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Helping Themselves: Optimal Virus-Specific CD4 T Cell Responses Require Help via CD4 T Cell Licensing of Dendritic Cells

Matthew R. Olson,*1 Shirley G. K. Seah,† Jolie Cullen,* Marie Greyer,* Kathryn Edenborough,* Peter C. Doherty,*‡ Sammy Bedoui,* Andrew M. Lew,† and Stephen J. Turner*

Although CD4⁺ T cell help (Th) is critical for inducing optimal B cell and CD8⁺ T cell responses, it remains unclear whether induction of CD4⁺ Th responses postinfection are also dependent on CD4⁺ T cell help. In this study, we show that activation of adoptively transferred Th cells during primary influenza A virus (IAV) infection enhances both the magnitude and functional breadth of endogenous primary IAV-specific CD4⁺ T cell responses. This enhancement was dependent on CD154-CD40–dependent dendritic cell licensing and resulted in a greater recall capacity of IAV-specific CD4⁺ and CD8⁺ T memory responses after heterologous IAV infection. These data suggest that engaging pre-existing CD4 responses at the time of priming may be a strategy for improving cellular immunity after vaccination. The Journal of Immunology, 2014, 193: 5420–5433.

The involvement of Ag-specific CD4⁺ Th cells is a key step to ensuring the maturation of robust B cell and T cell immunity following infection. Activated CD4⁺ T cells function to help Ag-specific B cells via the production of cytokines that impact on Ab isotype class switching. The responding Th set also provides essential costimulatory signals through cognate interactions (such as CD154/CD40) that promote B cell proliferation, survival, and, ultimately, differentiation to Ab-producing plasma cells (1–3).

Though not involving a direct interaction between the two T lymphocyte subsets, Ag-specific CD4⁺ T cells can also help responding CD8⁺ T cells by producing cytokines, such as IL-2. This cytokine is considered to act as a growth factor for rapidly dividing CD8⁺ CTLs while also driving full CTL differentiation and the acquisition of effector function (4–6). Th cells also serve to promote CD8⁺ T cell responses via licensing of Ag-presenting dendritic cells (DCs) via CD154 ligation of CD40 expressed on activated DCs (7–9). The consequence of this interaction is more efficient cross-presentation of Ag (9, 10) and the production of inflammatory cytokines (such as IL-12) by DCs (11) that facilitate the full maturation of effector CTL responses.

The dependence of CTL responses on CD4⁺ T cell help after primary immunization or infection is highly dependent on the nature of the immunogen. For example, acute CTL responses to pathogens or immunogens that are poorly inflammatory are dependent on CD4⁺ T cell help (8, 12, 13). In contrast, CD4⁺ T cell–independent primary CTL responses are readily induced in the context of more robust inflammatory microenvironments resulting from acute viral (12, 14–16) and bacterial infections (17). A likely scenario is that these pathogens directly activate DCs via the engagement of TLR pathways, circumventing the need for CD4⁺ Th-dependent CD40L to promote primary virus-specific CTL responses (18). Even so, it seems that CD4⁺ T cell help during the initial priming phase invariably plays a critical role in the generation of optimal CD8⁺ CTL memory (14, 15, 17), likely via the production of IL-2 (19). A more recent report demonstrated that CD154 expression in CD4⁺ T cells also contributes to maintaining DC activation at later stages of primary influenza infection helping sustain virus-specific CD8⁺ T cell (20).

Aside from providing help to B cell and CD8⁺ CTL responses, activated CD4⁺ T cells can also have a direct role in controlling influenza A virus (IAV) infection (21–23). It has also been shown that previous exposure with seasonal influenza generates CD4⁺ T cell memory populations capable of recognizing conserved peptide targets from heterologous influenza subtypes (24). Furthermore, recent data suggest that pre-existing CD4⁺ T cell memory T cell responses underpin heterostypic immunity after influenza challenge (25).

Despite the critical role of CD4⁺ T cells in multiple facets of antiviral immunity, little is known about the underlying mechanisms that contribute to the generation of robust, and highly functional, primary and secondary CD4⁺ T cell responses post-
infection. Given the requirement for CD4+ T cell help for optimal B cell and CD8+ CTL-mediated immunity, there remains the key question of whether CD4+ Th cells help themselves to promote effective CD4+ T cell responses. This question has been difficult to address, because the most common approach for assessing Th dependence of immunity is to either eliminate all of the CD4+ T cells by mAb depletion or use MHC class II (MHC II)−/− mice that lack the CD4+ set. We have addressed this issue by adoptive transfer of a distinct population of TCR-transgenic (Tg) OTII (26) CD4+ T cells specific for H2-Aβ OVA232–339 (IAA OVA232) peptide into naive mice. Recipient mice were then infected with a recombinant IA V engineered to express the OVA232–339 peptide (IAV-OVA) (27) in the viral hemagglutinin (HA), and the impact of this augmented help on the concurrent, endogenous influenza-specific (rather than IAA OVA232 specific) CD4 Th response (28) was assessed. The addition of CD4+ T cell help markedly enhanced the quantity and quality of primary IAV-specific CD4+ T cell responses postinfection. The results thus establish that the presence of large numbers of naive OTII helper cells indeed promotes the enhanced expansion and differentiation of concomitant primary and secondary CD4+ T cell responses, with the effect on the CD8+ set being seen only for the recall of CT-ligated immunity. Analyzing the underlying mechanism showed a prominent role for DC licensing via CD40 ligation.

