

# A Lung-specific Neo-antigen Elicits Specific CD8<sup>+</sup> T Cell Tolerance with Preserved CD4<sup>+</sup> T Cell Reactivity

## Implications for Immune-mediated Lung Disease

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### Abstract

The A/Japan/57 influenza hemagglutinin (HA) was expressed in BALB/c mice under the transcriptional control of the surfactant protein C (SP-C) promoter, resulting in expression of HA in type II alveolar epithelial cells, as well as low level variable expression in other tissues, including the thymus in some of the founder lines. Transgenic animals were able to recover from infection with A/Japan/57 influenza, and they were able to mount antibody responses to A/Japan/57 HA in titers similar to wild type. We therefore tested their CD4<sup>+</sup> T lymphocyte responses to HA and found them to be similar to wild type responses. However, CD8<sup>+</sup> T cells from A/Japan/57-infected transgenic animals were unable to express cytolytic activity against target cells expressing the A/Japan/57 HA. The CD8<sup>+</sup> T cell tolerance was also extremely specific, since transgenics immunized with an influenza strain containing a single amino acid substitution in a dominant HA epitope were able to mount full cytolytic responses to that epitope, but not the wild-type epitope. Adoptive transfer of CD8<sup>+</sup> T cell clones into transgenic animals resulted extensive interstitial pneumonitis that was antigen-specific and associated with significant morbidity and mortality. We conclude that a lung-specific transgene may lead to specific CD8<sup>+</sup> T cell tolerance, with CD4<sup>+</sup> T cell and B cell reactivity to the antigen, and that CD4<sup>+</sup> T cell reactivity may remain intact to an antigen expressed in the thymus, even when CD8<sup>+</sup> T cell tolerance exists. This observation may have profound implications concerning immune-mediated lung diseases, particularly those mediated by CD4<sup>+</sup> T cells. (*J. Clin. Invest.* 1996; 98:914–922.) Key words: lymphocyte • tolerance • transgene • alveolar cell • lung injury

### Introduction

Encounters between lymphocyte receptors and antigens do not inevitably result in activation of the lymphocyte, but may

actually result in its death or inactivation, the net effect of which is the biologic phenomenon referred to as immunologic tolerance. At least two such effects have been described: thymic (deletional) tolerance, and peripheral (nondeletional) tolerance. A variety of models have been developed for the study of central (1–3) and peripheral (4, 5) tolerance. The immunophenotypic appearance of these models is quite varied, and it is unclear what factors dictate when the expression of a particular self antigen results in central deletion (for review see reference 6), or in anergy, deletion, or neither in the periphery (for review see reference 5).

The first example of clonal deletion was reported by Kappler et al. (1), who found that the vast majority of T cells reactive with I-E class II molecules carried TCR containing the V $\beta$ 17 chain. Mice not expressing I-E possessed a large fraction of the V $\beta$ 17-positive cells, whereas I-E-positive strains showed deletion of these potentially autoreactive T cells in the thymus. Similarly, Kisielow et al. (2), used TCR transgenic animals, whose TCR were directed against the male HY antigen restricted by H-2D<sup>b</sup>, and demonstrated thymic deletion of CD4<sup>+</sup> CD8<sup>+</sup> thymocytes in the males, but not in the females.

Since many tissue-specific antigens may not be represented in the thymus, the establishment of peripheral tolerance has been the subject of much recent interest, and an abundance of data has emerged from models using tissue-specific expression of MHC transgenes (7). For example, Miller et al. expressed H-2K<sup>b</sup> under the control of the rat insulin promoter and found that in vitro cytotoxic responses to the transgene could not be elicited (8). However, Hammerling et al. (5) showed in vitro responses (mixed lymphocyte reaction) in transgenic animals carrying K<sup>b</sup> expressed in keratinocytes, Schwann cells, or small intestine, but not in animals carrying K<sup>b</sup> expressed in hepatocytes.

In contrast to transgenic MHC alloantigens, non-MHC antigens must be processed and presented for TCR recognition, so the mechanisms for tolerance induction may be different. Several different models have been used to study mechanisms of tolerance to viral transgenes expressed in the pancreas (4, 9, 10), in an effort to understand mechanisms underlying type I diabetes, but no models exist for the study of how lung-specific antigens elicit cellular and humoral tolerance. The lung is a much less privileged immunological site subject to constant antigenic exposure and lymphocyte traffic (11).

We have used a transgenic model system in which an influenza hemagglutinin (HA)<sup>1</sup> has been placed under the tran-

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1. Abbreviations used in this paper: APC, antigen presenting cell; HA, influenza hemagglutinin; NP, influenza nucleoprotein; SP-C, surfactant protein C; vv(HA), recombinant vaccinia virus expressing influenza hemagglutinin; vv(NP), recombinant vaccinia virus expressing influenza nucleoprotein; vv(pSC11), recombinant vaccinia virus expressing the control plasmid pSC11; HAI, hemagglutination inhibition.

scriptional control of the surfactant protein C (SP-C) promoter, in order to study the responses to an endogenously synthesized gene product expressed in the lung (12, 13). We have discovered that some founder lines have leaky expression in the thymus, and using these animals we have been able to demonstrate simultaneous CD8<sup>+</sup> T cell antigen-specific "tolerance" (absence of *in vitro* cytotoxic response) and CD4<sup>+</sup> T cell "ignorance" (antigen-specific proliferative response *in vitro* in the absence of *in vivo* spontaneous recognition and injury). We have also shown that adoptive transfer of CD8<sup>+</sup> T cell clones induced extensive interstitial pneumonitis that was antigen-specific and associated with significant morbidity and mortality. These data suggest that some endogenous (non-endosomal) antigens in the thymus may be expressed in such a way as to lead to presentation by class I major histocompatibility antigens and negative selection (deletion) of reactive CD8<sup>+</sup> T cells, while failing to gain access to the class II presentation pathway, resulting in a failure of negative selection of CD4<sup>+</sup> T cells. This implies that the dichotomous rules of antigen presentation elucidated with respect to T cell effector function also apply to thymic (negative) selection, and carries profound implications concerning immune-mediated lung diseases, particularly those mediated by CD4<sup>+</sup> T cells.

