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Many helper T cell subsets have Fas ligand-dependent cytotoxic potential

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Abstract

$CD4^+$ helper T cells can have cytotoxic activity against cells displaying relevant peptide-MHCII (p:MHCII) ligands. Cytotoxicity may be a property of Th1 cells and depend on perforin and the Eomes transcription factor. We assessed these assertions for polyclonal p:MHCII-specific $CD4^+$ T cells activated *in vivo* in different contexts. Mice immunized with an immunogenic peptide in adjuvant or infected with Lymphocytic choriomeningitis virus or *Listeria monocytogenes* bacteria induced cytotoxic Th cells that killed B cells displaying relevant p:MHCII complexes. Cytotoxicity was dependent on Fas expression by target cells but independent of Eomes or perforin expression by T cells. Although the priming regimens induced different proportions of Th1, Th17, regulatory T cells, and T follicular helper cells, the T cells expressed Fas ligand in all cases. Reciprocally, Fas was upregulated on target cells in a p:MHCII-specific manner. These results indicate that many different Th subsets have cytotoxic potential that is enhanced by cognate induction of Fas on target cells.

Introduction

T cells have traditionally been categorized as cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells. Both types exist as quiescent naïve cells in the pre-immune repertoire, but proliferate and differentiate into effector cells following TCR binding to the relevant MHC-bound peptides on APCs. $CD8^+$ effector T cells utilize Fas ligand (FasL), TNFα, perforin, and/or granzyme (1, 2) to kill cells displaying the relevant MHC-bound peptide. In contrast, $CD4^+$ effector T cells secrete cytokines after interaction with cells expressing the relevant MHCII-bound peptide and typically enhance the microbicidal functions of the interacting cells. For example, Th1 cells migrate to sites of infection where they produce TNFα, IFN-ɣ, and CD40L, which activate macrophages to clear intracellular infections (3-7).

Recently, however, it has become clear that $CD4^+$ T cells, usually Th1 cells, can also have direct cytotoxic activity (8-13) *in vivo* following infections and cancer (14, 15). Cytotoxic Th cell frequency correlated with viral control in influenza- and HIV-infected humans indicating a role for these cells in immunity (16, 17). In mouse models, cytotoxic Th cells depend on perforin for cytotoxic function and express KLRG1 and Ly6C, which are also

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expressed preferentially on Th1 cells (11, 18-20). In addition, cytotoxic Th cells can express the Th1-associated transcription factors Blimp1, T-bet, and Eomes, and Eomes has been proposed as a master regulator of cytotoxic Th cell formation (20-26). It is unclear, however, whether Th1 cells are the only Th cells with cytotoxic activity.

Here, we characterized p:MHCII-specific cytotoxic $CD4^+$ Th cell populations induced by immunization or acute infections. In these cases, cytotoxic Th formation did not require Eomes or perforin, was not limited to T-bet-expressing Th1 cells, and acted in part through a mechanism requiring Fas expression by target cells. Multiple Th subsets including T follicular helper (Tfh), regulatory T (Treg) cells, and Th17 cells expressed FasL, suggesting all could have cytotoxic potential. TCR-dependent interactions between p:MHCII-specific T cells and p:MHCII-bearing target cells induced Fas on the target cells. These results suggest that many different kinds of Th cells have the capacity to stimulate the target cell to express the surface protein that causes its demise.

Materials and Methods

Mice

Six- to 8-week-old C57BL/6 (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1), B6.129S-*Tnfrsf1a*^{tm1Imx} *Tnfrsf1b*^{tm1Imx}/J (TNFR I & II KO), B6.129P2-*Cd40*^{tm1Kik}/J (CD40 KO), C57BL/6-*Prf1*^{tm1Sdz}/J (Perforin KO), B6.MRL-*Fas*^{lpr}/J (Fas KO), and B6.129S6-*Tbx21*^{tm1Glm}/J (T-bet KO) were purchased from the Jackson Laboratory or the National Cancer Institute Mouse Repository (Frederick, MD, USA). B6 IFN-γR1-deficient mice were a gift from M. Farrar (University of Minnesota). B6 *Cd4-Cre Eomes*$^{fl/fl}$ and B6 *Eomes*^{GFP} mice were a gift from S. L. Reiner (Columbia University). B6 DR5 (TRAIL-R KO) deficient mice were a gift from T. S. Griffith (University of Minnesota). *Rag1*$^{-/-}$ B3K508 TCR transgenic mice (27) and *Rag1*$^{-/-}$ SM1 *Tbx21*^{ZsGreen} TCR transgenic mice were bred and housed in specific pathogen–free conditions in accordance with guidelines of the University of Minnesota Institutional Animal Care and Use Committee and National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Minnesota approved all animal experiments.

Infections and Immunizations

The ActA-deficient *Listeria monocytogenes* (*Lm*)-2W and *Lm*-3K strains were described previously (28, 29). Mice were injected intravenously with 1×10^7 colony-forming units of *Lm* bacteria or intraperitoneally with 2×10^5 plaque-forming units of the LCMV Armstrong strain. For immunizations, mice were injected intraperitoneally with 10 μg of peptide mixed with 20 μg Poly I:C or 100 μL CFA. In some experiments mice received 10 μg of peptide mixed with 20 μg Poly I:C subcutaneously, intravenously, or intramuscularly. In some experiments, peptide/Poly I:C-immunized animals were challenged with an i.v. injection of 100 μg of 2W peptide and sacrificed 2 h later for analysis of IFN-γ production.

Tetramers

Biotin-labeled soluble I-A^b molecules containing 2W, 3K, or LCMV glycoprotein (GP)66–77 peptides covalently attached to the I-A^b beta chain were produced with the I-A^b alpha

chain in Drosophila melanogaster S2 cells, then purified and made into tetramers with streptavidin (SA)-phycoerythrin (PE) or (SA)-allophycocyanin (Prozyme, San Leandro, CA, USA) as described previously (30, 31).