Materials and Methods

Mice and virus infections

The wild-type (wt) C57BL/6 Ly5.2 mice and B6 background (CD40-deficient Ly5.2, CD154-deficient Ly5.2, CD1 cDC-TG/FOP, µMT Ly5.2, OTII Ly5.1, CD154KO OTII Ly5.1, TCR-Tg OTII, and gDT-II Ly5.1) mice were bred and housed in specific pathogen-free conditions at the Department of Microbiology and Immunology at the University of Melbourne (Parkville, VIC, Australia) or the Walter and Eliza Hall Institute (Parkville, VIC, Australia). This study was done in strict accordance with recommendations made by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice were maintained in specific pathogen-free environment with all animal experiments approved by the University of Melbourne Animal Ethics Committee (ethics license number 1011846.5).

In most experiments, wt or gene-deficient (CD8−/−) mice were dosed i.v. with PBS (as a control) or 103–104 × 106 TCR-Tg OTII CD4+ T cells specific for the chicken OVA 323–339 peptide. The OTII populations used for adoptive transfer were isolated from the lymph nodes (LN) of naive OTII TCR-Tg mice and enriched to give >80% CD4+ T cells by incubation at 37°C on plates coated with goat anti-mouse IgG and IgM Abs (Jackson Immuno-Research Laboratories) followed by anti-PE beads (Miltenyi Biotec) and a PE-conjugated Ab mixture (B220 [30-F11], CD11c [N418], CD8α [53.6.7] and NK1.1 [PK136] [All from BioLegend]). To analyze primary virus-specific CD4+ and CD8+ T cell response, PBS or OTII recipient mice were infected intranasally (i.n.) with 106 PFU wt IAV/X31 (IAV), IAV/ X31OVA232–339, (IAV-OVA), or IAV/X31gD232–233. To examine virus-specific T cell responses after secondary IAV infection, mice were first infected i.n. with 106 PFU HIN2 IAV-OVA and then challenged i.n. 45–60 d later with 2 × 103 PFU serologically different HIN1 PBS IAV.

Tissue sampling and isolation of lymphocytes and DCs

Single-cell suspension of spleen LNs from infected mice were pressed between frosted glass slides. Lungs were finely chopped and incubated at 37°C in 1 mg/ml collagenase A (Roche) for 30–45 min. After incubation, the lung samples were pressed through a 70-μm nylon mesh screen and washed with media containing 10% FCS. Bronchoalveolar lavage (BAL) cells were harvested by flushing the lungs three times with HBSS (University of Melbourne Media Preparation Unit). RBCs from spleen and lung samples were lysed by incubating for 5 min at room temperature with ammonium Tris chloride buffer (0.14 M NH₄Cl and 0.017 M Tris). DCs were enriched by Nycodenz (Progen, Heidelberg, Germany) gradient centrifugation as previously described by Bedoui et al. (29). Briefly, mediastinal LNs (mLNs) were collected 3 d after primary IAV infection and dispensed into microfuge tubes followed by collagenase/DNase/EDTA treatment for 20 min with constant agitation. The resulting cell preparation was layered onto Nycodenz (Progen), followed by a layer of FCS (Thermo Fisher, Wembley, Western Australia, Australia). After centrifugation, both upper density fractions were collected and pelleted. The cells were then stained with a mixture of Abs from eBioscience, anti-MHC II conjugated to AF700 (clone M5/114.15.2) and anti-CD11c conjugated to PE-Cy7 (clone N418); and from BD Pharmingen, anti-CD205 conjugated to aliphophycocyanin (clone 205yekta), anti-CD103 conjugated to PE (clone M290), anti-CD8α conjugated to Pacific Blue (clone 53.6.7), anti-CD40 conjugated to FITC (clone 3/23), and anti-CD11b conjugated to aliphophycocyanin-Cy7 (clone 557657). DC populations were gated on CD11c “MHC II” cell populations. Data were acquired on a BD FACS Aria (BD Pharmingen), and data were analyzed using FlowJo v8.8.5 (Tree Star) software.

Intracellular cytokine staining

The numbers of IAV-specific CD4+ and CD8+ T cells were determined by intracellular cytokine staining (ICS). Briefly, single-cell suspensions from the spleen, mLN, lung, and BAL were incubated with peptides (CD4: HA321–335 [YQQASGRVTSTFRS, 2 μg/ml] and PA311–325 [QYYSLIRPENPAHK, 2 μg/ml]; CD8: nucleoprotein [NP]366–374 [ASNEN- METM, 1 μM] and PA224–233 [SSLENFRAYV, 1 μM]; all from Auspep) in the presence of 1 μg/ml GolgiPlug (BD Biosciences) and 10 U/ml human IL-2 (Roche) at 37°C. After 6 h, cells were washed with FACS buffer (PBS containing 1% BSA and 0.02% sodium azide) and stained with either anti-CD4 (GK1.5; BioLegend) or anti-CD8α (53.6.7, BD Biosciences), and anti-CD44 (FM7; BioLegend) before fixation with BD fixation/permeabilization buffer (BD Biosciences). Cells were washed with permeabilization buffer and stained for intracellular IFN-γ (XMG1.2, eBio- science), TNF-α (MP6-XT22; BioLegend), IL-2 (JES5-5H4; BioLegend), or in some experiments CD154 (MR1; BioLegend). Flow cytometric analysis was performed on an FACS Canto II (BD Biosciences), and data were analyzed using FlowJo v8.8.5 (Tree Star) software.

In vitro generation of virus-specific CD4+ memory T cells

Naïve OTII CD4+ T cells were isolated from the LNs of uninfected OTII TCR-Tg mice, as above, and cultured in RPMI 1640 (Life Technologies) in the presence of plate-bound anti-CD3 (145-2C11; 10 μg/ml; BD Biosciences), anti-CD28 (37.51; 5 μg/ml; BioLegend), and recombinant human IL-2 (10 IU/ml) for an additional 5 d.