## Methods

**Generation of transgenic mice.** Four lines of influenza HA transgenic mice were generated by microinjection of a linearized form of a plasmid containing the open reading frame of the A/Japan/305/57 influenza hemagglutinin (HA), flanked by the SV40 t intron and polyadenylation signal. The surfactant protein C (SP-C) promoter was used in order to achieve lung specific expression (12, 13), and homozygous H-2<sup>d</sup> lines were established. Transgene expression in various organs was studied using reverse transcriptase followed by the polymerase chain reaction (RT/PCR). Briefly, RNA was isolated from harvested organs using acid/phenol/guanidinium isothiocyanate (14), reverse transcribed using MMLV reverse transcriptase, and PCR was performed with Taq polymerase using primers located at the 5' end of the t-intron and the 3' end of the HA open reading frame. All of the data shown refer to studies performed on line 8 animals, except where noted.

*In situ* hybridization for HA mRNA was performed using well characterized methods (15–17). Sense and antisense <sup>3</sup>H-labeled riboprobes were generated and hybridization was carried out at 25°C. After hybridization the specimens were washed and then overlaid with Kodak NTB-2 autoradiography emulsion, and exposed for 4 wk.

**B cell reactivity.** B cell reactivity was assessed with the hemagglutination inhibition assay (HAI). Transgenic and nontransgenic mice were immunized and boosted with A/Japan/57 virus IP. Preimmune sera were drawn, as well as sera after primary and after booster immunization. Serial dilutions of sera were plated along with a defined quantity of A/Japan/57 virus; after 30 min, a small amount of chicken erythrocyte suspension is added. The titer of serum at which hemagglutination is blocked was compared with a target virus of the H2N2 subtype (A/Japan/57) versus a target virus of the H3N2 subtype (A/Memphis/71).

**CD4<sup>+</sup> T cell reactivity.** Transgenic and nontransgenic animals were immunized IP with either vv(HA) or a control strain, vv(pSC11), a vaccinia recombinant carrying a control plasmid, and splenocytes were harvested after 3 wk. They were restimulated *in vitro* with APC loaded with inactivated A/Japan/57 virus (UV Jap; ultraviolet irradiated), and the specificity of the T cells was analyzed in a proliferation assay, in which cells were stimulated with either UV Jap/APC or APC alone, and incorporation of tritiated thymidine was measured. Antibody to CD4 (GK1.5) was added to some of the wells.

In separate experiments splenocytes were generated in the same fashion, and their proliferation was assayed using APC infected with two reassortant strains of live influenza virus, A/JAP/BEL (H2N1) and A/X7/F1 (H1N2).

**CD8<sup>+</sup> T cell responses.** Transgenic animals and control littermates were sublethally infected intranasally with A/Japan/57, and splenocytes were harvested and restimulated 3 wk later *in vitro* with A/Japan/57-infected antigen presenting cells (APC). 5 days after stimulation they were tested for cytolytic activity using a <sup>51</sup>Cr release assay against P815 target cells infected with one of 3 viruses: A/Japan/57, a recombinant vaccinia virus expressing the A/Japan/57 HA (vv[HA]), or a recombinant vaccinia virus expressing the A/Japan/57 nucleoprotein (vv[NP]). Responses were also tested against target cells loaded with 10<sup>-9</sup> M synthetic peptide representing the immunodominant 204–212 epitope.

**Fine specificity analysis.** Transgenic animals and controls were immunized with either A/Japan/57 or A/GV17/57, in order to examine tolerance to an epitope with a single amino acid substitution, and to study the fine specificity of negative selection. A/GV17/57 HA is identical to A/Japan/57 HA except for an N to K substitution at position 207 in an immunodominant 9-mer epitope (a T cell receptor contact residue in HA204–212). 3 wk after infection, splenocytes were harvested and restimulated with A/Japan/57-infected or A/GV17/57-infected APC, respectively. 5 d after stimulation the cells were tested for cytolytic activity using a standard <sup>51</sup>Cr release assay against P815 target cells whose MHC molecules were loaded with synthetic peptides, corresponding to one of the K<sup>d</sup>-restricted epitopes in A/Japan/57 (LYQNVGTYV), A/GV17/57 (LYQKVGTYV), an epitope found in A/Zhang/57 (LYQDVGTYV), or the nucleoprotein (NP147–155) epitope shared by both strains (TYQRTRALV), all at concentrations of 10<sup>-9</sup> M, and E:T of 20:1.

**Adoptive transfer.** CD8<sup>+</sup> and CD4<sup>+</sup> T cell clones used in these experiments were generated by limiting dilution as previously described (18). The clones were restimulated *in vitro* with irradiated autologous splenocytes that were infected with A/Japan/57 influenza. On day 5 after stimulation, clones were separated from stimulators by density gradient centrifugation and injected via the tail vein into transgenic animals. 5 d after transfer, the animals were killed with phenobarbital, their trachea intubated, and their lungs inflated with 10% buffered formalin prior to excision. The lungs were embedded, sectioned and stained with hematoxylin and eosin.

## Results

We expressed the A/Japan/57 hemagglutinin (HA) as a transgene under the transcriptional control of the human surfactant protein C (SP-C) promoter in order to achieve HA expression in mouse alveolar epithelium (12, 13), and four H-2<sup>d</sup> lines were established. Fig. 1 shows the presence of a 426-bp HA fragment amplified by RT/PCR from lung RNA of representative mice from each line. Line 1 mice show very weak pulmonary expression of the transgene, while lungs of line 2 show high level expression of the HA transgene. Line 4 and 8 animals showed intermediate levels of expression. Tissue homogenates of other organs from each line were also tested for HA transcripts by RT/PCR. Noteworthy was the finding that HA expression was detectable in thymic RNA from lines 4 and 8 but not lines 1 or 2. We also sampled RNA from the liver, brain, heart, skeletal muscle, and kidneys of line 2 and 8 mice. As in the thymus, HA gene expression was demonstrable in homogenates of all organs from line 8 mice, but not from line 2 mice (not shown).

