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Direct IL-6 Signals Maximize Protective Secondary CD4 T Cell Responses against Influenza

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Memory T cells can often respond against pathogens that have evaded neutralizing Abs and are thus key to vaccine-induced protection, yet the signals needed to optimize their responses are unclear. In this study, we identify a dramatic and selective requirement for IL-6 to achieve optimal memory CD4 T cell recall following heterosubtypic influenza A virus (IAV) challenge of mice primed previously with wild-type or attenuated IAV strains. Through analysis of endogenous T cell responses and adoptive transfer of IAV-specific memory T cell populations, we find that without IL-6, $CD4^+$, but not $CD8^+$, secondary effector populations expand less and have blunted function and antiviral impact. Early and direct IL-6 signals to memory CD4 T cells are required to program maximal secondary effector responses at the site of infection during heterosubtypic challenge, indicating a novel role for a costimulatory cytokine in recall responses. ***The Journal of Immunology*****, 2016, 197: 3260–3270.**

Innate inflammatory responses are critical for protection against primary (1°) influenza A virus (IAV) infection. Triggering of innate immune recognition pathways with pathogen lysates or specific pathogen products to boost acute inflammation (1–5) dramatically improves the outcome of IAV challenge in unprimed mice. In contrast, mice deficient for TLR adaptor proteins MyD88 and Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF) (6, 7), components of inflammasome pathways (8, 9), or treated with anti-inflammatory agents (10) are all far more susceptible to 1° IAV challenge. A key function of innate immune recognition pathways is to induce production of costimulatory cytokines, but how individual components of the inflammatory response contribute to protection is not clear, at least in part because the impact of many cytokines and chemokines is multifactorial. For example, unprimed IL-6–deficient ($Il6^{-/-}$) mice succumb to doses of IAV that are sublethal for wild-type (WT) mice, an outcome correlated in different studies with either impaired Ab production, reduced neutrophil activity, or dysregulated immunopathology (11–13). Similarly, IL-1 (8, 14) and TNF (15–19) each have been shown to contribute to protection against 1° IAV infection through multiple mechanisms.

Mice and humans primed with, or vaccinated against, one strain of IAV generate neutralizing Ab that prevents reinfection with the same strain providing long-lived "homotypic" immunity. Priming also generates strong protection against IAV strains not recognized by neutralizing Ab. This "heterosubtypic" immunity is largely mediated by memory T cells that recognize internal IAV protein epitopes that are shared between the priming and challenge virus (20). In contrast to the critical role of TLR pathways in protecting naive animals against IAV, heterosubtypic protection does not require TLR signaling, as IAV-primed WT and MyD88/TRIF-deficient mice are equally resistant to supralethal heterosubtypic challenge (6). This has been taken to indicate that innate inflammation plays only a minimal role in heterosubtypic immunity. However, memory CD4 T cells responding against IAV rapidly induce the production of high levels of several cytokines, including IL-6, IL-1, and TNF, from several innate immune cell subsets through a MyD88/TRIF-independent mechanism (21). This T cell–driven inflammatory response correlates with viral control (22, 23) and suggests that production of some innate cytokines, triggered through pathways other than the traditional pattern recognition receptor pathways, may be critical for heterosubtypic protection (24).

In this study, we analyze whether and how IL-6 expression impacts heterosubtypic immunity. $Il6^{-/-}$ mice primed with highly virulent or attenuated mouse-adapted IAV strains displayed impaired viral clearance and enhanced infection-associated morbidity following secondary (2°) challenge with heterosubtypic IAV. Analysis of endogenous polyclonal and adoptively transferred TCR transgenic (Tg) CD4 and CD8 T cell responses reveal that the generation of IAV-specific T cell memory is not impacted by IL-6. However, the magnitude and functional potential of recall CD4 T cell responses are dramatically and selectively impaired. We confirm that protection against IAV mediated by adoptive transfer of memory $CD4^+$, but not $CD8^+$, T cells is severely compromised in $Il6^{-/-}$ hosts, as well as in WT hosts treated with IL-6–neutralizing Ab.

Mechanistically, the critical IL-6 signals required for optimal memory CD4 T cell–mediated protection are delivered only during

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Abbreviations used in this article: 1°, primary; 2°, secondary; dLN, draining mediastinal lymph node; dpi, d postinfection; EID_{50}, 50% egg infective dose; HA, hemagglutinin; IAV, influenza A virus; ICCS, intracellular cytokine staining; NP, nucleoprotein; PA, acidic polymerase; PB, basic polymerase; $TCID_{50}$, 50% tissue culture infective dose; Tg, transgenic; Treg, regulatory T cell; TRIF, Toll/IL-1R domain–containing adapter inducing IFN-β; WT, wild-type.

the first few days after infection. Early IL-6 drives maximum expansion of primed CD4 T cells and enhances production of the cytokines IL-2, TNF, and IFN-γ, especially in the cohort of cells responding in the lung. Finally, by analyzing IL-6 receptor–deficient memory CD4 T cells responding in WT hosts, we show that direct IL-6 signals to memory CD4 T cells are responsible for promoting maximal 2° effector expansion and function.