Generation of bone marrow chimeras

Mixed bone marrow (BM) chimeras were generated to assess the role of CD40 and CD154 on DCs. Femoral BM from CD40−/− or CD154−/− mice was mixed 1:1 with BM from CD11cDC-TG/FOP donors and used to reconstitute lethally irradiated (550 Gy; γ-irradiation; 66Co source, 3 h apart) wt B6 mice. Neomycin sulfate was also added to the drinking water for 4 wk following irradiation. The recipients were further injected i.p. 1 d after irradiation with 100 μl anti-Thy1 Ab (T24) to deplete endogenous T cells. After 8 wk, four mice per group were harvested and checked for reconstitution of CD4+ and CD8+ T cells, B cells, and DCs by FACS analysis. The DCs were enriched from these mice by finely dicing the spleens and incubating in the presence of collagenase/DNase and EDTA, followed by negative magnetic enrichment (29).

Determination of lung viral titers

Lungs from infected mice were harvested and washed immediately in cold HBSS. Lungs were placed in 2 ml RPMI 1640 containing 100 U/ml penicillin and 24 μg/ml gentamycin (both from Invitrogen) and homogenized using a tissue homogenizer (Polytron 2000). Lungs homogenates were centrifuged for 10 min at 4°C to pellet debris, and supernatants were aliquoted and frozen at −80°C for later viral titer determination on Madin-Darby canine kidney cells as previously described (30).

Data analysis

All data were analyzed using Prism v4.0a software (GraphPad). The statistical analysis used either a Student t test or ANOVA followed by a Tukey posttest where indicated, with p < 0.05 being considered significant.

Results

Heterologous CD4+ Th enhances primary virus-specific CD4+ T cell responses

Despite previous work demonstrating the CD4+ T cell dependency of high-quality virus-specific B cell and CD8+ T cell responses, it is unclear whether activated CD4+ T cells help themselves to
enhance antiviral CD4+ T cell-mediated immunity. To determine whether the addition of exogenous CD4+ Th serves to promote influenza-specific CD4+ T cell responses to the native IAV HA and NP peptides (28), graded numbers of OTII TCR-Tg CD4+ T cells (26) were adoptively transferred into naive C57BL/6 (B6, H2b) recipients and infected the following day with the IAV-OVA virus that expresses the OVA323–339 peptide within the head of the viral HA molecule (27). The total numbers of splenic IFN-γIAHA211- or IANP311-specific CD4+ T cells were then enumerated 10 d postinfection. Small, but measurable, IAV-specific CD4+ T cell responses were observed in controls that were pretreated with PBS, with there being little increase in the magnitude of IAHA211- and IANP311-specific CD4+ T cell responses in mice given up to 10^5 adoptively transferred OTIII Th cells. However, there was a significant increase (p < 0.05) in magnitude for the IAHA211- and IANP311-specific splenic CD4+ populations in mice that had received 10^6 OTII T cells when compared with the unaugmented (without OTIIIs) response (Fig. 1A, 1B). Moreover, the provision of additional CD4+ Th promotes greater IANP311-specific CD4+ T cell responses in the mLN and lung tissue of IAV-OVA–infected mice (Fig. 1C). As confirmation of the Th effect, the increase in endogenous IAV-specific CD4+ T cell spleen numbers was also observed for mice given 10^6 herpesvirus-specific gDT-II TCR-Tg CD4 T cells (29), followed by infection with a recombinant IAV expressing the cognate gD peptide (Supplemental Fig. 1A, 1B). Taken together, these data indicate that the provision of excess CD4+ Th enhances virus-specific CD4+ T cell responses.

To determine if the adoptive transfer of different numbers of OTII cells resulted in differences in activation status postinfection, we examined the expression of CD69, CD25 and Foxp3 on OTII T cells from mice that had received either 10^4 or 10^6 OTII cells prior to infection (Supplemental Fig. 1C). Greater numbers of OTII cells were present in mice that had received 10^6 OTII compared with those that had received 10^4 OTII cells in both the mLN and spleen. Moreover, the OTII cells in mice that had received a larger number of adoptively transferred cells exhibited higher levels of CD69 in the mLN, with lower levels of CD25 and Foxp3 in both the mLN and spleen (Supplemental Fig. 1C). This suggests that adoptive transfer of a greater number of OTII results in greater activation status of the transferred OTIIIs and may reflect diminished suppression of the CD4+ T cell responses due to the development of fewer regulatory T cells (Tregs).

We next examined the IAOVA323-specific CD4+ T cell response for both the adoptively transferred OTIIIs (CD45.1+) and endogenous CD4 T cell repertoire (Supplemental Fig. 1D). After adoptive transfer of PBS or 10^6 OTII cells into naive recipients, mice were infected 1 d later i.n. with 10^5 PFU of IAV/OVA323. Lymphocytes were isolated from the spleen, mLN, and BAL 10 d postinfection and stimulated with or without OVA323–339 peptide in the presence of brefeldin A for 6 h. These data show that OVA323–339 peptide stimulation did not induce IFN-γ production by OTII cells (Supplemental Fig. 1D). Moreover, the BAL, between 40 and 50% of these cells were IAb HA211- or IAb NP311-specific CD4+ T cells (Supplemental Fig. 1D). These data show that significant OTII CD4+ T cell reactivity is predominant at the site of infection rather than in the secondary lymphoid tissues. Moreover, these data suggest that the endogenous OVA323 CD4+ T cell response, at least in the context of IAV infection, is at best a subdominant response even in the absence of any competing OTII CD4+ T cell responses.

