CD4 T cells inhibit the CD8 T cell response during low-dose virus infection

Stephen Cose1, Clair Brammer, David J. Zammit, D. A. Blair and Leo Lefranc

Division of Immunology, Department of Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

1Present address: School of Clinical Veterinary Science, University of Bristol, Langford, BS40 5DU, UK

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Abstract
CD4 T cells are not thought to play a significant role in generating an effective primary CD8 T cell response to most viral infections. We have challenged this view by demonstrating that antigen-specific CD4 T cells can indeed suppress the proliferation of antigen-specific naive CD8 T cells in response to low doses of vesicular stomatitis virus. This finding is in contrast to the established observations that at high antigen loads CD4 T cells play little role in generating CD8 T cell responses, and that in non-infectious model systems CD4 T cells actually help the CD8 T cell response. Our results suggest that at low infectious doses, CD4 T cells play a much larger role in controlling infections than previously appreciated.

Introduction
Several studies have recently defined the role of CD4 help in activating CD8 T cells through the interaction of CD40 and its ligand (1–3). These important studies were performed in non-infectious systems, however, and little evidence exists to show a similar role for CD4 T cells during a live infection. Indeed, CD4 T cells have been shown to play little or no role in generating a CD8 T cell response to viral infection (4, 5), with the exception of herpes simplex virus-1 infection of the footpad (6). This lack of requirement for CD4 T cell help in resolving viral infections may be due to the fact that many viruses can bypass CD4 help by providing their own inflammatory signals (7, 8). However, CD8 T cells generated in the absence of CD4 T cells are defective in the memory phase of the immune response (9–11), indicating that although CD4 T cells may be superfluous for the activation phase of a viral challenge, they are required for the memory phase. It therefore remains uncertain as to what role CD4 T cells play during the primary phase of a viral challenge.

Part of the explanation may lie with viral dose, since large viral loads contribute to the massive expansion of CD8 T cells (12, 13), and may thus diminish the role of CD4 T cells during such infections. Such a mechanism may certainly explain the discrepancy that exists between infectious and non-infectious systems regarding CD4 T cell help. The question remains though as to whether the two systems can be reconciled. Under low-dose infection conditions, the relative paucity of initial viral proteins may mean that direct Toll-like receptor (TLR) signaling is somewhat diminished, and thus CD4 T cells may well play a more active role in generating the CD8 T cell response. Since the initial antigen encounter is small, one might expect that CD4 T cells would serve to help the CD8 T cell response in a manner similar to that seen in non-infectious systems. We have examined this issue by looking at the role that antigen-specific CD4 T cells play in resolving a low-dose vesicular stomatitis virus (VSV) infection. In contrast to previous studies which showed that CD4 T cells play little role in generating an effective primary CD8 T cell response, even to VSV (4, 5, 9–11, 14, 15), we show that, during a low-dose infection, CD4 T cells can directly affect the outcome of the CD8 T cell response. This is not through conventional CD4 help since addition of antigen-specific CD4 T cells actually inhibited the generation of CD8 T cells. These results are surprising, and suggest that CD4 T cells play a much larger role in the early stages of natural viral infection (where viral antigen may be initially limiting) than previously realised.

Methods
Mice
All mice were housed on-site under specific pathogen-free conditions. C57BL/6-Ly5.2 mice (CD45.1) were purchased...
from CR-NCl. OT-I, OT-II and TEx transgenic mice (CD45.2) on a Rag-1- background were bred in-house. The TEx mice are a transgenic line whose CD4 T cells are specific for the E32-68 peptide from the MHC class II protein I-E, presented in the context of MHC class II I-Ak (16), and were a kind gift of Prof. Alexander Rudensky, Department of Immunology, University of Washington, Seattle, WA, USA.