These findings highlight striking differences in how IL-6 impacts the outcome of 1° versus 2° IAV challenge, and in how IL-6 affects recall responses of $CD4^+$ versus $CD8^+$ T cells during acute viral infection. Our studies indicate a unique role for early IL-6 in promoting protective CD4 T cell memory responses and suggest that upregulated expression of IL-6 during this phase of the recall response might dramatically improve heterosubtypic protection against seasonal and pandemic IAV.

Materials and Methods

Mice

BALB/c, C57BL/6, C57BL/6.Thy1.1, J_HD (BALB/c background), and *Il6*$^{-/-}$ (BALB/c and C57BL/6 background) mice were at least 8 wk old at time of infection. Naive $CD4^+$ cells and $CD8^+$ T cells were obtained from 5- to 8-wk-old HNT.Thy1.1 mice or clone 4.Thy1.1 mice on a BALB/c background, respectively. Naive CD4 T cells were also obtained from OT-II.Thy1.1.Thy1.2 mice or OT-II cells deficient for IL-6Rα (CD126) that were obtained by crossing C57BL/6 mice carrying a floxed IL-6Rα gene (The Jackson Laboratory) to OT-II mice expressing Cre driven by the CD4 promoter. HNT mice express a TCR recognizing aa 126–138 (HNTNGVTAACSHE) of A/Puerto Rico/8/34 (PR8, H1N1) hemagglutinin (HA), OT-II mice express a TCR recognizing aa 323–339 (ISQAVHAAHAEINEAGR) of chicken OVA, and clone 4 mice express a TCR recognizing aa 518–528 (IYSTVASSL) of PR8 HA. J_HD and HNT TCR Tg mice were obtained from the animal breeding facility at the Trudeau Institute or the University of Massachusetts Medical School. Some mice were originally obtained from The Jackson Laboratory and were bred at the Trudeau Institute or the University of Massachusetts Medical School. Experimental animal procedures were conducted in accordance with Animal Care and Use Committee guidelines.

Naive cell isolation, memory T cell generation, cell transfer, and Ab treatments

Naive T cells from TCR Tg mice were obtained from pooled spleen and lymph nodes as previously described (25). Resulting TCR Tg cells were routinely >97% TCR^+ and expressed a characteristic naive phenotype (small size, $CD62L^{hi}$, $CD44^{lo}$, and $CD25^{lo}$). Th1 or Tc1 effectors were generated from naive TCR Tg T cells cultured as previously described (25–27). In vitro–generated memory cells were obtained from effector cultures that were washed several times and rested for at least 3 d in media free of Ag or exogenous cytokine. In some experiments, cells were labeled with CFSE (Molecular Probes) prior to adoptive transfer. All cell populations were adoptively transferred to unprimed mice in 200 μl of PBS by i.v. injection.

Mice were injected i.p. with IL-6–neutalizing Ab (MP5-20F3) or isotype control Ab (HRPN) (Bio X Cell) as indicated. In some experiments, mice were treated i.p. with 0.5 mg of anti–Ly-6G Ab to deplete neutrophils (1A8) or isotype control (2A3) (Bio X Cell) at 0, 2, 4, and 6 d postinfection (dpi).

Virus stocks and infections

A/Puerto Rico/8/34 (PR8, H1N1) originating from stocks at St. Jude Children's Hospital, A/PR8-OVA_{II} (H1N1) (provided by P. Doherty), A/Philippines/2/82/x-79 (A/Phil, H3N2), and the cold-adapted attenuated strain A/Alaska/6/77 CR-29 (H3N2) (28) (both provided by S. Epstein, National Institutes of Health) were grown in the allantoic cavity of embryonated hen eggs at the Trudeau Institute and the 50% egg infective dose (EID_{50}) or 50% tissue culture infective dose ($TCID_{50}$) were characterized. Mice were infected intranasally under light isoflurane anesthesia (Webster Veterinary Supply) with stated doses of virus in 50 μl of PBS. Mice that received adoptively transferred T cells were infected on the same day as cell transfer. All infected mice were monitored daily for percentage weight loss, hunched posture, ruffled fur, and lack of movement as previously described (29).