### FIGURE 1.
Addition of exogenous CD4+ Th cell enhances primary virus-specific CD4+ T cell responses. Naive OTII CD4+ T cells (Nil or 10^3–10^6) were adoptively transferred into naive recipients and infected i.n. the following day with 10^5 PFU IAV/X31 OVA323 (IAV-OVA). Spleens were harvested on day 10 and analyzed for the proportion and total number (per spleen) of IAHA211- and IA NP311-specific CD4+ T cells by IFN-γ ICS. (A) Representative dot plots gated on splenic CD4+ lymphocytes, with the number in each plot representing the proportion that were IFN-γ in the total CD4+ set. (B) The total number of IAV-specific CD4+ T cells calculated based on the frequencies shown in (A). Error bars represent SEM. *Significantly different by Student t test (p < 0.05) as compared with mice that received no (Nil) exogenous CD4+ Th cells. These experiments represent one of four individual experiments with three to four mice per group. (C) The total number of IA NP311-specific CD4+ T cells were determined for the spleen, mLN (MedLN), and lung 10 d after primary infection. Data are mean ± SEM. These data represent three individual experiments with at least eight mice per group. *p < 0.05 by Student t test as compared with mice that received PBS.
Published evidence variously suggests that CD4+ Th for CD8+ T cell responses can be independent of, or require, cognate peptide–MHC II (pMHC II) stimulation of the Th set (13, 31). We thus analyzed IAV-specific CD4+ T cells responses in mice given adoptively transferred OTII CD4+ T cells that were then infected i.n. with the wt IAV/X31 strain that lacks the OVA peptide. As might be expected, the OVA TCR-Tg Th enhancement of the IAbHA211- and IAbNP311-specific CD4+ T cell responses was lost under these conditions, with native IAV-specific CD4+ T cell numbers being equivalent for IAV wt-infected mice that were first given PBS or OTII CD4+ T cells (Supplemental Fig. 2). These numbers being equivalent for IAV wt-infected mice that were first compared with mice that received no (Nil) exogenous CD4+ OTII CD4+ T cells had heightened IAbHA211- and IAbNP311-specific CD4+ T cell responses. At all time points examined, the mice that received in mice with augmented CD4+ Th, the total number of double (IFN-γ/IL-2+) CD4+ T cells and particularly triple cytokine–producing (IFN-γ/IL-2+/TNF-α+) CD4+ T cells were markedly increased (Fig. 3D; p < 0.05). Moreover, this enhanced functional capacity of IAV-specific CD4+ T cells generated with OTII Th help was maintained into long-term memory (Fig. 3E). Thus, additional CD4+ Th at the time of priming augments IAV-specific CD4+ T cell responses during both the acute and memory phases of IAV-specific CD4+ T cell response.

**Augmentation of IAV-specific CD4+ T cell responses is dependent on CD40–CD154 interactions**

The provision of CD4+ T cell help to B and CD8+ T cells is dependent on the ligation of CD40 on the surface of DCs and/or or B cells by CD154 expressed on the CD4+ Th set (2, 3, 8). We thus reasoned CD4+-mediated virus-specific CD4+ T cell augmentation might also depend on this CD40–CD154 interaction. To test this hypothesis, mice given either PBS or OTII CD4+ T cells were treated 1 d prior to IAV-OVA infection with either a control IgG or an anti-CD154 (MR1) Ab to inhibit CD40–CD154 binding. As expected, the marked increase in the proportion and total numbers of IAbHA211- and IAbNP311-specific CD4 T cells in mice given OTII CD4 T cells as compared with the controls (Fig. 4A, 4B; p < 0.05) was abolished by the MR1 treatment (Fig. 4A, 4B; p > 0.05). Thus, the augmented IAV-specific CD4+ T cell responses observed after the addition of the OTII Th set are CD40–CD154 dependent. Moreover, the augmented functionality of helped CD4+ T cells (Fig. 3) is also lost upon treatment with MR1 (Fig. 4C). These data indicate that CD40–CD154 interactions are critical for the increase in both numbers and functional capacity promoted for IAV-specific CD4+ T cells by the exogenous CD4+ Th set.

**CD154 expression by the OTII Th set is key for optimal IAV-specific CD4+ T cell responses**

To definitely show the aforementioned effect was due to CD4 expression of CD154 on activated OTII CD4+ T cells, CD4+ T cells from conventional (CD154+/+) OTII or CD154−/− OTII donors were adoptively transferred into wt B6 recipients and infected i.n. with the IAV-OVA virus. As expected (Figs. 1, 2), giving wt B6 recipients the normal OTII-CD154+/+ T cells resulted in greater

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**FIGURE 2.** Additional CD4+ Th help enhances primary CD4+ but not CD8+ IAV-specific responses. Mice were given PBS (Nil) or 10^6 OTII CD4 T cells and infected i.n. with IAV-OVA as per Fig. 1. Mice were then harvested at intervals and assessed by IFN-γ ICS for the total numbers (see Fig. 1) of responding IAbHA211- (A) and IAbNP311-specific (B) CD4+ T cells and DNP366-specific (C) and DP324-specific (D) CD8+ CTLs. Results are mean ± SEM. Each data point represents at least six mice, from two to three individual experiments. *p < 0.05 as compared with mice that received no (Nil) exogenous CD4+ Th. L.O.D., limit of detection.
IA$^h$HA$_{211}$- and IA$^h$NP$_{311}$-specific CD4$^+$ T cell responses in the spleen (Fig. 5A, left panels, 5B, 5C) and mLNs (Fig. 5D, 5E) at 10 d postinfection, with the effect being reduced to background levels (PBS control) in mice given equivalent numbers of CD154$^{-/-}$ OTII cells (Fig. 5). Clearly, CD154 expression on the adoptively transferred OTII Th cells is required for optimal IAV-specific CD4$^+$ T cell responses.