Adoptive transfer and immunization
Single-cell suspensions of pooled spleen and lymph nodes from OT-I and/or OT-II or TEx transgenic mice were lysed of RBCs, washed, counted and passed through nylon mesh (40 μm) before injection intravenously (IV) into recipient (C57BL/6-Ly5.2) mice, in the numbers stated. Before injection, the cells were labeled with 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) as previously described (17). One day later, the mice were immunized IV with recombinant VSV-ovalbumin peptide (OVA) or VSV–OED (O = SIINFEKL peptide, E = E32-68 peptide, D = DS-RED II) with the indicated amount of virus, or immunized with OVA-loaded splenocytes. OVA-loaded splenocytes were prepared as previously described (18), and 25 × 10^6 OVA-loaded splenocytes injected IV into recipient mice. Recombinant VSV constructs were produced as previously described (19, 20). VSV–OVA (21) is a recombinant VSV expressing the ovalbumin gene, and VSV–OED was produced using the same protocols as above.

Flow cytometry
Single-cell suspensions were prepared from the spleens of mice. After lysis of the RBCs, splenocytes were stained with combinations of the following antibodies: PE-labeled αCD11a, PerCP-labeled αCD8 or αCD4 and Cy5-labeled αCD45.2 (eBioscience, San Diego, CA, USA). To detect OVA-specific or VSV nucleoprotein-specific CD8+ T cells, splenocytes were stained for 1 h with allophycocyanin (APC)-labeled H-2Kb tetramers containing either the OVA-derived peptide SIINFEKL or the N protein-derived peptide RGYYQQGL and washed prior to addition of the above antibodies. After washing, the relative fluorescence intensities were then measured using a FACS Calibur (BD Biosciences, San Jose, CA, USA) and data analysed using FlowJo software (Tree Star, Ashland, OR, USA). For some experiments, splenocytes were first stained with PE-labeled H-2Kb tetramers containing the OVA-derived peptide SIINFEKL and APC-labeled H-2Kb tetramers containing the N protein-derived peptide RGYYQQGL. After washing, the cells were then stained with PECy7-labeled αCD11a, Alexa405-labeled αCD4, PerCP-Cy5.5-labeled αCD8 and APC-Cy7-labeled αCD45.2 (eBioscience). After washing, the relative fluorescence intensities were measured using an LSRII flow cytometer (BD Biosciences) and data analysed using FlowJo software (Tree Star).

Neutralizing antibody assay
Serum from mice was serially diluted, in duplicate, in a 96-well flat-bottomed plate and incubated with 100 plaque-forming units (p.f.u.) of VSV–OVA for 1 h. Following addition of 5 × 10^4 baby hamster kidney (BHK) cells per well, the plates were incubated for a further 2 days. Antibody titers were then determined by the lowest dilution of serum where the BHK cells showed no viral cytopathic effect.

Results
Recruitment of CD8 T cells depends on the viral dose
High doses of virus can generate primary CD8 T cell responses in the absence of CD4 T cell help, probably through engagement of TLRs (7, 8). To test the role of CD4 T cells in response to lower doses, we immunized mice with titrated doses of VSV–OVA and measured the frequency of responding CD8 T cells at the height of the CD8 T cell response (day 6) using MHC class I tetramers (Fig. 1). CD11a was used in conjunction with the tetramer staining because both activated and memory CD8 T cells express high levels of CD11a (22), and staining with this molecule in conjunction with tetramer identifies bona fide antigen-specific CD8 T cells (23). Lower doses of VSV–OVA resulted in lower numbers of CD8 T cells being recruited into the primary immune response, for both the N-specific (Fig. 1A) and OVA-specific (Fig. 1B) CD8 T cells. Note that the OVA-specific CD8 T cell response was ~3-fold lower than that of the N-specific CD8 T cells at all doses lower than 1 × 10^6 p.f.u. VSV–OVA (compare Fig. 1A with B). This difference in CD8 T cell recruitment may reflect differences in expression of viral proteins in VSV infections and/or in naive CD8 T cell precursor frequency, such that at 1 × 10^6 p.f.u. VSV–OVA, the OVA-specific CD8 T cells could not be distinguished above background staining (Fig. 1B). In all subsequent experiments, we therefore used the lowest dose of VSV–OVA in which we could detect a significant population of OVA-specific CD8 T cells (1 × 10^5 p.f.u.), and compared our results at this dose with the infectious dose of 1 × 10^6 p.f.u. VSV–OVA.