*In situ* hybridization for HA expression in transgenic lung sections clearly demonstrates expression in cuboidal to low columnar cells of alveolar epithelium. Expression was most

## EXPRESSION OF HA TRANSGENE

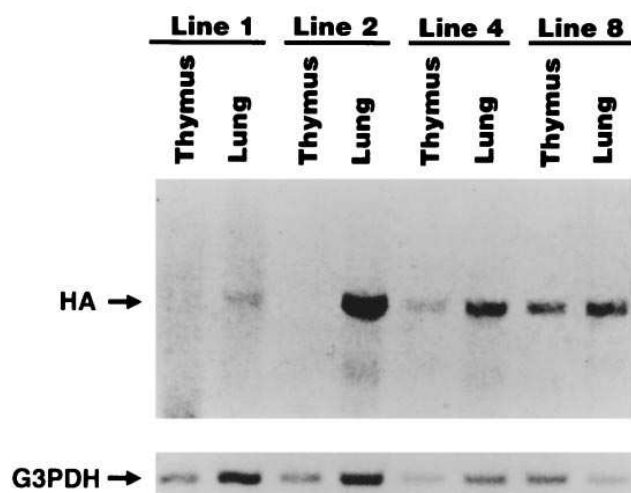


Figure 1. Ethidium bromide stained agarose gel electrophoresis of RT/PCR products produced using RNA extracted from organ homogenates as template. HA-specific primers were used to amplify transgene-specific signals, and control primers were used to amplify a fragment of the glucose-3-phosphate dehydrogenase gene.

prominent at the junction of alveolar walls (Fig. 2) and was thus morphologically correlated with the type II alveolar epithelial cells.

The three transgenic lines tested (2, 4, and 8) showed very similar cellular and humoral immune responses to the A/Japan/57 HA, as well as to A/Japan/57 virus infection, and there were no differences observed between animals heterozygous or homozygous for the transgene. Because of the apparent difference between line 2 and 8 mice in terms of thymic expres-

sion, a more extensive analysis was done to compare responses in these two lines. Both transgenic lines showed identical responses, and therefore, except where noted, the data presented reflects studies performed on line 8 animals.

**Humoral immune responses.** Transgene positive and negative mice showed no difference in susceptibility to virus infection or virus clearance (not shown). This finding was not unexpected. Expression of the HA transgene in the thymus and/or lung of the developing mouse could result in immunological tolerance to the HA by  $CD4^+$  and  $CD8^+$  T lymphocytes, but since T lymphocyte responses to other influenza viral polypeptides in the mouse have been described (19–21) a cellular immune response to one or several of these determinants could account for virus clearance and recovery from infection in the HA-transgenic animals. However, humoral antibody responses also play a role in recovery from influenza infection (22–24), and HA is the major target protein for neutralizing antibody to influenza virus (21, 25, 26), so it was of interest to determine if  $HA^+$  mice could mount an antibody response to HA. To evaluate this we measured the hemagglutination inhibition (HAI) titer of immune sera, which correlates with neutralizing antibody activity (27). Table I shows HAI titer of sera from transgenic and nontransgenic mice bled at day 21 after primary immunization followed by boosting and bleeding 10 d after secondary immunization. Both transgenics and controls showed a 4- to 8-fold increase in HAI titer after the booster immunization, by day ten of the secondary response, when tested against A/Japan/57 virus, but no such response was seen against A/Memphis/71 virus, supporting the conclusion that these represent HA-specific responses, and that there was no difference between the transgenic and nontransgenic responses. These results were also confirmed by ELISA (not shown).

**$CD4^+$  T lymphocyte responses in HA transgenic mice.** The presence of antibody to the HA in transgenic mice indicated an absence of (or at least incomplete) tolerance to the HA in their B lymphocyte compartment. These mice also dem-

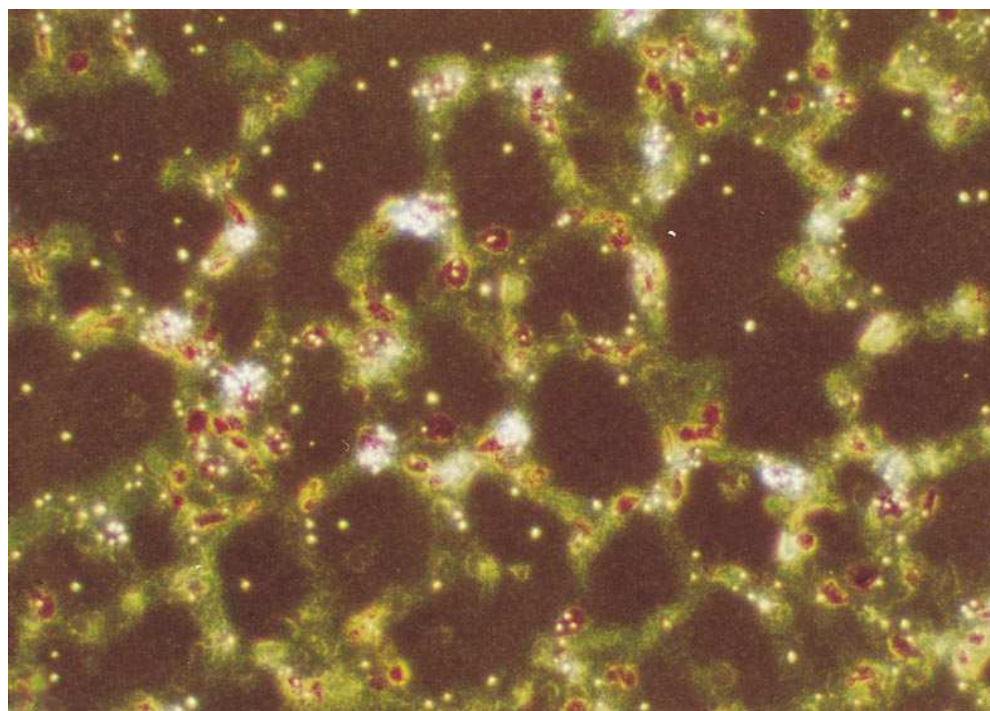


Figure 2. In situ hybridization of lung sections, taken from a transgenic animal (line 1 is shown), with an HA-specific probe. Expression is apparent in cuboidal to low columnar cells of alveolar epithelium, most visibly at the junction of alveolar walls, which is the usual location of type II pneumocytes.