At the same time, we asked if CD154 expression on the exogenous OTII Th set is sufficient to augment the IAV-specific CD4$^+$ T cell responses (Fig. 5) by doing the same experiment using CD154$^{-/-}$ or CD154$^{+/+}$ BM mixed with CD11c-DTR-GFP BM (Supplemental Fig. 6). Transfer of CD154$^{+/+}$ OTII Th cells into these mice dramatically enhanced both the IA$^h$HA$_{211}$- and IA$^h$NP$_{311}$-specific CD4$^+$ T cell response in the spleen (Fig. 6C, 6D, 6E, 6F) as compared with PBS-treated controls ($p < 0.05$). In DT-treated, CD40$^{-/-}$ or CD154$^{-/-}$ BM mixed at a 1:1 ratio with BM from CD11c human diphtheria toxin receptor (DTR) Tg mice, in which all CD11c$^+$ cells express the DTR and GFP (33). Efficient chimerism was established in mice receiving either CD40$^{-/-}$ or CD154$^{-/-}$ BM, mixed with CD40$^{+/+}$CD154$^{+/+}$CD11c-DTR-GFP BM (Supplemental Fig. 3).

OTII TCR-Tg CD4$^+$ T cells were adoptively transferred into chimeric recipient mice, infected the next day with the IAV-OVA, and then treated with either DT or PBS for 1–5 postinfection. Subsequent to DT treatment, significant CD11c$^+$ DC depletion (compared with PBS controls) was seen for both sets of chimeric mice (Fig. 6A, 6B, $p < 0.05$). In DT-treated, CD40$^{-/-}$ mixed BM chimeras that had received OTII CD4$^+$ T cells, there was a 70–80% decrease (versus PBS control) in both frequency and total numbers for the IA$^h$HA$_{211}$- and IA$^h$NP$_{311}$-specific CD4$^+$ T cells (Fig. 6C, 6D, $p < 0.05$). However, these IAV-specific CD4$^+$ T cell responses were unaffected by DT in the CD154$^{-/-}$ mixed BM chimeras (Fig. 6E, 6F, $p > 0.05$). Interestingly, although elimination of the CD40-expressing DCs had a minimal impact on the capacity of IA$^h$NP$_{311}$-specific set to produce IL-2 (Fig. 6C, 6D, $p > 0.05$), the data described above suggested CD154 expression was critical on CD4$^+$ Th cells to promote optimal antiviral CD4$^+$ T cell responses (Fig. 6), suggesting that CD40 signaling is an important component of this mechanism.
right panel), loss of the CD40-expressing DCs reduced the proportion of IL-21IAHNP311-specific CD4+ T cells (Fig. 6E, 6F, right panel; \( p < 0.05 \)). Taken together, these data strongly suggest that CD154-expressing CD4 T cells interact directly with CD40-bearing DCs to induce maximal IA V-specific CD4+ T cell responses, whereas CD154 expression on DCs may contribute to ensuring optimal IL-2 production by these same IA V-specific CD4+ T cells.

Although priming of IAV-specific CD8+ T cell responses is essentially limited to CD8α+ (LN) and/or CD103+ (lung)-derived DCs, a broader range of DC subsets are capable of presenting Ag to CD4+ T cells (34). In an attempt to further delineate the precise CD11c+ DC subset that was being affected by the addition of exogenous CD4+ T cell help, we examined cell-surface expression of MHC II and CD40 on DCs populations isolated from the mLNs 3 d after adoptive transfer of OTII and IA V-OVA infection (Fig. 7). There was no difference in the levels of cell-surface expression of MHC II on CD11c+CD103+ DCs among mice that received none, 103, or 106 OTII (Fig. 7A–D). Although no difference in MHC II levels were observed on CD8+ DCs (Fig. 7E, 7G), there was increased CD40 expression on CD8+ DCs isolated from mice that had received 106 OTII compared with 103 or no OTII T cells (Fig. 7F, 7H). This supports the notion that additional CD40L–CD40 interactions are contributing to augmented primary CD4+ T cell responses after IAV infection.

**Rested effector CD4+ T cells can provide help to augment influenza-specific primary Th responses**

An implication from the above data are that pre-existing memory CD4+ T cells could be used to boost naive Th responses during or after vaccination. In an attempt to address this point, we generated rested effector CD4 T cells by activating naive OTII CD4 T cells in vitro for 2 d with plate-bound anti-CD3ε and soluble anti-CD28 mAbs and then resting these cells for an additional 5 d in IL-2. These cells were ~95%+ OTII CD4+ T cells (as identified by expression of TCR chains Vα2 and Vβ5.1/5.2), and virtually all expressed CD44 and CD62L, which are typically used to identify memory CD4 T cells (Fig. 8A) (35). Graded numbers of rested CD44hiCD62Lhi OTII Th cells were adoptively transferred into naive recipient mice that were infected the following day with the IAV-OVA virus. The total numbers of splenic IFN-γ+ IAbHA211- or IAbNP311-specific CD4 T cells were then enumerated 10 d postinfection as per Fig. 1. Similar to Fig. 1, only mice that had received 106 previously activated CD44hiCD62Lhi CD4 T cells exhibited an increased proportion and total number (\( p < 0.05 \)) of IAbHA211- and IAbNP311-specific CD4 T cells as compared with PBS (nil)–treated mice (Fig. 8B, 8C). Furthermore, both IAbHA211- and IAbNP311-specific CD4 T cells exhibited greater functional capacity (\( p < 0.05 \)) to produce IFN-γ (MFI) and IL-2 (percentage, right panel) (Fig. 8D; \( p < 0.05 \)). These data indicate that targeting of established memory CD4+ T cells could potentially promote de novo virus-specific CD4 T cell responses.