Cell enrichment and flow cytometry

Single cell suspensions were generated by dissociating spleens with the GentleMACS dissociator (Miltenyi Biotec) or by hand in some experiments. Single cell suspensions were stained for 1 h at room temperature with allophycocyanin-conjugated tetramers. 0.1 μg of FITC labelled CD90.1 (OX-7; Biolegend) was added to the staining cocktail if the experiment utilized TCR transgenic cells and 2 μg of CXCR5-BUV395 (2G8; Becton-Dickinson) was added for experiments examining Tfh cells. Biotinylated anti-FasL or FasL-PE antibody (MFL3; eBioscience) was also added to the staining cocktail for many of the experiments. Samples were then enriched for bead-bound cells and enumerated as described previously (Moon et al., 2007) with minor modifications. In brief, cells were stained with EasySep Mouse APC Positive Selection Kits (Stemcell Technologies) or EasySep Mouse FITC Positive Selection Kits (Stemcell Technologies) and enriched with EasySep magnets (Stemcell Technologies).

For identification of surface markers, the sample was stained on ice with various combinations of the following antibodies: PE-Cy7 or APC-ef780 labelled B220 (RA3–6B2; eBioscience), APC-ef780 labelled CD11b (MI-70; eBioscience), APC-ef780 labelled CD11c (N418; eBioscience), PE-Cy7 labelled PD-1 (J43; eBioscience), PE labelled CD3ε (145-2C11; eBioscience), BV786 or eFluor 450 labelled CD4 (RM4–5; Becton-Dickinson or eBioscience), AF700 labelled CD44 (IM7; eBioscience), BV510 labelled CD45.1 (A20; Becton-Dickinson), PE or V500 or BV510 labelled CD8α (53-6.7; eBioscience or Becton-Dickinson), FITC labelled Fas (Jo2; Becton-Dickinson), AF700 labelled MHC Class II (I-A/I-E; M5/114.15.2; eBioscience), BV650 labelled IFN-γ (XMG1.2; Biolegend), PE labelled Eomes (Dan11mag; eBioscience), Percp-Cy5.5 labelled Foxp3 (FJK-16s; eBioscience), BV421 labelled Rorγt (Q31-378; Becton-Dickinson), BV605 labelled T-bet (4B10; Biolegend), AF488 labelled Bcl6 (K112-91; Becton-Dickinson) and FITC labelled CD90.2 (53-2.1; eBioscience). All samples were also stained with a fixable viability dye (eFluor780; eBioscience). In experiments using biotinylated anti-FasL antibodies, PE-conjugated streptavidin (eBioscience) was included in the staining cocktail. When analyzing T cell differentiation, intracellular transcription factors, or IFN-γ production after peptide challenge, staining was performed as described previously (31). To calculate cell numbers, Fluorescent AccuCheck counting beads (Invitrogen) were added to each sample after the final wash step. Cells were then analyzed on an LSR II or Fortessa (Becton Dickinson) flow cytometer. Data were analyzed with FlowJo (TreeStar).

Cell transfer

LNs were collected from *Rag1*$^{-/-}$ B3K508 TCR transgenic mice and CD4^{+} T cell numbers and purity were analyzed on an LSR II (Becton Dickinson) flow cytometer after staining with CD4-eFluor 450 antibody. Twenty-thousand TCR transgenic cells were injected intravenously into C57BL/6 (B6) 24 h before infection with *Lm*-3K.

In Vivo Cytotoxicity Assay

The target cells for the *in vivo* cytotoxicity assays were prepared by labeling bulk splenocytes from CD45.1 mice with CellTrace Violet (CTV; Life Technologies). Briefly, spleen cells were suspended in PBS at 3×10^7 cells/ml and incubated at 37°C for 10 minutes in 2 μM (hi) or 0.4 μM (lo) CTV. The CTV was quenched by adding RPMI with 10% FBS. The CTV^{lo} cells received 100 μg of 2W or GP66 peptide in the RPMI with 10% FBS for a final peptide concentration of 10 μg/ml. The CTV^{hi} and CTV^{lo} cells were then incubated for 1 h at 37°C before being washed twice with PBS. The CTV^{hi} and CTV^{lo} cells were mixed at a 1:1 ratio and transferred into mice, with each mouse receiving 2×10^7 total cells. The mice were sacrificed 20 h after the cell transfer and the spleens were processed for cell enrichment as previously described (32). The unbound fraction from the cell enrichment was collected and 5% of this fraction was assayed by flow cytometry to determine the CTV^{lo}/CTV^{hi} ratio of the B cells in the target cell population in addition to their expression of Fas and MHC II. Specific Lysis = 100 − (CTV ratio in experimental mice/CTV ratio in naive mice) × 100.

In Vitro Cytotoxicity Assay

B cells were isolated from CD45.1 mice using a B cell negative selection kit (Stemcell Technologies) and cultured overnight at 1×10^7 cells/ml at 37°C in complete IMDM media and 10 μg/ml of Pam3CSK4 (Invivogen). The cells then were labelled with 0.2 μM CTV or 0.2 μM CFSE (Life Technologies) for 10 minutes at 37°C in PBS. The labelling reactions were quenched by 1 h incubation at 37°C in complete IMDM media. FliC peptide was added to the CFSE-labeled samples during the quenching period. 10 μg/ml. CFSE- and CTV-labelled cells were washed twice with complete IMDM media, mixed at a 1:1 ratio, and added to 96 well cell culture plates. To obtain cytotoxic T cells for the culture, 1×10^5 SM1 $Tbx21^{ZsGreen}$ TCR transgenic $CD4^+$ T cells were transferred into B6 mice, which were immunized with FliC/CFA. Secondary lymphoid organs were harvested 7 d post-immunization and SM1 cells were isolated by allophycocyanin-labelled CD90.1 antibody-based magnetic enrichment (Stemcell Technologies). The SM1 cells were then stained with CCR6-PE (FAB590P; R and D biosystems), CXCR5-BUV395, and CD4-eFluor 450 antibodies at room temperature for 1 h before sorting on a FACS Aria (Becton Dickinson) cell sorter. Sorted SM1 T cells expressing CCR6, $Tbx21^{ZsGreen}$, CXCR5, or lacking all three markers were added to B cell-containing wells for a final ratio of 10 T cells to 1 CFSE labelled B cell. Some wells received B cells alone to determine the baseline CFSE/CTV ratio. The cells were cultured for 20 h and assayed by flow cytometry to determine the CFSE/CTV ratio of the B cells. Specific lysis = 100 – (CFSE/CTV ratio for T cell containing well / CFSE/CTV ratio for B cell alone well) × 100.