Extra ‘help’ during a low-dose infection decreases the CD8 T cell response
To determine whether the primary CD8 T cell response after low-dose infection was dependent on help from CD4 T cells, we adoptively transferred into recipient mice OVA-specific CD4+ transgenic (OT-II) T cells prior to infection with either a high (1 × 10^5) or low (1 × 10^3) dose of VSV–OVA (Fig. 2). In non-infectious systems, cognate CD4 help is required to generate a CD8 T cell response (1–3, 24), and addition of exogenous antigen-specific CD4 T cells increases the antigen-specific CD8 T cell response (25). We reasoned that the inclusion of additional help prior to a low-dose infection might mirror the results seen in such non-infectious systems. Consistent with earlier reports that the CD8 T cell response to high viral doses is largely independent of CD4 help (4, 5, 9–11, 14, 15), the addition of 1 × 10^5 OT-II CD4 T cells prior to a high-dose infection had no effect on the resulting CD8 T cell response (Fig. 2). This was true for both the N-specific and the OVA-specific CD8 T cell response (N specific, 27% without OT-II cells versus 22.9% with; OVA specific, 12.5% without OT-II cells versus 13.4% with; upper panels, Fig. 2A and B, respectively). However, when the OT-II cells were adoptively transferred prior to a low-dose infection, the CD8 T cell response actually decreased when compared with the no-transfer controls. This was the case for both the N-specific and
OVA-specific CD8 T cells (N specific, 1.9% without OT-II cells to 0.1% with; OVA specific, 1% without OT-II cells to 0.1% with; lower panels, Fig. 2A and B, respectively). This was a surprise finding, and suggested that the role of CD4 T cells in responding to low-dose infection was distinct from that previously described for either non-infectious (1–3) or infectious systems (4, 5, 9–11, 14, 15).

To show that such an effect was a general characteristic of CD4 T cells during a low-dose infection and not an artifact of the specific TCR transgenic system used, we repeated the above experiments using different transgenic CD4 T cells. In this case, the transgenic T cells were isolated from TEα mice, a transgenic line whose CD4 T cells recognize the EA52–68 peptide from the MHC class II I-E protein (16). Immunization of recipient mice after adoptive transfer of TEα cells with a high dose of VSV–OED, a recombinant VSV encoding the SIINFEKL (OVA) and EA52–68 peptides, again revealed no requirement for the TEα cells in generating an effective CD8 T cell response. Both N-specific and OVA-specific CD8 T cells were generated at similar levels regardless of whether TEα cells were present or not (N specific, 33.8% without TEα cells and 30.8% with; OVA specific, 9.1% without TEα cells and 7.6% with; upper panels, Fig. 2C and D, respectively). However, as with the OT-II transfer system, when TEα cells were adoptively transferred prior to a low-dose infection, a reduction in both the N-specific and OVA-specific CD8 T cells was seen compared with the no-transfer controls (N specific, 6.7% without TEα cells to 2.9% with; OVA specific, 4.2% without TEα cells to 0.7% with; lower panels, Fig. 2C and D, respectively). This response is antigen specific since transferred TEα cells do not divide when mice are given VSV–OVA, a virus that does not express the cognate antigen for TEα cells.

To show that the adoptively transferred CD4 T cells were responding to the infection, we measured the fold increase of transferred OT-II or TEα cells following infection compared with uninfected controls (Fig. 2E; open bars, OT-II cells; closed bars, TEα cells). When mice were infected with a low dose of virus (VSV–OVA for OT-II transfer, open bars, left-hand side; VSV–OED for TEα transfer, closed bars, left-hand side), both sets of transgenic CD4 T cells increased by >100-fold. Examination of the CFSE dilution of the transferred CD4 T cells showed that >95% of the CD4 T cells had undergone division, regardless of the viral dose used (data not shown). It is interesting to note that despite the greater expansion of the transgenic CD4 T cells following high-dose infection, inhibition of the CD8 T cell response was only seen following low-dose infection, where the expansion of the CD4 T cells was more limited. These results show that the effect on the CD8 T cell response to infection was a general characteristic of CD4 T cells and occurred only in response to a low-dose infection.