**Augmented CD4 Th during the primary response enhances secondary IAV-specific CD4+ and CD8+ T cell responses after wt IAV challenge**

Earlier studies showed that although CD4+ Th is not necessarily required for primary CD8+ CTL responses, it is crucial for the recall of optimal CTL memory (14, 15, 17). Given that the provision of excess CD4+ Th help enhances primary IAV-specific CD4+ T cell responses, we next asked whether there is any effect on the recall of immunity, a key issue for any vaccination strategy that might use these findings. Naive wt mice that were
CD154 expression on CD4+ T cells is necessary and sufficient for optimal IAV-specific responses. Naive wt and CD154−/− recipients were given PBS (Nil) or 2 × 10^6 wt or CD154−/− OTII CD4+ Th cells and infected with IAV-OVA as before. Spleen and mLN (MedLN) populations were harvested on day 10, and the proportions and total numbers of IAbHA211- and IAbNP311-specific CD4+ T cells were analyzed by IFN-γ ICS. (A) Representative dot plots of splenic CD4+ gated lymphocytes after in vitro stimulation with no peptide or the HA211- or NP311-peptides. Numbers on each plot represent the frequency of IFN-γ+ CD4 T cells after stimulation. The total numbers (per organ) of IAbHA211- (B and C) and IAbNP311-specific (C, D) T cells are shown for the spleen (B and C) and mLNs (D and E). Data are mean ± SEM. Representative of four experiments with at least 10 mice per group. *Significantly different by ANOVA (p < 0.05). KO, knockout.
given PBS or OTII CD4+ T cells were infected with IAV-OVA (HKx31, H3N2) and rested for 45–60 d before secondary i.n. challenge with wt IAV/PR8 (H1N1). This gives a second round of infection in the respiratory tract, as the H3N2 and H1N1 viruses are serologically distinct, and there is limited Ab-mediated neutralization, without activating memory CD4+ OT-II Th cells established after the primary infection. Comparable numbers of IAbNP311-specific CD4+ T cells were observed until day 5 in mice primed in the absence or presence of OTII CD4+ Th, but the recall of the helped IAV-specific memory CD4+ set was massively increased by days 7.5 and 12 (Fig. 9A, left panels, p < 0.05). This was due, in part, to the greater numbers of IAV-specific CD4+ memory T cells, an effect that is most apparent in the spleen (Fig. 9), and also resulted in a significantly larger number of these lymphocytes localizing to (or being retained in) the site of virus-induced pathology (BAL, Fig. 9).

Although the numbers of IAV-specific CD8+ CTLs generated at the acute phase of the primary response in mice primed with IAV-OVA (H3N2) were identical in the presence (OTII) or absence (nil) of augmented Th, there were significantly more DbNP366-specific (though not DbPA224-specific) CD8+ memory T cells at day 60 in mice that had received OTII cells (Fig. 2C). Both the DbNP366- and DbPA224-specific CD8+ sets were, however, significantly increased in size for every site analyzed on days 7.5 and 12 after the wt IAV (H1N1) challenge (Fig. 9A, right panels; p < 0.05). Furthermore, the relative fold expansion of these cells, from day 0 (“resting” memory) to day 7.5 was two to four times higher for all locations in the mice that were helped during the primary infection (Fig. 9B). Given that the secondary D8NP566-specific CD8+ T cell response is already an immunodominant response compared with other IAV-specific secondary responses (36), it is particularly impressive that the
excess CD4+ Th during the initial priming phase leads to further augmentation.

Importantly, the enhanced secondary IA^BNP311-specific CD4+ T cell response was completely absent (p > 0.05) from the spleen and BAL populations of mice given OTII CD4+ Th and MR1 (anti-CD40L) treatment at the time of initial priming (Fig. 10A, 10B), with the same being true for the secondary D^BNP66-specific CD8+ CTL expansion (Fig. 10C, 10D). Clearly, the CD154–CD40 interactions observed between activated OTII T cells and DCs at the time of priming are key for establishing memory IAV-specific T cell populations with enhanced recall capacity. Interestingly, mice that had augmented secondary Th responses cleared secondary IAV infection by day 7 after, irrespective of MR1 treatment (Fig. 10E). Thus, although anti-CD40 blockade at the time of initial priming resulted in diminished IAV-specific secondary T cell responses in mice that received OTII T cells, this response is still capable of effective viral clearance. Interestingly, mice that had CD40L–CD40 interactions blocked at the time of initial priming exhibited greater immunopathology after A/PR8 challenge, as measured by greater weight loss over the course of the secondary infection (Fig. 10F). This was regardless of whether mice had received OTII T cells at the time of initial priming or not (Fig. 10F, compare open squares to open triangles). So despite being able to clear a secondary infection, CD40L–CD40 interactions likely result in altered effector functions that impact immune regulation in the infected lung.

Discussion

The present analysis shows very clearly that the provision of exogenous, virus-specific CD4+ T cell help at the time of T cell priming serves to enhance both primary and secondary IAV-specific CD4+ T cell responses. Strikingly, this enhancement of IAV-specific CD4+ T cell responses was completely dependent on CD40–CD154 interactions. Previous studies with infectious disease models have demonstrated that activated CD4+ T cells promote optimal B cell and CD8+ T cell responses via cell-surface CD154 that interacts with CD40 on activated B cells (2, 3) or DCs (8, 13, 32), respectively. Moreover, Ballesteros-Tato et al. (20) have recently demonstrated that CD154 expression on CD4+ T cells also plays a role in helping maintain DC activation late in infection. Thus, in a manner analogous to optimal priming of the CD8+ CTL response (10, 13), our data support a model by which cognate CD4–DC interactions and subsequent CD154–CD40 ligation underpin the induction of optimal virus-specific CD4+ T cell responses. Although we cannot discount the possibility that CD4+ T cell activation results in the production of cytokines that indirectly contributes to conditioning of DCs promoting either survival and/or proliferation of DCs, particularly early postinfection, the fact that the OVA323 peptide stimulation induces poor cytokine responses by OTII, especially in secondary lymphoid tissues (Supplemental Fig. 1D), we prefer the notion that Th augmentation of DC activation results from direct cell–cell contact and subsequent sustained DC licensing.

