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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,*† Shirly 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+ T 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 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 responses.

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 heterosubtypic 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 (IAβOVA323) peptide into naive mice.Recipient mice were then infected with a recombinant IAV 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 IAβOVA323 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 concomitantly primary and secondary CD4+ T cell responses, with the effect on the CD8+ set being seen only for the recall of CD8-T-mediated 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/6J Ly5.2 mice and B6 background (CD40-deficient Ly5.2, CD154-deficient Ly5.2, CD11cDTR/GFP, μMT Ly5.2, OTI LY5.1, CD154KO OTI LY5.1, TCR-Tg OTII, and gdt-II Ly5.1) mice were bred and housed in specific pathogen-free conditions at the Department of Animal Health 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 1018465).

In most experiments, wt or gene-deﬁcient (−/−) mice were dosed i.v. with PBS (as a control) or 10^3−1 × 10^3 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 (LNs) 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 ImmunoResearch Laboratories) followed by anti-PE beads (Miltenyi Biotec) and a PE-conjugated Ab mixture (B220 [30-F11], CD11c [N418], CD8a [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 10^6 PFU wt IAIV31 (IAV), IAV/X31OVA323–339 (IAV-OVA), or IAV/X31gdl3b−/−. To examine virus-specific T cell responses after secondary IAV infection, mice were first infected i.v. with 10^4 PFU HIN2 IAV-OVA and then challenged i.n. 45–60 d later with 2 × 10^3 PFU serologically different H1N1 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 disrupted with a scalpel 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 allopheco-cyanin (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 allopheco-cyanin-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: HA211–225 [YQQASGRVTYRSTRS, 2 μg/ml] and PA311–323 [QVYSLRNPENPAHK, 2 μg/ml]; CD8: nucleoprotein [NP]266–274 [ASNENMETM, 1 μM] and PA224–233 [SSLNFRAYV, 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 (GK.1; BioLegend) or anti-CD8a (53.6.7, BD Biosciences), and anti-CD44 (F(ab’)_2), BioLegend) before fixation with BD fixation/permeabilization buffer (BD). Cells were washed with permeabilization buffer and stained for intracellular IFN-γ (XM1G12, eBioscience), TNF-α (MP6-XCT2; BioLegend), IL-2 (JES5-5H4; BioLegend), and in some experiments CD154 (MR1; BioLegend). Flow cytometric analysis was performed on a 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**

Naive 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-CD3ε (145-2C11; 10 μg/ml; BD Biosciences), anti-CD28 (29F1; 5 μg/ml; BioLegend), and reciprocal human IL-2 (10 U/ml) until primary (2 d) and secondary (4–5 d) CD4+ T cell responses were established. Data were acquired on a BD FACS Aria (BD Pharmingen), and data were analyzed using FlowJo v8.8.5 (Tree Star) software.

**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 CD11cDTR/GFP donors and used to reconstitute lethally irradiated (550 Gy) recipient mice (group 2, 3 weeks 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-Thyl 1 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 gentamicin (both from Invitrogen) and homogenized using a tissue homogenizer (Polytron 1200 PT). Lung 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 IA V 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 IA V-OVA virus that expresses the OVA323–339 peptide within the head of the viral HA molecule (27). The total numbers of splenic IFN-γ⁺ IAHA211⁺- or IA³NP311⁺-specific CD4⁺ T cells were then enumerated in mice given up to 10⁵ adoptively transferred OTII Th cells. However, there was a significant increase (p < 0.05) in magnitude for the IA³HA211⁺- and IA³NP311⁺-specific splenic CD4⁺ populations in mice that had received 10⁶ OTII T cells when compared with the unaugmented (without OTIIs) response (Fig. 1A, 1B). Moreover, the provision of additional CD4⁺ Th when compared with the unaugmented (without OTIIs) response increased greater IA³NP311⁺-specific CD4⁺ T cell responses in the OTII Th cell recipients and infected the following day with the IA V-OVA virus (27). The total numbers of IAbHA211⁺ and IAbNP311⁺-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 IA³NP311⁺-specific CD4⁺ sets in mice given up to 10⁵ adoptively transferred OTII Th cells. However, there was a significant increase (p < 0.05) in magnitude for the IA³HA211⁺- and IA³NP311⁺-specific splenic CD4⁺ populations in mice that had received 10⁶ OTII T cells when compared with the unaugmented (without OTIIs) response (Fig. 1A, 1B). Moreover, the provision of additional CD4⁺ Th promoted greater IA³NP311⁺-specific CD4⁺ T cell responses in the mLN and lung tissue of IA V-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 10⁶ 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 cells that had received either 10⁴ or 10⁶ OTII cells prior to infection (Supplemental Fig. 1C). Greater numbers of OTII cells were present in mice that had received 10⁶ OTII compared with those that had received 10⁴ 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 OTIIs 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 IA³OVA323⁺-specific CD4⁺ T cell response for both the adoptively transferred OTIIs (CD45.1⁺) and endogenous CD4⁺ T cell repertoire (Supplemental Fig. 1D). After adoptive transfer of PBS or 10⁶ OTII cells into naive recipients, mice were infected 1 d later i.n. with 10⁵ 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, CD45.1⁺) in the spleen and only modestly by cells in draining mLN (<1%). However, in the BAL, between 40 and 50% of these cells made IFN-γ upon peptide stimulation. Importantly, we did not observe any significant endogenous IA³OVA323⁺-specific CD4⁺ T cell responses after peptide stimulation in mice that either received PBS or OTII CD4⁺ T cells (Supplemental Fig. 1D, CD45.1⁻). 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 OVA323–339 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 10³–10⁶) were adoptively transferred into naive recipients and infected i.n. the following day with 10⁶ PFU IAV/X31/OVA323 (IAV-OVA). Spleens were harvested on day 10 and analyzed for the proportion and total number (per spleen) of IA³HA211⁺- and IA³NP311⁺-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 based calculated from 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 IA³NP311⁺-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 data establish that the augmentation by CD4+ Th is Ag dependent.