Table I. Anti-Hemagglutinin Antibody Titer

Mouse	H2N2		H3N2	
	Pre	Post	Pre	Post
2.1 (HA-)	512	> 2056	512	512
2.2 (HA-)	256	> 2056	256	256
3.1 (HA+)	64	256	512	512
3.2 (HA+)	256	> 2056	512	512

Hemagglutination inhibition titers from a representative set of animals immunized and boosted with A/Japan/57 influenza: two transgenic and two nontransgenic controls. Values shown are specific titers against A/Japan/57 influenza virions (H2N2) or nonspecific against A/Memphis/71 (H3N2). The titers are measured before and after booster immunization.

onstrated an anamnestic anti-HA response to influenza challenge. Since the secondary antibody response is T lymphocyte dependent, we assessed the responsiveness of transgenic CD4<sup>+</sup> T lymphocytes to HA. Since immunization with type A influenza virus primes for CD4<sup>+</sup> T cell responses to multiple viral polypeptides (28, 29), it was important to selectively examine the HA-specific response of CD4<sup>+</sup> T cells. Our strategy was to first prime the mice by infection with vv(HA), a recombinant vaccinia expressing the cDNA encoding the A/Japan/57 HA. Three weeks later, splenocytes from primed transgene positive and negative mice were stimulated in vitro with non-infectious (UV-inactivated) A/Japan/57 virions. Non-infectious virions preferentially stimulate CD4<sup>+</sup> T cells to the A/Japan/57 virus because they inefficiently enter the MHC class I presentation pathway (29–32). As Fig. 3 shows, pooled splenocytes from transgenic mice mount as vigorous a proliferative response to non-infectious A/Japan/57 virus as nontransgenic splenocytes, after priming with vv(HA). Transgenic mice primed with a control vaccinia showed no response above background to A/Japan/57-infected stimulator cells. Both transgenic and non-

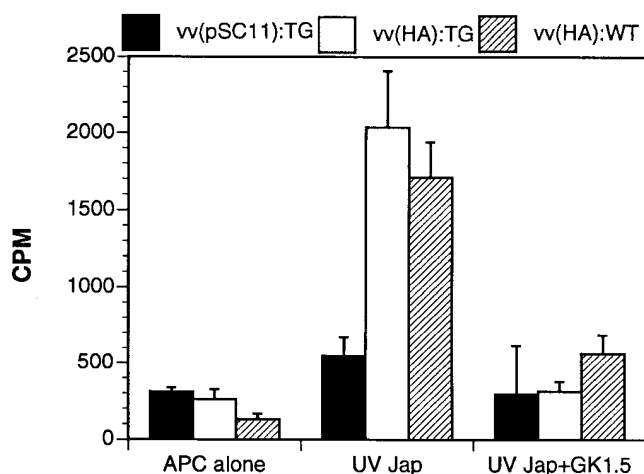


Figure 3. T cell proliferative responses by splenocytes harvested from transgenic (TG) and nontransgenic (WT) animals which immunized with either vv(HA) or a control vaccinia construct, vv(pSC11). The splenocytes were stimulated with APC, either untreated, or treated with UV-inactivated A/Japan/57 virions (with or without the GK1.5 anti-CD4 antibody).

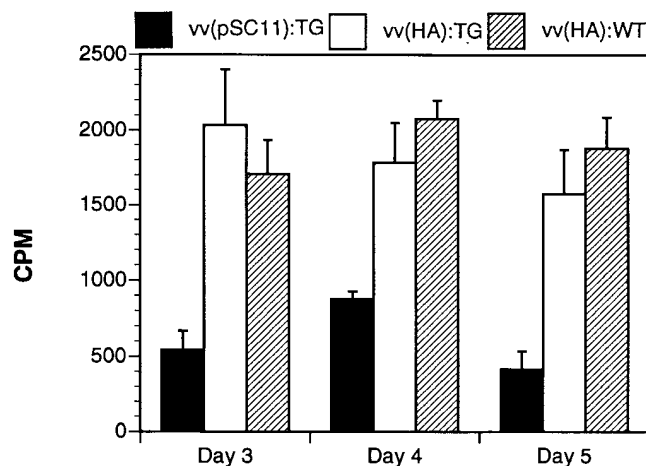


Figure 4. Proliferative responses (in counts per minute) on days 3, 4, and 5 after stimulation with UV-inactivated A/Japan/57 virions from transgenic (TG) and nontransgenic (WT) animals, after immunization with either vv(HA) or a control vaccinia construct, vv(pSC11).

transgenic responses were inhibited by the addition of a monoclonal antibody (GK1.5) specific for the murine CD4 molecule (Fig. 3). A control monoclonal antibody directed to the murine CD8 molecule did not inhibit proliferation (not shown). The kinetics of T lymphocyte proliferation to non-infectious virions were comparable for both HA transgene positive and negative mice (Fig. 4). The data in Figs. 3 and 4 are each representative of three separate experiments. Comparable results were also obtained using primed splenocytes from line 2 mice, with putatively negative thymic expression (data not shown). Supernatants from these bulk T cell cultures were also tested for the presence of IL-2, IL-4, IL-5, and  $\gamma$ -interferon by ELISA, and no differences were seen between the transgenic (of either line 2 or 8) and the nontransgenic cultures (data not shown).