One way that CD4-dependent licensing of DCs has been shown to promote CD8+ T cell responses is via enhancement the costimulatory capacity of licensed DCs (8, 9). Furthermore, IL-2 production by responding CD4+ T cells is a byproduct of enhanced costimulation (37), and we observed improved IL-2 production by IAV-specific CD4+ T cells in mice that had received additional CD40–CD154-dependent CD4+ Th. Thus, the CD40–CD154-dependent licensing of DCs by activated CD4+ Th cells and enhancement of DC costimulatory capacity may also serve to promote virus-specific CD4+ T cell responses. Moreover, CD40-dependent DC activation enhances MHC II expression and presentation, leading to more efficient CD4+ T cell activation (38).

The provision of exogenous CD4+ Th in the context of IAV infection may therefore act to increase and/or maintain the expression of pMHC II complexes on the surface of Ag-presenting DCs, thereby allowing extended immunogen presentation and full activation of the endogenous IAV-specific CD4+ T cell responses. Moreover, the generation of polyfunctional CD4+ T cells can be attributed to stimulation with increasing concentrations of cognate peptides on activated DCs (39). This supports our observations that the addition of exogenous CD4+ Th promoted the generation of
IAV-specific CD4+ T cells with enhanced functional capacity. Although we did not observe increased expression of pMHC II in our model, it is tempting to speculate that increased DC activation (measured by increased CD40 expression on CD8+ DCs), as well as more prolonged Ag presentation, contributes to both greater proliferation and a broader cytokine response profile within populations of responding IAV-specific CD4+ T cells, and this currently under investigation.

Earlier studies indicated that naive, CD154−/− CD4+ T cells proliferate less after in vivo immunization, despite showing a normal capacity to proliferate in vitro in response to polyclonal stimuli (40). Further, CD154−/− CD4+ T cells fail to provide help to B cells when primed in a CD40-deficient (CD40−/−) environment, suggesting a role for CD154 signaling on the T cell to induce full functional capacity (41). Based on these data, a conclusion might be that CD4+ T cells need to express CD154 to facilitate their own priming and/or to provide help for other cells of the immune system. Uniquely, our data establish that this is not the case. Although CD154−/− mice showed reduced CD4+ T cell responses after IAV infection (Fig. 5), CD154−/− recipients that received exogenous OVA-specific CD154+/+ help and were then infected with IAV-OVA generated robust and highly functional IAV-specific CD154−/− CD4+ T cells. These data show conclusively that CD154 expression by responding virus-specific CD4+ T cells is not mandatory if there is an alternative source of CD154. Rather, the diminished CD4+ T cell response observed in CD154−/− mice can be attributed to a reduced capacity of these cells to provide Th for other responding CD4+ T cells.
Polyfunctional T cells, as defined by their capacity to produce IFN-γ, TNF-α, and IL-2, have been identified as a positive correlate of immune protection against viral, bacterial, and parasitic infections in mice and humans (43–45). Developing a better understanding of the factors that promote the generation of polyfunctional T cell responses may thus provide insights into improving vaccine strategies designed to elicit robust cellular immunity. We demonstrate in this study that the addition of exogenous CD4+ Th increases both virus-specific CD4+ T cell numbers and markedly improves their polyfunctionality. This gain of function postinfection clearly indicates that a major factor controlling the generation of poorly versus highly functional CD4+ T cells is the extent of help available during the initial priming phase. Similar observations have been made for CD8+ T cells, in which CD4+ Th is critical for the capacity of these cells to produce IL-2 and respond following secondary infection (19).

We have previously demonstrated that addition of exogenous CD4+ Th cell help contributes to accelerated control of primary IAV infection (46). Interestingly, when we examined viral clearance after secondary A/PR8 IAV challenge, we observed no difference in the kinetics irrespective of whether mice had received augmented help at the time of initial priming or not. This likely reflects the fact that even in a context in which secondary T cell responses have been compromised, there is significant compensation by other immunological mechanisms that result in effective viral clearance. Importantly, when CD40L–CD40 interactions were blocked at the time of initial priming, we did observe greater immunopathology during the course of secondary A/PR8 IAV infection. It is intriguing that IAV-specific CD8+ effector T cells isolated from infected mouse lungs are capable of producing IL-10, a potent negative regulator of inflammation (47). It is tempting to speculate that the initial CD40L–CD40 interactions...
are important for setting up the appropriate regulatory mechanisms, particularly in the infected lung, which ensure that robust immune responses in sensitive tissues such as the lung, are appropriate.

The provision of exogenous CD4+ Th during primary infection clearly increased IAV-specific CD4+ T cell numbers and improved their polyfunctionality. There was, however, no comparable effect on the acute phase of the IAV-specific CD8+ CTL responses. These data are in line with previous reports that IAV-specific CD8+ CTL effectors are minimally, if at all, dependent on CD4+ Th during primary infection (14, 18). The likely explanation is that IAV infection directly activates DCs via engagement of TLR pathways, circumventing the need for a CD4+ T cell–DC interaction to elicit robust primary effector CD8+ T cell responses (18). That said, our data clearly show that activated CD4+ T cells are not redundant during the priming phase, as mice that had been given a source of exogenous CD4+ Th exhibited marked increases in IAV-specific CD4+ and CD8+ T cell numbers after secondary challenge. Furthermore, both were dependent on CD40–CD154 interactions during the initial primary response. This indicates that early conditioning of DCs by augmented CD4+ Th promotes the programming of both long-lived memory CD4+ and CD8+ T cells. To date, the precise mechanisms that result in the enhanced recall capacity of helped IAV-specific T cell responses remains to be determined. Given recent data suggesting that specific changes in chromatin landscape are key for maintaining an optimal recall capacity by virus-specific memory T cells (48–51), we hypothesize that the addition of exogenous CD4+ T cell help results in key alterations in the transcriptional and genomic signature of memory T cells that promote more robust recall capacity. We are currently using systems biology approaches to address this question.

