8 ± 2	6	24 ± 20	156 ± 12
TCR-HA Tg mice treated with:[a]					
Nil	IFN-γ	355 ± 1	23 ± 8	478 ± 70	250 ± 8
	IL-4	390 ± 48	102 ± 18	436 ± 88	80 ± 4
Rat IgG	IFN-γ	ND	ND	304 ± 95	ND
	IL-4	ND	ND	392 ± 56	ND
6.5 mAb	IFN-γ	131 ± 14	53 ± 3	162 ± 31	22 ± 5
	IL-4	355 ± 25	0	1000 ± 97	0
Anti-CD4 mAb	IFN-γ	163 ± 19	28 ± 3	354 ± 25	23 ± 3
	IL-4	42 ± 1	11 ± 3	27 ± 6	0
Anti-CD8 mAb	IFN-γ	109 ± 12	25 ± 3	52 ± 10	9 ± 2
	IL-4	167 ± 21	0	587 ± 113	0

[a] Six mice in each group were injected with mAb and challenged with PR8 virus on day 0. Mice were sacrificed on day 3 and 7 after infection, and cells were harvested from spleens and lungs.

[b] After 4 days of restimulation with PR8 virus, the cytokines were estimated by ELISA and expressed as mean of duplicates ± SD (pg/ml).

Tc1-type cells with the ability to migrate into the lung upon infection with PR8 virus, $CD4^+$ TCR-HA^+ T cells differentiate mostly to Th2 cells.

Lack of direct involvement in virus clearance of class II-restricted CTLs

To dissect out the role of the TCR-HA^+ T cell subsets in the protection against PR8 virus, we conducted a set of adoptive transfer experiments using as recipients BALB/c mice infused with unseparated or negatively selected $CD4^+$ or $CD8^+$ T cells from Tg mice. Recipient mice were infected with LD_{100} of PR8 virus. As shown in Table V, whereas the BALB/c mice infused with TCR-HA Tg cells completely cleared the virus by day 7, neither the mice receiving $CD4^+$ cells nor the mice receiving $CD8^+$ cells from Tg mice were able to clear the virus.

As expected, $\beta_2{}^{-/-}$ mice failed to clear the virus and to mount a CTL response. Infusion of unseparated or negatively selected $CD4^+$ or $CD8^+$ T cells from Tg mice into $\beta_2{}^{-/-}$ mice did not restore the ability to clear the virus (Table V). It is noteworthy that BALB/c mice infused with unseparated or $CD8^+$ T cells from Tg mice developed both class I- and class II-restricted CTLs in the spleen, while $\beta_2{}^{-/-}$ mice developed only class II-restricted CTLs.

These results indicate that neither $CD4^+$ nor $CD8^+$ TCR-HA^+ T cells alone can clear the virus and that both subsets are required for an effective defense reaction following the employment of class I-restricted $CD8^+$ T cells.

Table V. *Pulmonary virus titers and CTL activities of splenocytes from wild-type or $\beta_2{}^{-/-}$ mice infused with TCR-HA transgenic T cells*

Group		Pulmonary Virus Titer[a]	CTL Activity MHC-I restricted[b]	CTL Activity MHC-II restricted[c]
BALB/c		2.9 ± 0.5	32 ± 4[d]	7 ± 6
$\beta_2{}^{-/-}$		3.4 ± 0.7	5 ± 4	3 ± 1
TCR-HA Tg		<1.0	58 ± 6	59 ± 5
Infusion:	Recipient:			
TCR-HA Tg T	BALB/c	<1.0	40 ± 7	21 ± 5
TCR-HA $CD8^+$ T	BALB/c	2.7	57 ± 12	20 ± 3
TCR-HA $CD4^+$ T	BALB/c	2.2 ± 0.3	21 ± 3	7 ± 5
TCR-HA Tg T	$\beta_2{}^{-/-}$	3.4	9 ± 4	32 ± 3
TCR-HA $CD8^+$ T	$\beta_2{}^{-/-}$	3.6 ± 1.2	10 ± 2	24 ± 3
TCR-HA $CD4^+$ T	$\beta_2{}^{-/-}$	2.6 ± 0.2	4 ± 3	6 ± 4

[a] Mice infused or not with transgenic T cells were infected with 1 LD_{100} of PR8 influenza virus and the pulmonary virus titers were measured on day 7 following the challenge.

[b] Splenocytes were harvested on day 7 following the challenge and restimulated in vitro with PR8 virus. CTL activity was tested against P815 target cells infected with PR8 virus at an E:T ratio of 10.

[c] Splenocytes were restimulated with $HA_{110-120}$ peptide and tested against M12 target cells coated with $HA_{110-120}$ peptide at an E:T ratio of 10.

[d] Percentage of specific lysis.