Cell Depletion with mAbs

Monoclonal antibodies were used to deplete CD4 or CD8α expressing cells in mice immunized with 2W peptide and PolyI:C. Each mouse was intravenously injected with 500 μg of CD4, CD8α, or isotype control antibody (GK1.5, 53-6.72, or 2A3 respectively; BioXcell) at the time of injection of target cells.

Statistical analysis

Statistical significance was determined using Prism (Graphpad) software for unpaired two-tailed Student's t test, one-way ANOVA, and two-way ANOVA tests.

Results

Generation of cytotoxic Th cells

We sought to identify an immunization regimen that drives a robust cytotoxic Th cell response. The 2W peptide was chosen as a model Ag because it is highly immunogenic in C57BL/6 (B6) mice and the TLR3 agonist Poly I:C was chosen as an adjuvant because it induces strong Th1 immunity of the kind thought to be associated with Th-mediated cytotoxicity (33, 34). A fluorochrome-labeled 2W:I-A^b tetramer and a magnetic bead-based cell enrichment method was used to track 2W:I-A^b-specific $CD4^+$ T cells (34). As reported previously, unimmunized B6 mice contained about 200 2W:I-A^b-specific $CD4^+$ T cells in their spleens (32) (Fig. 1A, 1B). This naïve precursor population increased to 3,000 $CD44^{high}$ effector cells seven days after injection of 2W peptide alone, or 30,000 such cells after injection of 2W peptide and Poly I:C (Fig. 1A, 1B). The large increase in the number of 2W:I-A^b-specific $CD4^+$ effector T cells induced in the presence of Poly I:C confirmed the adjuvant activity of this molecule. As expected (34), these T cells expressed CD4 and not CD8 (35)(Fig. 1C).

$CD4^+$ T cell-mediated cytotoxicity was then assessed using an *in vivo* assay. Target cells were prepared by labeling splenocytes with CellTrace Violet (CTV) at two concentrations. Cells labeled with a lower concentration were incubated with 2W peptide while cells labeled with a higher concentration were incubated without peptide. These cells were mixed and injected into naive or 2W peptide/PolyI:C-immunized mice. The target cells displaying 2W:I-A^b complexes were preferentially lysed, as indicated by a decrease in the percentage of cells with low CTV labeling (Fig. 2A). CD4 or CD8α depleting antibodies were then administered at the same time as the target cells to identify the cytotoxic population. The specific lysis was then calculated by taking the ratio of 2W-incubated to no peptide-incubated target cells in 2W primed mice, and dividing this ratio by that in naïve mice (14). CD4 but not CD8α antibody treatment depleted 2W:I-A^b-specific $CD4^+$ T cells (Fig. 2B) and greatly reduced 2W-target cell lysis (Fig. 2C). These results demonstrate that 2W peptide/Poly I:C immunization generates a 2W:I-A^b-specific $CD4^+$ cytotoxic Th cell response.

Analysis of the cytotoxic pathways utilized by cytotoxic Th cells

We sought to define the pathways utilized by cytotoxic Th cells to kill target cells. CTV-labeled target cells deficient in expression of CD40, IFN-γ receptor, TNF receptor I and II, TRAIL receptor, or Fas were injected into wild-type (WT) mice that were primed with 2W peptide/Poly I:C (14, 36-39). The perforin pathway was tested by injecting CTV-labeled WT target cells into 2W peptide/Poly I:C-immunized perforin-deficient mice (19). WT and perforin-deficient mice generated similar numbers of 2W:I-A^b-specific $CD4^+$ T cells after priming (Fig. 3A). Of the six pathways tested, only deficiency in the Fas pathway caused a significant decrease in specific target cell lysis (Fig. 3B). The dependence of target cell lysis

on Fas correlated with induction of FasL expression on the 2W:I-A^b-specific $CD4^+$ effector T cells that formed in response to 2W peptide/Poly I:C immunization (Fig. 3C, 3D). These results suggest that peptide/Poly I:C-induced Th cells kill target cells by inducing FasL to engage Fas expressed on the target cells.

Cytotoxic Th cells have been shown to be protective against viral infections in humans and mice, and $CD4^+$ T cells expressing cytotoxic cell associated markers have been identified following bacterial infections (16, 17, 19, 20). Therefore, we determined whether the Fas pathway is important for cytotoxic Th cell-mediated killing under these conditions. Mice were infected with Lymphocytic Choriomeningitis Virus (LCMV) or *Listeria monocytogenes*-expressing 2W peptide (*Lm*-2W) and Ag-specific $CD4^+$ T cell responses were examined using LCMV GP66:I-A^b or 2W:I-A^b tetramers, respectively. Both infections induced expansion of the relevant $CD4^+$ T cell populations although LCMV induced more than *Lm*-2W (Fig. 4A). In both cases, the relevant $CD4^+$ T cell populations expressed FasL with *Lm*-2W infection driving significantly higher expression (Fig. 4B). Both the LCMV and *Lm*-2W infected mice showed substantial specific target cell lysis *in vivo*, which was significantly but partially reduced in the case of Fas-deficient target cells (Fig. 4C, 4D). The *Lm*-2W infected mice did not exhibit as robust specific cell lysis as the LCMV infected mice in line with the sizes of their $CD4^+$ T cell responses (Fig. 4A, 4C, 4D). Taken together, these results suggest that the Fas pathway is involved in the killing of target cells by cytotoxic Th cells induced by acute viral and bacterial infections.

The relationship between different T helper subsets and cytotoxic function

The Th subsets induced by peptide/Poly I:C immunization were examined to determine which ones could have been responsible for cytotoxicity. Th1 and Treg cells were identified based on expression of the lineage defining transcription factors T-bet and Foxp3, respectively, while Tfh cells were identified based on expression of the chemokine receptor CXCR5 (Fig. 5A; (21, 40-43). The 2W:I-A^b-specific naïve $CD4^+$ T population in unimmunized mice contains about 10% Treg cells, but no Th1 or Tfh cells (29, 35). In contrast, the 2W:I-A^b-specific effector cell population generated by 2W peptide/Poly I:C immunization consisted of 60% Th1, 3% Treg, 8% Tfh, and 30% cells of unknown lineage (Fig. 5B). T-bet-deficient mice, which do not generate Th1 cells, were then examined to test the cytotoxic potential of this subset (44). The clonal expansion of T-bet-deficient 2W:I-A^b-specific $CD4^+$ T cells was equivalent to their WT counterparts following immunization with 2W peptide/Poly I:C (Fig. 5C). As expected, T-bet-deficient 2W:I-A^b-specific $CD4^+$ T cells did not become Th1 cells (Fig. 5B) or produce IFN-γ after peptide challenge (Fig. 5D) and instead became unknown effector cells distinct from Tfh or Treg cells (Fig. 5B). Remarkably, 2W-pulsed target cells were killed as efficiently in T-bet-deficient mice as they were in WT mice (Fig. 5E). These results demonstrate that although 2W peptide/Poly I:C immunization induces primarily Th1 cells, the function of these cells is not necessary for Th cell-mediated killing.