The inhibitory effect of anti-CD4 antibody on T cell proliferation suggested that the response to HA was mediated by

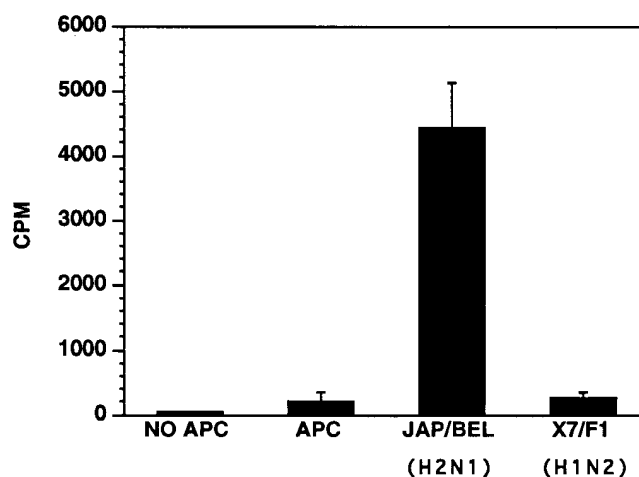


Figure 5. Proliferative responses (in counts per minute) on day 5 after stimulation with A/Japan/57-derived reassortant strains of influenza: A/JAP/Bel and A/X7/F1. All responses were inhibitable with GK1.5, an antibody to CD4 (not shown).

Table II. CD8<sup>+</sup> T Cell Cytolytic Activity

T cells	E:T	P815 Targets (percent specific lysis)					Peptide
		Uninfected	A/Japan/57	vv(HA)	vv(NP)	204-212	
Line 8	5:1	0.9	95.5	1.0	57.8	*	
	10:1	2.1	98.2	1.1	75.7	1.9	
	20:1	3.7	98.4	3.4	79.9	2.9	
Line 2	5:1	0.7	90.1	0.9	72.0	*	
	10:1	2.3	97.5	2.6	81.0	3.6	
	20:1	5.3	100	3.7	84.2	3.9	
Control line	5:1	0.6	93.2	69.8	31.5	*	
	10:1	2.1	98.2	86.2	53.0	64.9	
	20:1	4.4	95.4	87.6	73.0	80.8	
11-1	10:1	12.8	96.6	66.9	7.7	*	
	20:1	*	97.5	67.8	8.6	93.8	

CD8<sup>+</sup> T cell cytolytic activity on P815 targets infected with A/Japan/57 influenza, vv(HA), vv(NP), or treated with 10<sup>-9</sup> M synthetic peptide (HA 204-212), by bulk splenocyte populations. The effectors were derived from line 8 transgenic animals, line 2 transgenic animals, or non-transgenic control animals. Also shown is the cytolytic activity of a CD8<sup>+</sup> T lymphocyte clone (11-1) whose specificity is directed towards the HA 204-212 K<sup>4</sup>-restricted epitope. The effector to target ratios were 5:1, 10:1, or 20:1, as indicated (\* not determined).

CD4<sup>+</sup> T cells. To further confirm this, we examined the proliferative response of splenocytes from VV(HA)-immunized transgenic mice to stimulator cells infected with one of two A/Japan/57-derived reassortant viruses: A/JAP/BEL (which contains the A/Japan/57 HA, but an antigenically unrelated neuraminidase) and A/X7/F1 (which has a neuraminidase identical to that of A/Japan/57 virus, but an antigenically distinct HA). Splenocytes from vv(HA)-immunized mice were harvested and secondarily stimulated in vitro with non-infectious A/Japan/57 virus to selectively expand primed CD4<sup>+</sup> T cells. After ten days of culture, the residual viable cells were restimulated with APC infected with either A/JAP/BEL or A/X7/F1 and proliferative responses were measured. As Fig. 5 shows, splenocytes from transgenic mice showed significant proliferation only to the A/JAP/BEL-infected APC (which express the A/Japan/57 HA). This response was also inhibited by the addition of anti-CD4 antibody to the proliferation assay (not shown).

**CD8<sup>+</sup> T lymphocyte responses of HA transgenic mice.** Although transgenic mice mount both an antibody and a CD4<sup>+</sup> T cell response to the A/Japan/57 HA, these animals failed to mount HA-specific CD8<sup>+</sup> CTL responses. Table II shows a representative response in a standard <sup>51</sup>Cr release cytotoxicity assay of splenocyte effectors from transgene positive and negative mice (lines 2 and 8). Spleens from three mice of each group were removed two weeks after intranasal infection with A/Japan/57 virus and secondarily stimulated in vitro with infectious A/Japan/57 virus. After 6 d in culture, splenocytes were tested for cytolytic activity on P815 (H-2<sup>d</sup> haplotype) cells infected with either A/Japan/57 virus, vv(HA), or vv(NP). Splenocytes from both transgene positive and negative mice efficiently lysed target cells infected with A/Japan/57 or vv(NP). As expected, nontransgenic splenocytes also recognized target cells infected with vv(HA), as well as uninfected

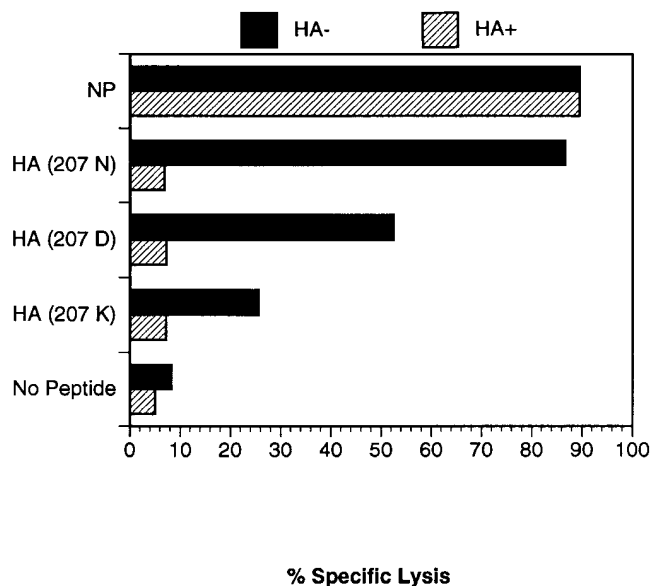
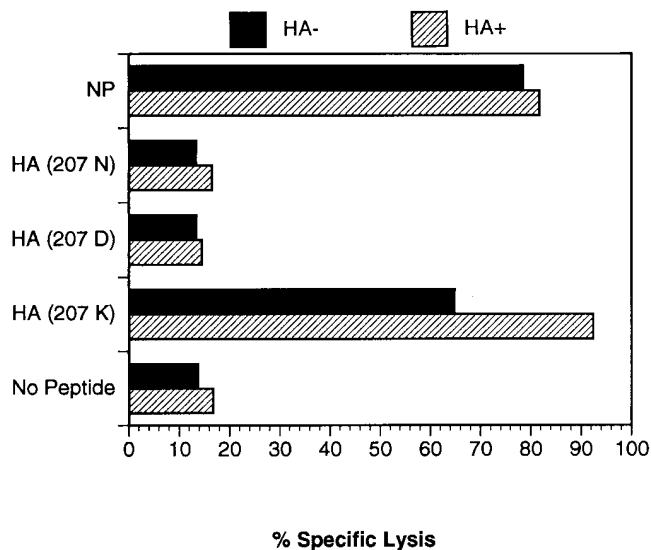