Increasing the number of CD4 T cells does not affect the kinetics of the CD8 T cell or B cell response

Since CD4 T cells are known to help the priming of CD8 T cells, at least in non-infectious systems, it was possible that the CD4 T cells in our system were in fact priming the CD8 T cells to more rapidly resolve the infection. If this was the case, the CD8 T cell response may actually have peaked before day 6, and we were simply looking at the end stages of the resolution of primary infection. To rule this out as a possibility, we
Fig. 2. High numbers of antigen-specific CD4 T cells inhibit the CD8 T cell response following low-dose infection. Mice received either 0 OT-II cells (A and B; left-hand panels) or 1 \times 10^5 OT-II cells (A and B; right-hand panels) or 0 Teα cells (C and D; left-hand panels) or 1 \times 10^5 Teα cells (C and D; right-hand panels). One day later mice received either 1 \times 10^6 or 1 \times 10^3 p.f.u. VSV–OVA (A and B; top and bottom panels, respectively) or 1 \times 10^6 or 1 \times 10^3 p.f.u. VSV–OED (C and D; top and bottom panels, respectively). Numbers inside the panels represent the percentage of gated CD8 T cells falling within the top right-hand quadrant. Data are representative plots from five separate experiments, with two mice per experiment. (E) Contribution of transferred CD4 T cells in the above panels, represented as a fold increase in the transferred transgenic CD4 T cells (open bars, OT-II; closed bars, Teα) following infection, compared with uninfected controls. Error bars represent the standard error of the mean.
conducted a time course experiment looking every 2 days for the presence of N-specific and OVA-specific CD8 T cells (Fig. 3A and B). Only results for the OVA-specific CD8 population are shown; however, similar results were found with the N protein-specific CD8 T cells. When mice were given a high-dose infection (1 \times 10^6 p.f.u.), we saw the standard bell-shaped curve of the responding OVA-specific CD8 T cells, peaking at day 6 regardless of whether OT-II cells were adoptively transferred or not (Fig. 3A; closed circles, no OT-II; open circles, 10^5 OT-II; note that the two sets of data are virtually superimposed upon each other). When mice that had received no OT-II cells were given a low-dose infection, the peak of the responding OVA-specific CD8 T cells was also on day 6 (Fig. 3B, closed circles), indicating that the CD8 T cell response to a low-dose infection runs a similar course to that of a high-dose infection. However, when 1 \times 10^5 OT-II cells were transferred into recipient mice prior to a low-dose infection, an earlier peak in the responding OVA-specific CD8 T cells was not seen (Fig. 3B, open circles), suggesting that the extra help afforded by the OT-II cells was not generating a quicker CD8 T cell response. The CD8 T cell response was not delayed past day 8 since increases in either N-specific or OVA-specific CD8 T cells were detected at any time point up to day 14 post-infection (data not shown).

It was also formally possible that rather than helping the CD8 T cell response, the OT-II cells were affecting the B cell response. To examine this, we determined the neutralizing serum antibody titer from the same mice as above. We found no difference in the neutralizing antibody titers in mice infected with 1 \times 10^6 p.f.u. VSV-OVA, regardless of whether OT-II cells were adoptively transferred or not prior to infection (Fig. 3C; closed circles, no OT-II; open circles, 10^5 OT-II). In this case, the titers rose at similar levels between the two groups of mice each day up to day 8. When mice were infected with 1 \times 10^3 p.f.u. VSV-OVA, neutralizing antibody titers could only be detected in mice that did not receive OT-II cells and rose to the level of detection at day 6 (Fig. 3D, closed circles). As with the CD8 T cell response, no neutralizing antibody could be detected at any time point when mice were given 1 \times 10^5 OT-II cells prior to low-dose infection (Fig. 3D, open circles). No neutralizing antibody titer could be detected as far out as day 14 post-infection (data not shown). These data suggest that during a low-dose infection, high numbers of antigen-specific CD4 T cells can act independently to clear infection without the CD8 T or B cell response. This is in accordance with a recent study which showed that virus-specific CD4 T cells actually impaired the induction of neutralizing antibodies, and consequent deliberate removal of CD4 T cells improved the neutralizing antibody response (26). That the CD4 T cells were working to independently clear virus is evidenced by the fact that no antigen-specific CD8 T or B cell response could be detected during the acute phase of the immune response.