We next examined the kinetics of the IAV CD4+ T cell response postinfection for mice that had received nil (PBS) or 10^6 OTII CD4 T cells. At all time points examined, the mice that received OTII CD4+ T cells had heightened IAbHA211- and IAbNP311-specific CD4+ T cell responses when compared with the PBS controls (Fig. 2A, 2B; p < 0.05), though the DbNP366- and DpPA224-specific CD8+ CTL responses were virtually identical for both groups of mice (Fig. 2C, 2D). These data indicate that the addition of exogenous CD4+ Th boosts primary virus-specific CD4+, but not CD8+ T cell responses.

**Exogenous CD4+ Th enhances the functional capacity of IAV-specific CD4+ Th cells**

We next asked if providing additional CD4+ Th cells at the time of priming altered the functional capacity of the endogenous IAV-specific CD4+ set. Subsequent to short-term in vitro peptide stimulation (Fig. 3), a significantly greater proportion of IAV-specific CD4+ T cells from mice that had received OTII T cells versus the PBS-treated unhelped mice were found to coexpress IFN-γ, TNF-α, and IL-2 (Fig. 3A, 3B; p < 0.05). Furthermore, both IAbHA211- and IAbNP311-specific CD4+ T cells observed in mice with augmented CD4+ Th, the total number of double (IFN-γ+/TNF-α+) and particularly triple cytokine–producing (IFN-γ+/TNF-α+/IL-2+) 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...
IA^{HA}_{211-} and IAnP_{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^{−/−} 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 at a 1:1 ratio with BM from CD11c^{−/−} mice (Fig. 6). Further, the data described above suggested that CD40 expression on DCs is important for the OTII Th-dependent enhancement of IAV-specific CD4+ T cell responses, we generated mixed BM chimeras using donor CD40^{−/−} or CD154^{−/−} or CD154^{+/−} BM mixed with WT BM, or CD11c^{−/−} BM, to determine if CD40 expression on DCs is important for the OTII Th-dependent enhancement of IAV-specific CD4+ T cell responses (Fig. 6), suggesting that CD40 signaling is an important component of this mechanism.

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CD40 expression by CD11c^{+} cells is required for optimal IAV-specific CD4+ T cell responses

Licensing of activated DCs results from cognate CD40–CD154 interactions between the DC and activated, Ag-specific CD4+ T cells (10, 13, 32). Further, 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. Further, the data described above suggested that CD154 expression on DCs was critical on CD4 Th cells to promote optimal antiviral CD4+ T cell responses (Fig. 6C, 6D, p < 0.05). In DT-treated, CD40^{−/−} or CD154^{−/−} BM mixed at a 1:1 ratio with BM from CD11c^{−/−} 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 WT BM, or CD11c^{−/−} BM, to determine if CD40 expression on DCs is important for the OTII Th-dependent enhancement of IAV-specific CD4+ T cell responses (Fig. 6), suggesting that CD40 signaling is an important component of this mechanism. Further, the data described above suggested that CD154 expression on DCs was critical on CD4 Th cells to promote optimal antiviral CD4+ T cell responses (Fig. 6C, 6D, p < 0.05). In DT-treated, CD40^{−/−} or CD154^{−/−} BM mixed at a 1:1 ratio with BM from CD11c^{−/−} 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 WT BM, or CD11c^{−/−} BM, to determine if CD40 expression on DCs is important for the OTII Th-dependent enhancement of IAV-specific 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-2*IAbHA211-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 IAV-specific CD4+ T cell responses, whereas CD154 expression on DCs may contribute to ensuring optimal IL-2 production by these same IAV-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 IAV-OVA infection (Fig. 7). There was no difference in the levels of cell-surface expression of MHC II on CD8+ DCs (Fig. 7E, 7G), there was increased CD40 expression on CD8+ DCs isolated from mice that had received 10^6 OTII vs. 10^3 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.