Figure 6. CD8<sup>+</sup> T cell cytolytic activity of bulk splenocyte populations from transgenic animals (filled bars) or nontransgenic control animals (shaded bars) immunized with A/Japan/57 influenza on P815 targets treated with 10<sup>-9</sup> M synthetic peptide. The peptides used were the wild type HA 204-212, or one of two substituted epitopes: 207D (Asp to Asn at position 207), or 207K (Asp to Lys at position 207) by bulk splenocyte populations. The effectors were derived from line 8 transgenic animals, line 2 transgenic animals, or nontransgenic control animals. Also shown is cytolytic activity on untreated P815 target cells, as well as targets treated with a positive control peptide, NP 147-155. The effector to target ratio was 20:1.

P815 cells that were treated with synthetic peptide corresponding to the immunodominant HA epitope, HA 204-212 (33, 34). Neither line 8 nor line 2 transgenic splenocytes recognized vv(HA)-infected or HA 204-212 peptide treated target cells. Similar results were obtained when transgene positive and negative mice were immunized with vv(HA), and secondarily stimulated with A/Japan/57 virus: only transgene negative animals mounted HA-specific CTL responses (not shown).

CTL directed to the dominant HA 204-212 epitope of the A/Japan/57 virus exhibit varying degrees of cross-reactivity to the HA proteins of influenza virus strains that have amino acid substitutions in the HA 204-212 epitope (35). CTL directed to the A/Japan/57 204-212 epitope show strong cross-reactive lysis of target cells expressing the 204-212 epitope of the A/Zhang/57 HA, which differs from A/Japan/57 in a single N to D substitution at position 207. The A/GV17/57 HA has an N to K substitution at position 207 and the corresponding 204-212 epitope is weakly recognized by CTL directed to the HA 204-212 epitope of the A/Japan/57 HA. This selectivity in CTL recognition allowed us to determine if the tolerance to the A/Japan/57 HA in transgene positive mice was equally selective. As shown in Fig. 6, this was indeed the case. CTL from A/Japan/57-immunized transgene negative mice lysed target cells treated with synthetic peptides corresponding to the dominant HA 204-212 epitope and the dominant H-2<sup>d</sup> epitope in the influenza nucleocapsid protein, NP 147-155. These CTL also cross-recognized target cells treated with the HA 204-212D peptide (the A/Zhang/57 epitope), but exhibited weaker cross-reactivity on target cells treated with HA 204-212K peptide



**Figure 7.** CD8<sup>+</sup> T cell cytolytic activity of bulk splenocyte populations from transgenic animals (filled bars) or nontransgenic control animals (shaded bars) immunized with A/GV17/57 influenza on P815 targets treated with 10<sup>-9</sup> M synthetic peptide. The peptides used were the wild type HA 204-212, or one of two substituted epitopes: 207D (Asp to Asn at position 207), or 207K (Asp to Lys at position 207) by bulk splenocyte populations. The effectors were derived from line 8 transgenic animals, line 2 transgenic animals, or nontransgenic control animals. Also shown is cytolytic activity on untreated P815 target cells, as well as targets treated with a positive control peptide, NP 147-155. The effector to target ratio was 20:1.

(the A/GV17/57 epitope). In contrast, the CTL from the A/Japan/57-immunized transgenic mice recognized target cells pulsed with the NP peptide, but none of the HA peptide pulsed targets. In the reciprocal experiment, animals were immunized with the weakly cross-reactive A/GV17/57 virus. After *in vitro* stimulation with A/GV17/57 virus, CTL from both transgene positive and transgene negative mice were able to mount an HA-specific CTL response exclusively to target cells treated with a HA 204-212K synthetic peptide corresponding to the A/GV17/57 HA epitope (Fig. 7). Taken together, these observations suggest that the tolerance of CD8<sup>+</sup> CTL to the A/Japan/57 HA in SPC-HA transgenic mice is highly specific.

**Adoptive transfer.** The absence of a CD8<sup>+</sup> T lymphocyte response to HA in transgenic animals raised the possibility that these mice might be susceptible to pulmonary injury upon exposure to HA specific CD8<sup>+</sup> CTL. Previous experiments in our laboratory have shown that cloned populations of HA-specific CD8<sup>+</sup> CTL clear infectious virus and mediate recovery from influenza infection after adoptive transfer into lethally infected mice (36), and it was of interest therefore to assess whether the adoptive transfer of an HA-specific CD8<sup>+</sup> CTL clone into SP-C HA transgenic mice would result in the development of pulmonary injury. The adoptive transfer of the HA specific CD8<sup>+</sup> CTL clone, 40-2, into transgenic mice resulted in the rapid development (24 to 48 h) of labored breathing, lethargy, and death, by day 4 to 5 after transfer. On gross inspection, the lungs showed consolidation and patchy areas of hemorrhage. Other organs showed no gross abnormalities. Histologic examination of these recipient lungs (Fig. 8 A) revealed severe vasculitis, bronchiolitis, and, most strikingly, ex-