Small numbers of adoptively transferred CD4 T cells affect the CD8 T cell response

We now had a system where the number of adoptively transferred antigen-specific CD4 T cells directly affected the adaptive immune response following infection, and this effect could be tracked by looking at the virus-specific CD8 T cell response using MHC class I tetramers. We next wondered whether the effect of the additional CD4 T cells on the responding CD8 T cells was dose dependent or whether our results were merely an artifact of the high transfer numbers. We reasoned that if low numbers of transferred CD4 T cells were still capable of affecting the CD8 T cell response, then this mechanism of CD4-mediated inhibition may be a natural
phenomenon occurring at low pathogenic doses rather than an artifact of experimental design.

Figure 4 shows the results of titrating into recipient mice graded numbers of OT-II cells prior to low-dose infection with VSV-OVA. Without additional CD4 T cells, the N- and OVA-specific CD8 T cell response was ~5 and 3% of the total CD8 T cell population, respectively (Fig. 4; closed bars, N-specific CD8 T cells; open bars, OVA-specific CD8 T cells). The transfer of up to 750 OT-II cells prior to low-dose infection did not significantly alter the number of responding N- or OVA-specific CD8 T cells compared with the untransferred controls. However, the addition of 1000 OT-II cells significantly decreased the N-specific CD8 T cell response ($P < 0.05$) compared with the untransferred control. The presence of even larger numbers of OT-II cells resulted in a significant decrease in both the responding N- and OVA-specific CD8 T cells compared with the untransferred controls (Fig. 4; N specific, $P < 0.005$; OVA specific, $P < 0.05$ at $10^4$ and $10^5$ transferred OT-II cells, $P < 0.005$ at $10^6$ transferred OT-II cells). Thus, as few as 1000 transferred OT-II cells were capable of affecting the CD8 T cell response. This number approaches the level of detection for transferred T cells in uninfected controls, and is in line with other studies that have examined the functional capacity of CD8 T cells at low transfer numbers (27, 28).

**CD4-mediated CD8 inhibition: competition for antigen?**

Having established a system whereby small numbers of CD4 T cells could affect the responding CD8 T cell population following primary viral infection, we wanted to assess how the CD4 T cells were mediating this effect. As a positive control for CD4 help, we adoptively transferred either 0 or $1 \times 10^6$ OT-II CD4 T cells along with either 0 or $1 \times 10^5$ OT-I CD8 T cells and 1 day later primed mice with OVA-loaded splenocytes (Fig. 5A and B). This priming regimen renders the OT-I T cell response CD4 independent when the OT-I T cells are transferred in sufficiently low numbers (29). We found that, in the absence of any additional CD4 help, the OVA-specific CD8 T cell response was minimal regardless of whether OT-I cells were present or not before priming (0.1%; Fig. 5A, bottom and top left panels, respectively). The addition of $1 \times 10^6$ OT-II cells prior to priming increased the OVA-specific CD8 T cell response to 0.6% without additional OT-I cells (Fig. 5A, top right-hand panel) and 0.8% when $1 \times 10^5$ OT-I cells were co-transferred with the OT-II cells (Fig. 5A, bottom right-hand panel), showing again the requirement for CD4 help in priming the CD8 T cell response in this system. Additionally, the presence of the OT-II cells also helped the OT-I T cells dominate the OVA-specific response at the expense of the endogenous OVA-specific T cells. Fig. 5B shows the CD11a$^+$ (activated) CD8$^+$ gated T cell population (right-hand quadrants in Fig. 5A) plotted as the OT-I donor marker against OVA-specific MHC class I tetramer staining. When no OT-I T cells were present, the OVA-specific response was completely dominated by the endogenous CD8 T cells (Fig. 5B, upper panels, lower right-hand quadrants). When $1 \times 10^5$ OT-I T cells were transferred without additional OT-II cells, the resulting OVA-specific CD8 T cell population was equally split between responding OT-I T cells and endogenous OVA-specific CD8 T cells (Fig. 5B, bottom left-hand panel; compare upper right- and lower right-hand quadrants, respectively). However, when the small number of OT-I T cells were co-transferred with $1 \times 10^5$ OT-II T cells, the OT-I T cells dominated the OVA-specific CD8 T cell response at the expense of the endogenous OVA-specific CD8 T cells (Fig. 5B, bottom right-hand panel; compare upper right- and lower right-hand quadrants, respectively). Such competition for antigen between CD8 T cells is not a new finding, and has indeed been recorded for CD4 T cells as well (13, 30, 31).