When we transferred CD154KO OTII cells into recipients, we observed a diminished primary IAV-specific CD4+ T cell response compared with mice that did not receive OTII cells. Ballesteros-Tato et al. (20) have recently demonstrated that CD154 expression on CD4+ T cells is required to maintain DC activation and therefore sustain primary IAV-specific CD8+ T cell responses, particularly at latter stages of infection. A conclusion from these
studies was that CD154–CD40 T cell–DC interactions serve to counteract early contraction of the primary CD8+ T cell response by inhibiting the suppressive effects of Tregs at late stages of infection. Similarly to the CD8+ T cell response, it is conceivable that CD4+ Treg activation also serves to suppress the IAV-specific CD4+ T cell response during the primary response. This notion is supported by our observation that addition of higher numbers of OTII cells resulted in increased levels of activation as measured by CD69, and this correlated with a decrease in the Foxp3 expression and therefore decreased Treg development. Thus, it is possible that the addition of excess activated CD4+ T cells at the time of initial IAV infection serves to override endogenous CD4+ Treg suppression of IAV-specific CD4+ T cell responses due to increased CD4+ T cell responses.

Regardless of the mechanism, these findings are intriguing as they suggest that, although sufficient, normal levels of CD4+ Th are likely suboptimal for generating optimal primary and secondary virus-specific T cell responses. Thus, these data indicate that vaccine strategies capable of inducing strong primary CD4+ T cell responses, or, alternatively, rely on stimulating pre-existing CD4+ memory Th, may also function to promote the induction of optimal de novo vaccine-specific CD4+ and CD8+ T cell responses. For example, it may be useful to engineer inactivated viruses or subunit vaccines to include peptides from common human pathogens (such as EBV and human CMV) that are recognized by high frequency CD4+ T cells. Furthermore, the addition of exogenous CD4+ Th has been shown to rescue virus-specific CD8+ CTL function after chronic viral infection (52). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). Another approach would be to include vaccine conjugates or carriers, such as the diphtheria toxoid antigen, after chronic viral infection (53). 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Supplemental Figure 2. Supplemental Figure 1. Augmentation of CD4+ T cell response by a different TH set. Naïve mice were given either PBS (Nil) or 10^3-10^6 herpesvirus IA^b^DTII-specific TCR-Tg CD4+ T cells, then infected i.n. the following day with 10^5 PFU IAV/X31gD. Spleens were harvested 10d later and the proportion and total number of IA^b^HB^211^- and IA^b^NP^311^-specific CD4+ T cells was determined by IFN-γ ICS. (A) Representative dot plots gated on CD4+ T lymphocytes, with the numbers in each plot representing the proportion IFN-γ+ in the total CD4+ set. (B) The total number of IAV-specific CD4 T cells based on the frequencies shown in panel A. data is mean ± SEM * = p<0.05) as compared to mice that received no (Nil) exogenous CD4+ T H. These experiments represent one of 2 individual experiments with 3-4 mice per group. (C) Analysis of activation status of OTIIIs after adoptive transfer of different precursor frequencies. Naive mice were given either PBS, 10^4 or 10^6 OTIIIs and infected IAV/HKx31OVA^323^ and the MLN and spleen harvested 7 days later. Cells were stained for CD45.1 and the number of OTIIIs enumerated, as well as the levels fo CD69 expression, CD25 and Foxp3 expression. Shown are representative data (n=5 mice per group) from 2 independent experiments. (D) Analysis of the OTII and endogenous IA^b^OVA^323^-specific response after IAV/HKx31OVA^323^ primary infection. Naïve mice were given either PBS (Nil) or 10^6 OTII TCR-Tg CD4+ T cells, then infected i.n. the following day with 10^4 PFU A/HKx31OVA^323^. Spleens were harvested 10d later and the proportion of IA^b^OVA^323^-specific CD4+ T cells determined by IFN-γ ICS. Shown are representative dot plots gated on CD4+CD44hi T cells showing IFN-γ production of CD45.1+ populations.
Supplemental Figure 2. CD4 T cell help for virus-specific CD4 T cells is antigen-dependant. Naïve mice were given PBS (Nil) or $10^6$ OTII CD4 T cells and infected with $10^4$ PFU of wt IAV lacking the OVA<sub>323-339</sub> peptide. (A) Spleen, MLN and BAL were harvested and analysed for the total numbers of HA<sub>211</sub>- (left panel) and NP<sub>311</sub>-specific CD4 T cells (right panel) by IFN-γ ICS. (B) Shows the capacity of splenic IFN-γ<sup>+</sup> NP<sub>311</sub>-specific CD4 T cells to produce TNF-α (% left panel), and IFN-γ (MFI, right panel). These data represent 1 of 2 individual experiments with 3-4 mice per group. Data is mean ± SEM, n.s., not significantly different by Student’s t-test ($p>0.05$).
Supplemental Figure 3. Reconstitution of BM chimera mice. Bone marrow from CD40−/− or CD154−/− mice was mixed 1:1 with BM from CD40+/+CD154+/+CD11cDTRGFP mice and used to reconstitute naïve, lethally irradiated, T cell depleted, wt B6 mice. After 8 weeks, spleens from 4 CD40−/−:CD11cDTR mice, 4 CD154−/−:CD11cDTR mice and 1 each of CD40−/− and CD154−/− mice were harvested and analysed for reconstitution of the adaptive immune system. The proportion of CD40-expressing B cells isolated from the LNs was analysed in each group (A, representative dot plots, left panel and B, quantitation of CD40+B cells, left panel). DCs (CD11c+ MHCII+) enriched from the spleens of each group were examined for CD40 and GFP expression (A, representative contour plots, left panel and B, quantitation of GFP+ DCs in each group). CD154 expression by CD4 T cells isolated from the LNs after in vitro stimulation with PMA/ionomycin (A, right panel and B, quantitation of CD154-expression on CD4 T cells). *, significantly different (p<0.05) as determined by Student’s t-test.