Ab response of TCR-HA Tg and BALB/c mice challenged with PR8 virus

To address the role of the humoral response in the recovery of TCR-HA Tg mice from the primary infection with PR8 virus, we measured the serum titers of HI Abs on days 3 and 7 after challenge. The BALB/c and TCR-HA Tg mice exhibited similar titers of PR8-specific HI Abs on day 7, whereas the titers on day 3 were undetectable (Table VI). Synthesis of anti-HA Abs elicited by primary infection is $CD4^+$ T cell dependent in both wild-type and Tg mice. As shown in Table VI, pretreatment with anti-CD4 mAb precluded the synthesis of protective Abs. Interestingly, $\beta_2{}^{-/-}$ mice infused or not with TCR-HA Tg cells displayed significantly

Table VI. *HI titers of PR8-specific Abs from sera of mice inoculated with Abs or infused with TCR-HA T cells*

Group	Treatment Before Challenge[a]	HI Titer (GMT ± SE) Day 3	HI Titer (GMT ± SE) Day 7
BALB/c	Nil	<40	1280
$\beta_2{}^{-/-}$	Nil	<40	242 ± 171
TCR-HA Tg	Nil	<40	970 ± 61
	Inoculated with:		
TCR-HA Tg	rat IgG	<40	1280
TCR-HA Tg	6.5 mAb	<40	1280
TCR-HA Tg	anti-CD4 mAb	<40	<40
TCR-HA Tg	anti-CD8 mAb	<40	1280
	Infused with:		
BALB/c	TCR-Tg T cells	<40	1576 ± 60
$\beta_2{}^{-/-}$	TCR-Tg T cells	<40	<40

[a] Mice were challenged with 1 LD_{100} dose of PR8 virus, and the blood was harvested 7 days after infection.

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impaired humoral responses against influenza virus. This observation is in agreement with previously reported studies addressing the immunogenicity of recombinant vaccinia vectors expressing influenza Ag inoculated into $\beta_2^{-/-}$ mice (28). Together, these results show that the humoral response is not responsible for the enhanced protection of TCR-HA Tg mice, since it is not significantly increased compared with that in BALB/c mice, although its role among other mechanisms cannot be excluded.

Discussion

Many lines of evidence indicate that thymocytes must be able to recognize thymic class I and class II molecules to be positively selected for export from the thymus and to become $CD4^+CD8^-$ or $CD8^+CD4^-$, functionally mature T cells (29). However, exceptions to this rule exist (7, 24) and are consistent with a quantitative instructional model of CD4/CD8 lineage differentiation (9).

In this study, we used a Tg mouse strain in which both $CD4^+$ and $CD8^+$ T cells express the TCRα and -β transgenes and use I-E^d molecules as elements for positive selection, since they recognize in periphery the cognate $HA_{110-120}$ peptide in association with I-E^d (7). FACS analysis data showed that between 5 and 20% of T splenocytes from adult TCR-HA Tg mice express the TCR transgenes, most of them bearing the CD4 coreceptor. We could also consistently detect a T cell subset with the $CD3^+CD8^+CD4^-$ TCR^+ phenotype in the spleen, peripheral blood, and thymus of Tg mice, which represented between 0.5 and 3% of the $CD3^+$ population (data not shown).

Because of the relatively high frequency of class II MHC-restricted T cells specific for a single virus epitope, this Tg model allows us to study the role of these cells during viral infection. An important finding of our study is the striking ability of nonimmunized TCR-HA Tg mice to develop a vigorous immune response enabling them to survive the challenge with a virus dose that kills 100% of naive BALB/c mice. We found that the LD_{100} for PR8 influenza virus was 5 times higher for Tg mice than for their wild-type counterparts. This observation shows that an expanded T cell population expressing a TCR specific for a single immunodominant epitope of HA in context of MHC class II molecules was able to confer protection and recovery from influenza pneumonia. Previous studies showed that LCMV infection of Tg mice expressing a TCR specific for a class I-restricted peptide derived from the LCMV glycoprotein induced specific effector and memory cells (30). However, our data demonstrate for the first time that primary infection with a lethal dose of virus elicited a protective response in Tg mice expressing a TCR specific for an epitope recognized in association with class II molecules. This observation underlines both the functionality of TCR^+ T cells as well as the role of MHC class II-restricted immune response in the defense against influenza virus. Previous studies suggested that the limited protection conferred by virus-specific T cells is due to the relatively slow kinetics of the T cell response (11). Here, we show that the size of a virus-specific T cell clone effectively compensates for the slow kinetics of the T cell response, resulting in enhanced protection against lethal challenge.

The increased host defense reaction of TCR-HA Tg mice is due to the pleiotropic effects of the $HA_{110-120}$-specific class II-restricted T cell population. In contrast to wild-type mice, TCR-HA Tg mice infected with PR8 virus show 1) an increased virus-specific, MHC-I restricted cytotoxicity; 2) detectable virus-specific MHC class II-restricted CTLs; and 3) increased cytokine secretion by splenic and lung lymphocytes. No significant differences between TCR-HA Tg and BALB/c mice were noted in terms of protective Abs (Table VI), indicating that the cellular response plays the major role in the enhanced protection of the TCR-HA Tg mice.