Resting 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 restocking 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 Va2 and Vb5.1/5.2), 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 10^6 previously activated CD44hiCD62Lhi CD4 T cells exhibited an increased proportion and total number (p < 0.05) of IAbHA211- or 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 (%) (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
**FIGURE 5.** 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 D9NP366-specific (though not D8PA224-specific) CD8+ memory T cells at day 60 in mice that had received OTII cells (Fig. 2C). Both the D9NP366- and D8PA224-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 D9NP366-specific CD8+ T cell response is already an immunodominant response compared with other IAV-specific secondary responses (36), it is particularly impressive that the

**FIGURE 6.** CD40 expression on CD11c+ cells is critical for optimal IAV-specific CD4 T cell responses. Mixed BM chimeras (CD40−/−:CD11cDTR, CD154−/−:CD11cDTR) were given 1 × 10⁶ naïve wt OTII CD4+ T cells and infected i.n. with IAV-OVA. Additionally, these mice were treated with PBS or with diphtheria toxin (DT) from 1 to 5 d postinfection. Spleens were analyzed for depletion of IAb+ CD11c+ GFP+ cells on day 10. (A) Representative histograms depicting the proportion of GFP+ of IAb+ CD11c+ gated splenocytes. The proportion of GFP+ cells was quantitated in CD40−/−:CD11cDTR and CD154−/−:CD11cDTR BM chimera mice (B). Representative dot plots of splenic CD4+ gated lymphocytes from CD40−/−:CD11cDTR (C) or CD154−/−:CD11cDTR (E) BM chimera mice after in vitro stimulation with media alone (no peptide) or the HA211- or NP311-peptides. Numbers on each plot represent the frequency of IFN-γ+ CD4 T cells after stimulation. Additionally, representative histograms of the capacity of IFN-γ+ IAbNP311-specific CD4 T cells to produce IL-2 are shown for each set of chimeras (percentage, right panel). The total number of IFN-γ+ IAbNP311-specific (left panel) or IAbHA211-specific (middle panel) CD4+ T cells per spleen of CD40−/−:CD11cDTR (D) or CD154−/−:CD11cDTR (F) BM chimeras. Additionally, the frequency of IFN-γ+ IAbNP311-specific CD4 T cells producing IL-2 is depicted for CD40−/−:CD11cDTR mice (D, right panel) and CD154−/−:CD11cDTR mice (F, right panel). Data are mean ± SEM. These data are representative of two experiments with 10–12 mice per group. *Significantly different from PBS-treated mice (p < 0.05) by Student t test.
excess CD4⁺ Th during the initial priming phase leads to further augmentation.

Importantly, the enhanced secondary IA^NP311^-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^NPbcl-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 contribute to conditioning of DCs promoting either survival and/or proliferation of DCs, particularly early postinfection, the fact that the OVA932 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⁺ T 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-\(\gamma\), TNF-\(\alpha\), 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 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

![Image](http://www.jimmunol.org/Downloadedfrom/5430CD40LICENSINGOFDCSPROMOTESThCELLRESPONSES.png)
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

**FIGURE 10.** Blockade of CD40/CD154 interactions during primary infection limit IAV-specific T cell responses after secondary challenge. Mice were treated with anti-CD154 mAb and infected with IAV-OVA H3N2 as per Fig. 4. After 45 d, mice were challenged i.v. with IAV/PR8 (H1N1), and a spleen (A, C) and BAL (B and D) were harvested on day 7 and analyzed for the total number of IA3NP311-specific CD4+ T cells (A and B) and DNP366-specific (C and D) CTLs by IFN-γ ICS. *Significantly different by Student t test (p < 0.05) as compared with PBS-treated mice given no exogenous CD4+ Th, †significantly different by Student t test (p < 0.05 MR1-treated mice as compared with human IgG–treated mice. (E) Lung viral titers were determined for naive mice or mice that had received PBS (white bars) or 10⁶ OTII T cells (black bars) and were then infected with 10³ PFU A/PR8. Some mice were also treated with anti-MR1 at the time of initial infection as described above. (F) Weight loss was measured daily for the same groups of mice. Data are mean ± SEM. These data are representative of two experiments with at least five mice per time point. *Significantly different (p < 0.05 Nil MR1 compared to PBS) by Student t test; †significantly different (p < 0.05, OTII MR1 versus OTII PBS). L.O.D., limit of detection.
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 CD40L–CD40 interactions.

Regardless of the mechanism, these findings are intriguing as they suggest that, although sufficient, normal levels of CD4+ T cells 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, relying on stimulating pre-existing CD4+ T cell responses, or CD4+ Treg activation also serves to suppress the IAV-specific CD4+ T cell responses 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 CD40L–CD40 interactions.

Disclosures

The authors have no financial conflicts of interest.

References


CD40 LICENSED OF DCs PROMOTES T cell RESPONSES