The above experiment clearly showed a role for CD4 T cells in helping to initiate a CD8 T cell response (albeit in a non-infectious system), as well as highlighting the antigenic competition known to occur between CD8 T cells (12, 13, 31, 32). Additionally, the experiment showed that the adoptively transferred OT-II cells were not defective in themselves, since they could clearly help the CD8 T cell response following a specific priming regimen. We then repeated the above transfer experiments using the low-dose VSV–OVA priming regimen in order to determine the effect that additional OT-II cells would have on the responding OT-I T cells. As previously shown, additional OT-II T cells caused a reduction in the responding OVA-specific CD8 T cell population when compared with the control (0.4% from 0.8%; Fig. 2C, upper right- and left-hand panels, respectively). When no additional OT-I T cells were transferred, the endogenous OVA-specific CD8 T cells responded to infection (Fig. 5D, lower right-hand quadrants in upper panels; left-hand panel, 0 OT-II and 0 OT-I; right-hand panel, $10^6$ OT-II and 0 OT-I). Transfer of $1 \times 10^5$ OT-I T cells in the absence of additional OT-II cells resulted in an OVA-specific CD8 T cell population that consisted of both OT-I T cells and responding endogenous CD8 T cells (Fig. 5D, bottom left-hand panel; upper right quadrant, OT-I T cells; lower right quadrant, endogenous T cells). However, when $1 \times 10^5$ OT-II T cells were co-transferred with $1 \times 10^5$ OT-I T cells prior to infection, the OVA-specific CD8 T cell response was once again almost completely abrogated (Fig. 5D, bottom right-hand panel). Significantly, the number of donor OT-I T cells could not be distinguished above control staining in mice that had received $1 \times 10^6$ OT-II cells (Fig. 5D, compare right-hand panels; upper right, 0 OT-I cells; lower right, $10^3$ OT-I cells). Since the OT-I T cells are capable of responding

![Fig. 4](image_url)

**Fig. 4.** Low numbers of antigen-specific CD4 T cells affect the CD8 T cell response to low-dose infection. Mice were seeded with varying numbers of transgenic OT-II cells infected with $1 \times 10^5$ p.f.u. VSV-OVA 1 day later and the percentage of N-specific (filled bars) or OVA-specific (open bars) CD8 T cells in the spleen determined 6 days following infection. Error bars represent the standard error of the mean, and asterisks represent significance compared with the untransferred control ($*P < 0.05$, **$P < 0.005$).
to infection in the absence of additional OT-II cells (Fig. 5D, bottom left-hand panel), the absence of the OT-I T cells in the presence of transferred OT-II cells suggests that the transferred CD4 T cells are acting at some level to inhibit antigen-specific CD8 T cell proliferation. This figure therefore highlights that it is not only the priming regimen but also the dose of virus that determines how a CD4 T cell helps the immune system respond to infection.

**Discussion**

We have described here an infection model that has allowed us to examine the role that CD4 T cells play in priming the CD8 T cell response to primary infection. In contrast to other infection models, including high-dose VSV infections (4, 5, 14, 15), we have found that antigen-specific CD4 T cells play a prominent role in the outcome of the primary CD8 T cell response. This role, however, is not the traditional role of CD4 T cell help described in non-infectious models (1–3), and has allowed us to directly examine the effect of antigen-specific CD4 T cells on the generation of the primary CD8 T cell response. Exactly how the CD4 T cells are affecting the immune response to a low-dose infection remains to be determined, although they are clearly preventing antigen-specific CD8 T cells from expanding. We can envisage three (not necessarily mutually exclusive) events to account for our
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Abbreviations

APC allogeneic thymic epithelial cell
BHK baby hamster kidney
CFSE 5-(and-6)-carboxyfluorescein succinimidyl ester
IV intravenously
OVA ovalbumin peptide
p.f.u. plaque-forming units
TLR toll-like receptor
VSV vesicular stomatitis virus

References