The surprising Th cell-mediated cytotoxicity in T-bet-deficient mice warranted investigation of the role of Eomes, another transcription that has been implicated in cytotoxic function (20). About 10% of the 2W:I-A^b-specific $CD4^+$ T cells in 2W/PolyI:C-immunized WT mice

expressed Eomes (Fig. 6A, 6B), mainly in T-bet$^+$ Th1 cells (Fig. 6A). The 2W:I-A^b-specific CD4$^+$ T cells in *Cd4-Cre Eomes$^{fl/fl}$* mice did not express Eomes (Fig. 6A), proliferated (Fig. 6C) and differentiated normally into Th1 cells (Fig. 6A), and demonstrated normal cytotoxicity following 2W/PolyI:C immunization (Fig. 6D). These results demonstrate that Eomes is also not necessary for the generation of Th cell-mediated cytotoxicity after PolyI:C immunization.

The finding that cytotoxic Th function was not a unique property of Th1 cells prompted investigation of other Th subsets. Immunization with CFA was used for this purpose because it induces a diverse CD4$^+$ T cell response including Th17 cells, which were identified by expression of RORγt (45, 46). Mice primed with 2W peptide in CFA generated a large 2W:I-A^b-specific CD4$^+$ T population consisting of CXCR5$^+$ Tfh, T-bet$^+$ Th1, Foxp3$^+$ Treg, RORγt$^+$ Th17, or cells of unknown lineage (Fig. 7A) and displayed in vivo cytotoxic activity that was weaker than induced by peptide/Poly I:C immunization (Fig. 7B). Each of the subsets significantly upregulated FasL in comparison to naive CD4$^+$ T cells (Fig. 7C) suggesting that all subsets could be involved in Fas-dependent target cell lysis.

The cytotoxic function of the different T helper subsets was examined using an *in vitro* cytotoxicity assay. The various Th subsets were generated from congenically-marked naïve SM1 TCR transgenic cells (47) capable of expressing ZsGreen from the *Tbx21* promoter (48), after adoptive transfer into B6 recipients primed with CFA containing the Salmonella FliC peptide for which the SM1 TCR is specific. SM1 Th1, Th17, Tfh, and lineage$^-$ cells were sorted based on expression of ZsGreen, CCR6 (49), CXCR5, or lack of all three markers, respectively, and cultured with FliC-pulsed and unpulsed B cells *in vitro*. Each subset lysed target cells in an Ag-dependent manner (Fig. 7E). The lineage$^-$ cells, however, were the worst at inducing cell lysis, which is in line with their lower FasL expression relative to Th1 and Th17 cells (Fig. 7C, 7E). These results demonstrate that many helper T cell subsets have the ability to kill target cells, likely through the Fas pathway.

The role of TCR affinity in regulating cytotoxic Th cell-mediated killing

It was of interest to assess the amount of Fas expressed by target cells given that maximal Th-mediated cytotoxicity depended on this molecule. 2W peptide-pulsed and unpulsed splenocytes were injected into 2W/CFA-primed mice and assayed for Fas expression by flow cytometry after 20 h. B cells that remained in the target cell population that was pulsed with the 2W peptide expressed more Fas than comparable cells that were not pulsed with peptide (Fig. 8A, 8B). This result indicated that Fas was induced on the target cells via cognate interactions with 2W:I-A^b-specific CD4$^+$ T cells. This hypothesis was tested with CD4$^+$ T cells from the B3K508 transgenic strain, which express a TCR that is specific for I-A^b-binding peptides called 3K, P5R, and P2A with 11, 93, and 175 K_D affinities, respectively (Fig. 8C) (50). CD45.1$^+$ B3K508 T cells were transferred into CD45.2$^+$ B6 mice that were then infected with *Lm* expressing 3K peptide (Fig. 8C). By day 7 after infection, the approximately 20,000 transferred naïve B3K508 T cells produced 562,609 ± 388,270 effector cells. On this day, the mice received target cells pulsed with 3K, P5R, or P2A peptides for an *in vivo* cytotoxicity assay and Fas measurement on residual target cells (Fig. 8C). The specific lysis of the target cells correlated with B3K508 TCR affinity for the

various peptide:I-A^b complexes, that is, 3K-pulsed target cells were killed more efficiently than P5R-pulsed target cells, which were killed more efficiently than P2A-pulsed target cells (Fig. 8D). Fas induction on the residual peptide-pulsed target cells showed the same positive correlation with TCR affinity for the cognate p:MHCII (Fig. 8E). These results suggest that $CD4^+$ T cells recognize target cells through their TCR and regulate Fas expression on the target cells in a manner that is proportional to their TCR affinity.

Discussion

Cytotoxic Th cells have been described as either Th1-like cells with cytotoxic function, notable for their high IFN-γ production, Eomes expression, and perforin-dependent cytotoxicity, or as cells that have undergone re-programming to express CD8α and $CD8^+$ T cell-like functions (20, 51-53). Initial studies used 4-1BB agonist antibodies to induce Eomes-expressing cytotoxic $CD4^+$ T cells, while $CD4^+$ T cell re-differentiation into $CD8^+$ T cell-like cells is associated with mucosal tissue (20, 54). In contrast, our studies of peptide:MHCII-specific effector T cells induced by peptide and Poly I:C revealed a T-bet- and Eomes-independent pathway of cytotoxic Th cell formation that did not involve $CD8^+$ T cell-like re-differentiation. This cytotoxic pathway was also perforin-independent and instead partially relied on Fas expression by target cells. Since Th1, Th17, and Tfh cells expressed FasL and had cytotoxic function after immunization with peptide/CFA, each of these subsets could have been responsible for the cytotoxicity observed in this system. The broad expression of FasL could explain why the induction of Th cell-mediated cytotoxicity does not require the cytokines necessary for Th1, Th2, or Th17 polarization (24). It is important to note, however, that the Fas pathway only accounted for about half of the cytotoxic potential of the peptide/Poly I:C-induced Th cells. Thus, some pathway of cytotoxicity other than those mediated by IFN-γR, TNFRI and II, or TRAIL is also involved.