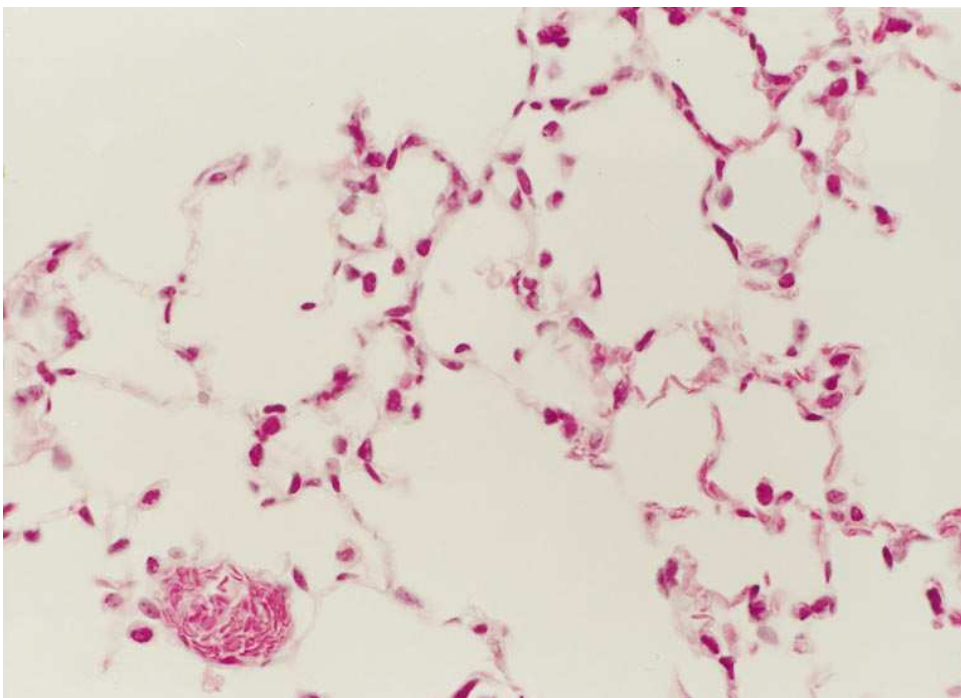
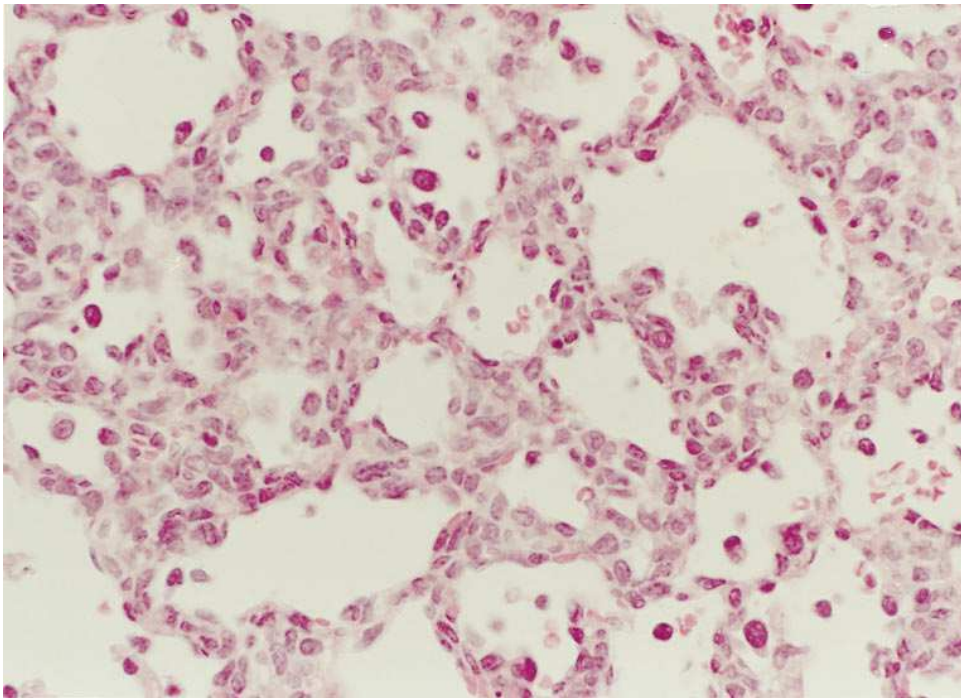
tensive infiltration of the alveolar septa and airspaces with mononuclear cells and concomitant focal disruption of alveolar wall integrity and intraalveolar hemorrhage. This pattern of injury was observed in six separate experiments and was similarly seen after clone transfer into mice of line 1 and 2 (not shown). In contrast, when the NP specific CD8<sup>+</sup> CTL clone 14-13 (37) was transferred into line 8 recipients there were no signs of morbidity or gross evidence of pulmonary injury at necropsy at day 5 after cell transfer. Histologic examination of the lungs of 14-13 recipients (Fig. 8 B) revealed only a modest accumulation of mononuclear cells around pulmonary vessels and bronchioles, but completely intact alveolar structures. This result suggests that a minor component of nonspecific bronchiolitis and vacuolitis appears to accompany adoptive transfer of activated T lymphocytes, but that the lymphocytic alveolitis and alveolar injury produced in transgenic mice was transgene-specific. Adoptive transfer of an HA specific CD4<sup>+</sup> (Th1) clone, V4, produced only mild bronchiolitis and vasculitis, similar to that seen with 14-13, without significant alveolar infiltration.

## Discussion

In this report we have examined the cellular and humoral immune responses to type A influenza virus and tissue injury in transgenic mice expressing the influenza A/Japan/57 HA gene in their lungs under the transcriptional control of the lung specific human SP-C promoter. Both HA transgenic and nontransgenic littermates exhibited comparable responses to intranasal infection with A/Japan/57 with no increased morbidity or mortality evident in the transgene positive animals, in short term experiments. Transgenic animals derived from three different founders mounted antibody and CD4<sup>+</sup> T lymphocyte responses to the HA protein after intranasal priming with the A/Japan/57 virus. Transgene positive mice were, however, unable to mount a detectable CD8<sup>+</sup> CTL response to the HA, and adoptive transfer of HA-specific CD8<sup>+</sup> CTL clones resulted in significant lung injury, morbidity, and mortality.