Both adoptive cell transfer experiments and treatment of Tg mice with anti-clonotype mAb demonstrated the critical role of $TCR\text{-}HA^+$ T cells for pulmonary virus clearance. However, it is noteworthy that neither the $CD4^+$ nor $CD8^+$ $TCR\text{-}HA^+$ T cells alone are sufficient for protection against influenza virus (Tables I and V). Apparently, the two main subsets of $TCR\text{-}HA^+$ T cells must cooperate during a protective response. The results of adoptive cell transfer experiments in $\beta_2^{-/-}$ mice (Table V) strongly suggest that the increased MHC class I-restricted cytotoxicity is an important element, required for the protection of Tg mice.

Despite the fact that the MHC class II-restricted $TCR\text{-}HA^+$ CTLs were not sufficient for protection, their role in the protective response of Tg mice cannot be ruled out, since the adoptive cell transfer experiments showed that $CD4^+$ $TCR\text{-}HA^+$ T cells cannot solely confer protection to BALB/c recipients (Table V). This contrasts with the results reported by Scherle et al. (31), who showed the protective ability of $CD4^+$ T cell clones when adoptively transferred into nude mice. The apparent discrepancy can be due to the fact that we used in our experimental protocols $CD4^+$ T cells from nonimmunized Tg mice, rather than Ag-stimulated cells. In Tg mice pretreated with anti-CD8 mAb, the cytokine secretion of the splenocytes is largely skewed toward a Th2 pattern, whereas mice injected with anti-CD4 mAbs showed a more pronounced Th1 pattern (Table IV). This suggests that the $CD8^+$ $TCR\text{-}HA^+$ T cells may be of the Tc1 phenotype. Recently, $CD8^+$ T cells were classified into two subsets, namely Tc1 and Tc2, based on the pattern of cytokine secretion (32). Previous studies underlined the importance of Th1 cells to the immunity against influenza virus as well as the detrimental role of excessive Th2 responses (33) and, in particular, of high levels of IL-4 (34). Conversely, $CD4^+$ $TCR\text{-}HA^+$ T cells, although predominantly differentiating to Th2 cells, are nevertheless required for a rapid expansion and local recruitment of both MHC class I- and MHC class II-restricted CTLs (Tables II and III). The ability to mediate protection of the MHC class II-restricted CTLs, rapidly recruited into the lungs of TCR-HA Tg mice (Table III), is eventually dependent on the expression of viral peptides in the context of MHC class II molecules on the infected cells. It is reasonable to accept the involvement of direct cytotoxicity because following viral infection, epithelial cells begin to express MHC class II molecules on their surface (35). Alternatively or in addition, $CD8^+$ $TCR\text{-}HA^+$ T cells may inhibit virus replication by secreting large amounts of IFN-γ or other cytokines. Indirectly, IFN-γ induces MHC type II expression and up-regulation of MHC type I on the infected cells (35).

The increased frequency of MHC class II-restricted CTLs after infection as well as following i.p. immunization with live virus or constructs bearing $HA_{110-120}$ peptide (36) showed that the $TCR\text{-}HA^+$ T cells of Tg mice received optimal stimulation through TCR, leading to their proliferation and differentiation into effector cells. The rapid induction of MHC class II-restricted cytotoxicity following in vitro or in vivo stimulation associated with the rather surprisingly significant immunogenic effect of the same peptide presented by self molecules as well as the prompt production of IFN-γ and IL-4 suggest that a subset of $TCR\text{-}HA^+$ T cells may behave like a primed cell population even in the absence of previous exposure to the nominal Ag. Furthermore, FACS analysis showed that 0 to 10% of $TCR\text{-}HA^+$ T cells from spleens of nonimmunized Tg mice expressed CD25 activation marker (data not shown). A recent study showed that a proportion of the $CD4^+$ $TCR\text{-}HA^+$ T cells expressed activation markers in the absence of intentional antigenic stimulation (37). It was shown that this activation was due to the binding of an endogenous ligand to TCR-HA

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rather than to occupancy of an alternate TCR. Interestingly, study of OVA-specific Tg mice also revealed a subset of activated TCR-OVA$^+$ T cells in nonprimed mice (38). In this case, a second TCR was a prerequisite for the presence of TCR-OVA$^+$ T cells with an activated phenotype in nonimmunized mice. Consequently, caution must be taken when TCR-HA$^+$ T cells from nonimmunized Tg animals are assumed to be naive.

In conclusion, the findings presented in this study show that the T cells specific for a single MHC class II-restricted immunodominant epitope of an influenza virus protein (HA) confer a strong protective immunity in nonimmunized Tg mice. The enhanced generation and recruitment of both class II- as well as class I-restricted CD8$^+$ CTLs is regulated by CD4$^+$ TCR-HA$^+$ T cells that are activated subsequent to virus challenge. The CD8$^+$ TCR-HA$^+$ T cells contribute to the protection and recovery of Tg mice from influenza pneumonia through IFN-γ production and, possibly, MHC class II-restricted cytotoxicity. Neither the CD8$^+$ nor the CD4$^+$ TCR-HA$^+$ T subset alone is sufficient in mediating complete virus clearance. MHC class I expression was required for complete virus clearance, indicating that the enhanced MHC class I cytotoxicity was crucial for the protection of TCR-HA Tg mice.

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