Our experiments suggest that a cognate T-B cell interaction-based amplification loop regulates Th cell-mediated cytotoxicity. Th cells induced Fas on target cells (5, 55, 56) in proportion to the affinity of their TCRs for the peptide:MHCII ligand displayed by the target cell. These T cells likely also expressed CD40L, which is a transient consequence of TCR signaling (57). Although Fas is induced on B cells by pattern recognition receptor signaling, expression could have been enhanced on the target B cells by signals from CD40 engaged by CD40L on the interacting Th cell (58-60). CD40 signaling enhances Fas-induced apoptosis in B cells that do not simultaneously receive signals through their surface Ig receptors (61). This could have occurred in our experiments since it is likely that very few of the 2W peptide-pulsed B cells that were used as target cells were specific for the 2W-peptide since they came from naïve mice. The capacity of Th cells to induce Fas on their targets cells could improve the efficiency of their cytotoxicity.

Cytotoxic $CD4^+$ T cells are induced during persistent infections (53, 62, 63). The findings presented here and in work by others (64, 65) demonstrate that cytotoxic Th cells are also induced during acute viral and bacterial infections. Although there is no doubt that cytotoxic $CD8^+$ T cells play a major role in the control of these infections (64, 65), the surprising potency of cytotoxic Th cells (66, 67) indicates that they may also contribute. Future work

will need to be carried out for a more complete understanding of cytotoxic Th cell activity in the context of different infections.

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Non-standard Abbreviations

pMHCII	peptide-MHCII
FasL	Fas ligand
Tfh	T follicular helper
Treg	T regulatory
CTV	CellTrace Violet
LCMV	Lymphocytic Choriomeningitis Virus
Lm-2W	Listeria monocytogenes-expressing 2W peptide
WT	Wild-type

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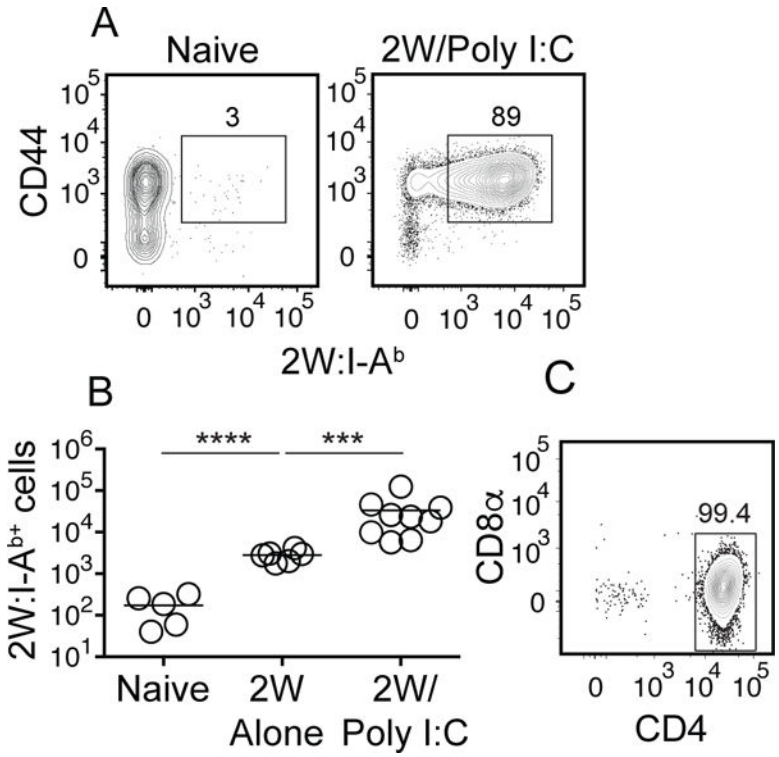

Figure 1. Poly I:C immunization induces robust CD4$^+$ T cell responses

(A) Flow cytometric analysis of B220$^-$ CD11b$^-$ CD11c$^-$ 2W:I-A^b tetramer-enriched cells from a naïve mouse or a mouse 7 days after 2W peptide/Poly I:C immunization. (B) Number of 2W:I-A^{b+} CD4$^+$ T cells in individual naïve or immunized mice, 7 days post-immunization with 2W peptide alone or 2W peptide/Poly I:C (n=5-9/group). (C) Representative flow cytometric analysis of CD4 and CD8α expression by 2W:I-A^{b+} CD4$^+$ T cells from 2W peptide/Poly I:C immunized mice. The bars in B represent the mean for each group. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance. *** = $p < 0.001$, **** = $p < 0.0001$.