Our initial finding that HA transgenic mice showed no increased susceptibility to influenza infection was not unexpected. Both CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes have both been shown to play important roles in recovery from murine influenza infection (25, 26). Since the CD8<sup>+</sup> CTL response to the influenza NP is intact in these animals, CD8<sup>+</sup> CTL effectors directed to NP (and possibly other influenza proteins) could function in virus clearance and recovery from infection. Also transgene positive mice were capable of mounting both an anti-HA antibody response and a CD4<sup>+</sup> T cell response to the HA implying that these immune effectors could as well participate in the recovery process. Thus, in contrast to several other transgenic models of tissue-specific expression of viral proteins (9, 10) where virus infection leads to destruction of the tissue expressing the transgene, we find no evidence for enhanced injury in the lungs of SP-C HA expressing mice after infection, in short term experiments. In this regard our results are similar to Lo et al. (4) who also failed to demonstrate enhanced pancreatic beta cell injury after influenza virus infection of transgenic mice expressing the HA gene in the pancreas. The reasons for this difference in the expression of injury between the HA and other viral proteins in transgenic mouse models is likely to be complex (7) and not discernible based on available data.





*Figure 8. (top)* A section of lung harvested from a transgenic animal 5 d after adoptive transfer of an HA-specific CD8<sup>+</sup> T cell clone (40-2). Severe vasculitis, bronchiolitis, and, most strikingly, extensive infiltration of the alveolar septa and airspaces with mononuclear cells and concomitant focal disruption of alveolar wall integrity and intra-alveolar hemorrhage are apparent. *(bottom)* A section of lung harvested from a transgenic animal 5 d after adoptive transfer of an NP-specific CD8<sup>+</sup> T cell clone, (14-13). Minimal periarteriolar and peribronchiolar mononuclear cell infiltration is apparent, with preservation of the structural integrity of the alveolar septa and spaces.

The failure of HA transgenic mice from lines 2, 4, and 8 to mount an HA specific CTL response suggests that there was tolerance to the HA transgene in the CD8<sup>+</sup> T cell compartment. This tolerance was highly selective since transgenic mice expressing the A/Japan/57 HA were able to mount a vigorous, specific CTL response to the HA 204-212 site of the A/GV17/57 virus, which differs from A/Japan/57 HA by a single amino acid. The most likely cause of the unresponsiveness of CD8<sup>+</sup> T cells to the HA in transgenic animals is specific deletion of HA-reactive CD8<sup>+</sup> T cells in the thymus of developing transgenic positive mice. In support of this view we could detect

HA gene expression by RT/PCR in thymus of line 4 and 8 mice. "Leaky" expression of transgenes driven off of tissue specific promoters in the thymus of transgenics have been reported in several transgenic models of T cell tolerance when sensitive detection techniques such as RT/PCR have been employed (8, 38). However, even RT/PCR may not be sensitive enough to detect biologically important low level transgene expression in the thymus. Indeed we have evidence that T cell reconstitution of nude mice(39) with thymus grafts from line 2 mice (which are negative for thymic HA gene expression by RT/PCR) results in selective CD8<sup>+</sup> T cell tolerance for the A/

Japan/57 HA (R.I. Enelow et al., manuscript in preparation). Therefore the CD8<sup>+</sup> CTL tolerance to the HA in line 2 mice can most likely be explained by a thymic deletion mechanism.

The absence of CD4<sup>+</sup> tolerance in the face of expression of both the HA gene and MHC class II molecules in the thymus is problematic. Self and foreign proteins are primarily processed and presented to CD4<sup>+</sup> T cells through an exogenous pathway (40). This pathway requires uptake of proteins into endocytic vesicles of antigen presenting cells and subsequent fragmentation of the protein in an endosomal processing compartment prior to loading of MHC class II molecules (33, 41, 42). While this exogenous MHC class II presentation pathway is believed to be involved in processing and presentation of soluble proteins, there is good evidence that both foreign and self membrane glycoproteins present on cell surfaces can gain access to the MHC class II presentation pathway. These membrane-bound molecules presumably enter the MHC class II pathway by cycling from the cell surface into the degradative endosomal compartment. One reason for the absence of central (thymic-dependent) CD4<sup>+</sup> T cell tolerance to the HA transgene may be related to the transport properties of the HA membrane glycoprotein. Internalization of many membrane proteins into endosomes requires the presence of an internalization sequence located in the cytoplasmic tail of the protein (43). Wild type HA protein lacks such a sequence and cycles from the plasma membrane into the endosomal compartment with a very low efficiency (44). Therefore inefficient processing and presentation of the HA transgene to MHC class II molecules in MHC class II expressing thymic cells could account for the split CD4<sup>+</sup>/CD8<sup>+</sup> T cell tolerance observed in the HA transgenic animals.

The lack of constitutive class II MHC expression on pulmonary epithelial cells may, in part, explain both the absence of spontaneous lung injury (in spite of the presence of HA-reactive CD4<sup>+</sup> T cells) as well as the lack of injury after adoptive transfer of a population of CD4<sup>+</sup> T cell clones. However, there are probably other factors as well which contribute to the control of expression of autoaggressive behavior in T cells.

Although sublethal infection of these mice with A/Japan/57 virus did not lead to enhanced pulmonary injury in short term experiments, the adoptive transfer results described in this report suggests that these animals may serve as a unique model for the dissection of the role of T lymphocytes in immune mediated pulmonary injury. In particular, the availability of HA-specific CTL clonal populations from mice genetically deficient in the production of specific cytokines (e.g.,  $\gamma$ -interferon [45]) and components of the CTL killing machinery (e.g., perforin [46]) should provide us with the unique opportunity to assess the contribution of each of these CTL effector molecules in the development of pulmonary injury. In addition, the presence of CD4<sup>+</sup> T cells reactive to the HA gene in mice expressing this neo-antigen in their lungs provides a potential model for the analysis of the factors leading to autoimmune pulmonary injury. This latter feature of the model should be directly relevant to human pulmonary diseases such as sarcoidosis, where autoreactive CD4<sup>+</sup> T lymphocytes may play a critical role in the development of pulmonary injury. Understanding how CD4<sup>+</sup> and CD8<sup>+</sup> T cells directed to lung specific proteins are generated and their effector activity regulated will be crucial in understanding the mechanisms of lung injury in pulmonary diseases where an autoimmune etiopathogenesis has been proposed, such as in many of the interstitial lung diseases.

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