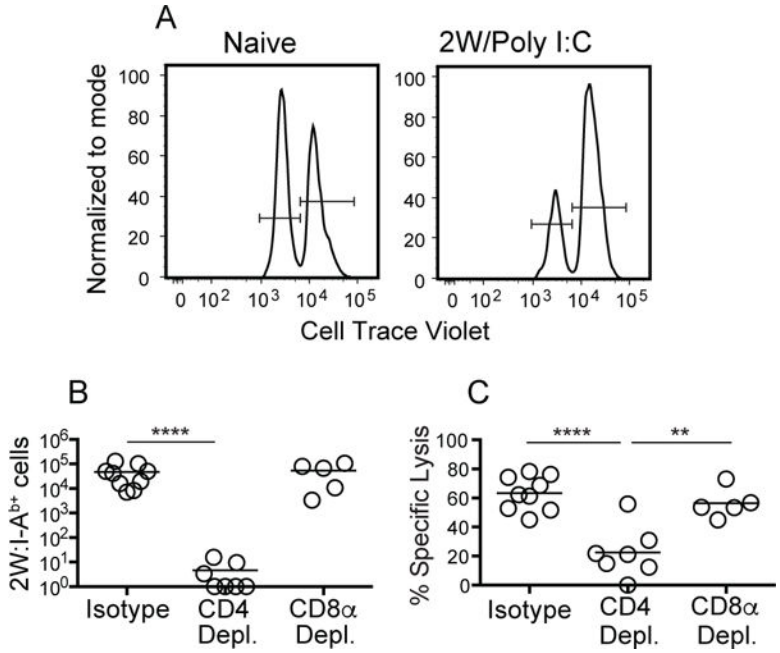

Figure 2. Poly I:C immunization generates cytotoxic Th cells

(A) Representative flow cytometric analysis of an *in vivo* cytotoxicity assay utilizing CTV to identify 2W peptide-presenting (CTV^{low}) and non-presenting (CTV^{high}) $B220^{+}$ target cells 20 h after their injection into naïve or 2W peptide/Poly I:C immunized mice. (B) Number of 2W:I-A^{b+} $CD4^{+}$ T cells and (C) specific cell lysis following an *in vivo* cytotoxicity assay in mice 7 days after immunization with 2W peptide/Poly I:C and after injection of isotype control, CD4, or CD8α depleting antibody (n=5–9/group). The bars in B and C represent the mean for each group. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance. ** = $p < 0.01$, **** = $p < 0.0001$.

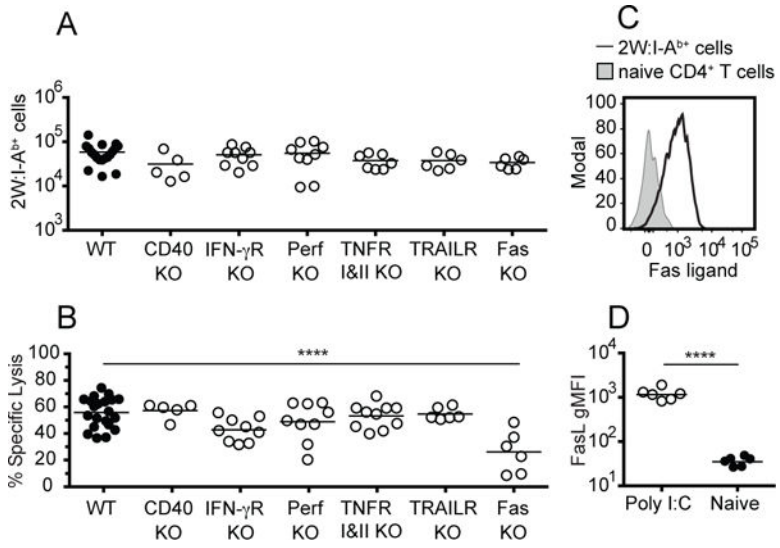

Figure 3. Cytotoxic Th cells generated with Poly I:C primarily utilize the Fas pathway for cytotoxicity

Mice were examined 7 days after immunization with 2W peptide/Poly I:C. (A) Number of 2W:I-A^{b+} $CD4^+$ T cells and (B) specific cell lysis following an *in vivo* cytotoxicity assay in WT mice that received WT, CD40-deficient, IFN-γ receptor-deficient, TNFα receptor I and II-deficient, TRAIL receptor-deficient or Fas-deficient target cells. Perforin-deficient mice that received WT target cells are also shown (n=5-21/group). (C) Representative flow cytometric analysis of FasL expression by 2W:I-A^{b+} $CD4^+$ T cells (line) or naïve $CD4^+$ T cells (grey). (D) Geometric mean fluorescence intensity of FasL on 2W:I-A^{b+} $CD4^+$ T cells from 2W peptide/Poly I:C immunized or naïve $CD44^{low}$ $CD4^+$ T cells (n=6/group). The bars in A, B, and D represent means. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance for A and B while Student's t test was used for D. **** = $p < 0.0001$.

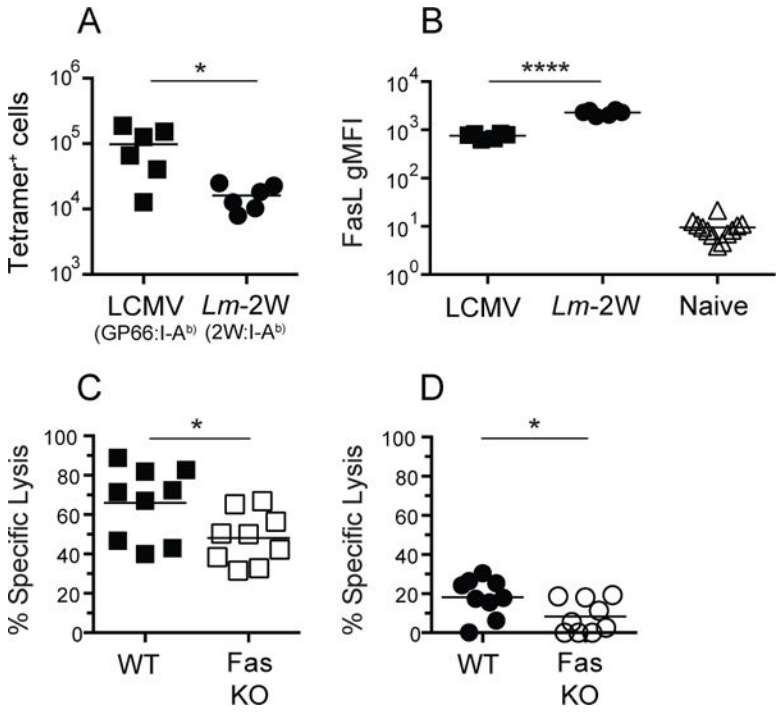

Figure 4. Cytotoxic Th cells primarily utilizing the Fas pathway are induced by LCMV and *Lm* infections

(A) Number of GP66:I-A^{b+} $CD4^+$ T cells following LCMV infection and 2W:I-A^{b+} $CD4^+$ T cells following *Lm*-2W infection (n = 6/group). (B) Geometric mean fluorescence intensity of FasL on GP66:I-A^{b+} $CD4^+$ T cells from LCMV infected mice, 2W:I-A^{b+} $CD4^+$ T cells from *Lm*-2W infected mice on day 7 post-infection, or naïve $CD44^{low}$ $CD4^+$ T cells (n=6-12/group). Specific cell lysis of WT or Fas-deficient target cells following in mice infected 7 days earlier with (C) LCMV or (D) *Lm*-2W (n=9/group). The bars in A, B, C, and D represent means. Pooled data from two independent experiments are shown. Student's t-test was used to determine significance. * = $p < 0.05$, **** = $p < 0.0001$.

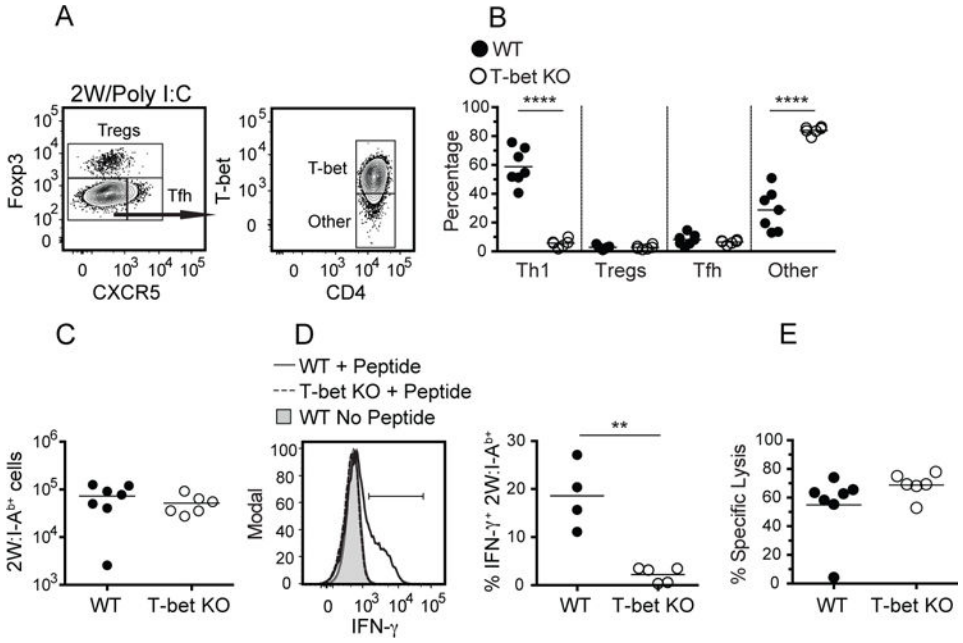

Figure 5. Poly I:C induced CD4$^+$ T cell cytotoxic function is T-bet independent

2W:I-A^{b+} CD4$^+$ T cells were examined from mice 7 days after immunization with 2W peptide/Poly I:C. (A) Flow cytometric analysis of 2W:I-A^{b+} CD4$^+$ T cell subsets with the gating strategy for Treg, Tfh, Th1, and other cells. (B) Percentage of 2W:I-A^{b+} CD4$^+$ T cells that differentiated into Th1, Treg, Tfh, or other cells in WT or T-bet-deficient mice (n=6-7/group). (C) Number of 2W:I-A^{b+} CD4$^+$ T cells in WT or T-bet-deficient mice (n=6-7/group) after immunization with 2W peptide/Poly I:C. (D) WT and T-bet-deficient mice were immunized with 2W peptide/Poly I:C and injected 7 days later with 2W peptide. The histograms depict IFN-γ production by 2W:I-A^{b+} CD4$^+$ T cells in WT mice (line) or T-bet-deficient mice (dashed line) 2 h after peptide challenge, or WT mice (grey) that were not challenged, with the gate identifying IFN-γ producing cells. The scatterplot shows IFN-γ production in 2W peptide-challenged mice in individual WT or T-bet-deficient mice (n=4-5/group). (E) Specific cell lysis following an *in vivo* cytotoxicity assay in mice 7 days after immunization with 2W peptide/Poly I:C. The bars in B, C, D, and E represent means. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance for B while Student's t test was used for C, D, and E. ** = $p < 0.01$, **** = $p < 0.0001$.

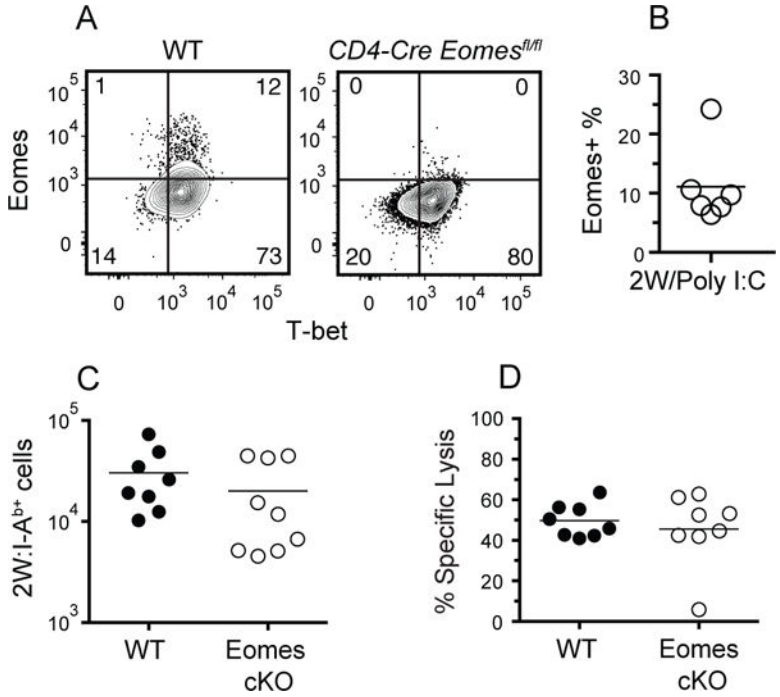

Figure 6. Poly I:C-induced CD4$^+$ T cell cytotoxic function is Eomes independent

2W:I-A^{b+} CD4$^+$ T cells were examined from mice 7 days after immunization with 2W peptide/Poly I:C. (A) Representative flow cytometric analysis of Eomes and T-bet expression by 2W:I-A^{b+} CD4$^+$ T cells from immunized WT or Eomes conditional knockout mice. (B) Percentage of 2W:I-A^{b+} CD4$^+$ T cells that express Eomes. (C) Number of 2W:I-A^{b+} CD4$^+$ T cells and (D) specific cell lysis following an *in vivo* cytotoxicity assay in WT or Eomes conditional knockout mice (n=8-9/group). The bars in B, C, and D represent means. Pooled data from two independent experiments are shown. Student's t test was used to determine significance for C and D.

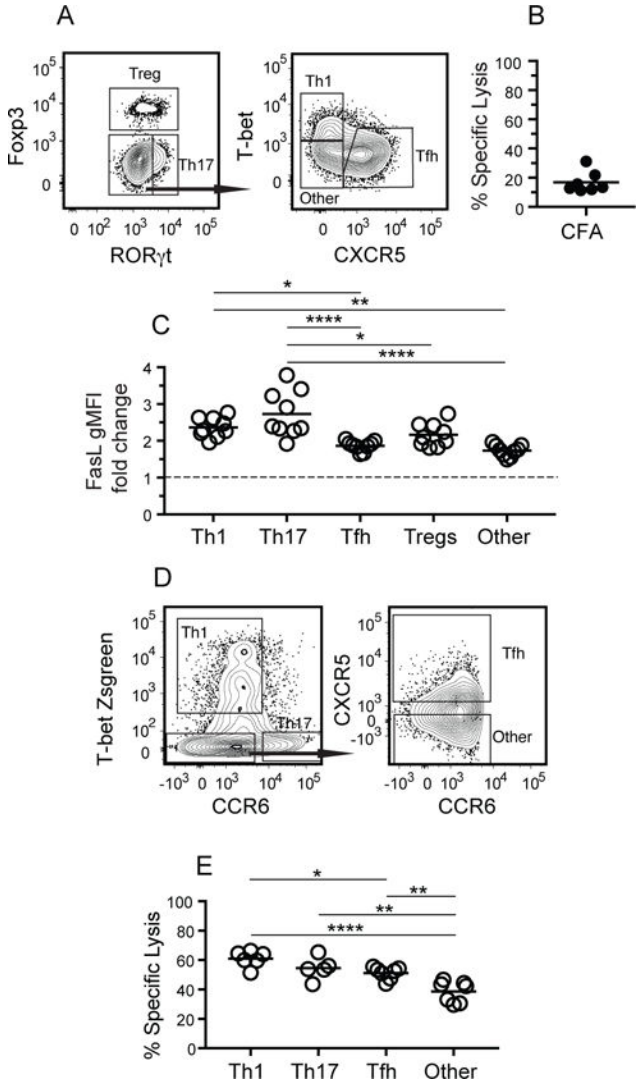

Figure 7. FasL expression is conserved across all T helper subsets generated by CFA immunization

(A) Flow cytometric analysis of 2W:I-A^{b+} CD4^{+} T cell subsets 7 days after 2W peptide/CFA immunization with the gating strategy for Treg, Th17, Th1, Tfh and other cells. (B) Specific cell lysis following an *in vivo* cytotoxicity assay in mice 7 days after immunization with 2W peptide/CFA (n=7). (C) Fold change in geometric mean fluorescence intensity of FasL on Th1, Th17, Tfh, Treg, or other 2W:I-A^{b+} CD4^{+} T cells relative to naïve CD44^{low} CD4^{+} T cells from 2W peptide/CFA immunized mice (n=11/group). (D) Gating strategy for sort-purifying Th1, Th17, Tfh and other SM1 *Tbx21*^{ZsGreen} TCR transgenic CD4^{+} T cells from adoptive recipient mice 7 days post-immunization with FliC/CFA. (E) Specific cell lysis following an *in vitro* cytotoxicity assay using sort-purified Th1, Th17, Tfh, or other SM1 cells. The bars in B, C, and E represent means. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

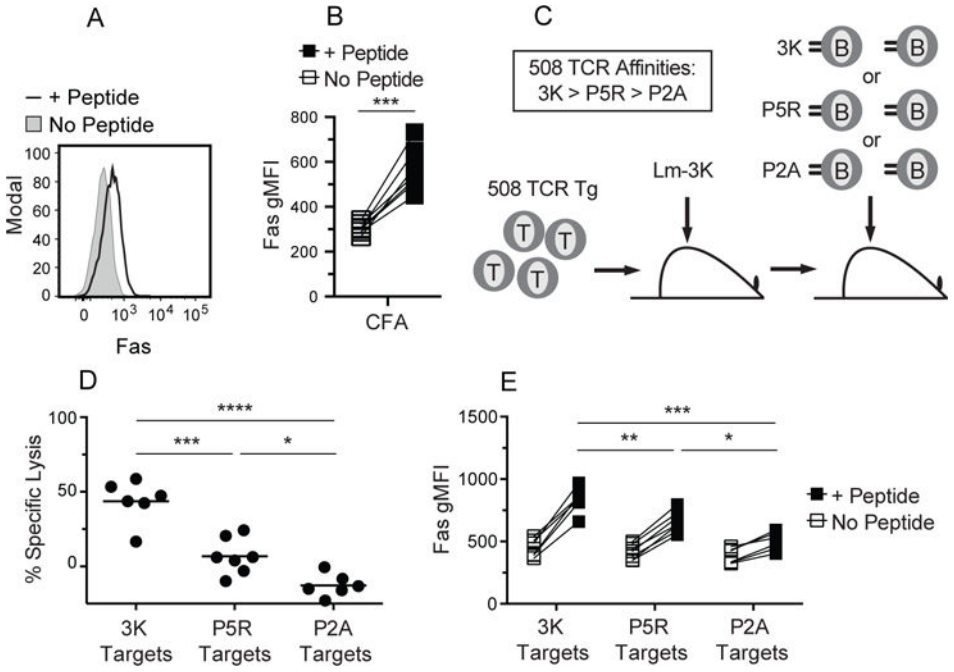

Figure 8. The induction of Fas on target cells correlates with the T cell receptor affinity of responding CD4$^+$ T cells

(A) Representative flow cytometric analysis of Fas expression and (B) Geometric mean fluorescence intensity of Fas staining on 2W peptide-presenting (line) or non-presenting (grey) target cells after an *in vivo* cytotoxicity assay in 2W peptide/CFA immunized mice. Lines in (B) indicate target cells from the same mouse. (C) Illustration of the adoptive transfer model system for examining the influence of TCR affinity on specific cell lysis and Fas expression. (D) Specific cell lysis following an *in vivo* cytotoxicity assay in mice infected with *Lm*-expressing 3K (n=6-7/group). (E) Fas geometric mean fluorescence intensity on peptide-presenting or non-presenting target cells after an *in vivo* cytotoxicity assay in *Lm*-expressing 3K infected mice. Lines indicate target cells from the same mouse. The bars in D represent means. Pooled data from two independent experiments are shown. One-way ANOVA was used to determine significance for D and E while Student's t test was used for B. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.