Tissue preparation

At different time points after virus infection, mice were euthanized by cervical dislocation followed by exsanguination by perforation of the abdominal aorta. Lungs were perfused by injecting 10 ml of PBS in the left ventricle of the heart. Lungs, spleen, and draining mediastinal lymph node (dLN) were prepared into single-cell suspensions by mechanical disruption of organs and passage through a nylon membrane.

For assessment of immunopathology following IAV infection, lung lobes were isolated and immediately fixed in 10% neutral buffered formalin. Lung samples were subsequently processed, embedded in paraffin, sectioned, placed on L-lysine–coated slides, and stained with H&E using standard histological techniques. Sections were graded blindly from 0 to 4 by a board-certified pathologist (S. Sell) on the basis of the extent of mononuclear cell infiltration and tissue damage.

Real-time PCR

Viral titers were determined by quantitation of viral RNA. RNA was prepared from whole-lung homogenates using TRIzol (Sigma-Aldrich), and 2.5 μg of RNA was reverse transcribed into cDNA using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed to amplify the acidic polymerase (PA) gene of PR8 and A/Phil using an ABI Prism 7700 sequence detector (Applied Biosystems) with 50 ng of cDNA per reaction and the following primers and probe: forward primer, 5′-CGGTCCAAATTCCTGCTGA-3′, reverse primer, 5′-CATTGGGTTCCTTCCATCCA-3′; probe, 5′-6-FAM-CCAAGTCATGAAGGAGAGGGAATACCGCT-3′. Data were analyzed with Sequence Detector v1.7a (Applied Biosystems). The copy number of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid of known concentration as a standard.

Flow cytometry

Cell suspensions were washed, resuspended in FACS buffer (PBS plus 0.5% BSA and 0.02% sodium azide; Sigma-Aldrich) and incubated on ice with 1 μg of anti-FcR (2.4G2) followed by saturating concentrations of the following fluorochrome-labeled Abs for surface staining: anti-Thy1.1 (OX-7), anti-Thy1.2 (53-2.1), anti-CD4 (RM4.5), anti-CD8 (H35-17.2), anti-CD69 (H12F3), anti-CD25 (PC61.5), anti-CD127 (A7R34), anti-CD44 (IM7), anti-CD45.2 (104), anti-CD11b (M1/70), and anti–Ly6-G (Gr-1, RB6-8C5) (BD Pharmingen, eBioscience, or BioLegend). To detect IAV-specific polyclonal CD4 T cells in IAV-primed mice, cells were stained for 1 h at 37°C with $I\text{-}A^b$/nucleoprotein $(NP)_{311–325}$ fluorochrome-labeled tetramer obtained from the National Institutes of Health Tetramer Facility prior to surface marker staining.

For intracellular cytokine staining (ICCS), CD4 T cells were stimulated for 4 h with 10 ng/ml PMA and 50 ng/ml ionomycin (Sigma-Aldrich) or for 18 h with 100 HA units of IAV (PR8) lysate. After 2 h, 10 μg/ml brefeldin A (Sigma-Aldrich) was added. Cells were then surface stained and fixed for 20 min in 4% paraformaldehyde followed by permeabilization for 10 min by incubation in 0.1% saponin buffer (PBS plus 1% FBS, 0.1% sodium azide, and 0.1% saponin; Sigma-Aldrich) and staining for cytokine by the addition of anti–IFN-γ, anti–IL-2, and TNF fluorescently labeled Ab (BD Pharmingen, eBioscience, or BioLegend) for 20 min. Granzyme B and Foxp3 (eBioscience) expression was determined by intracellular staining as per the manufacturer's instructions.

All FACS analyses were performed using LSR II and FACSCanto flow cytometers (BD Biosciences) and FlowJo (Tree Star) analysis software.

ELISPOT assay

ELISPOT analysis for detection of IFN-γ– and IL-2–secreting T cells was performed as previously described (21). Briefly, 96-well plates (Millipore) were coated overnight with purified anti-mouse IFN-γ or IL-2 Ab. Plates were washed and blocked with complete T cell media prior to the addition of 10^5 lung cells or 10^6 spleen or lymph node cells and 10^6 syngeneic APCs from the spleen of unprimed mice. Wells were stimulated with 100 HA units of IAV lysate to elicit CD4 T cell responses or with 10 μg of $PA_{224–233}/D^b$, $NP_{366–374}/D^b$, or basic polymerase $(PB)_{703–711}/K^b$ peptides to elicit CD8 T cell responses. Plates were incubated overnight with biotinylated detection Ab against IFN-γ or IL-2 followed by streptavidin alkaline phosphate substrate. Plates were developed with 5-bromo-4-chloro-indolyl phosphate substrate, and resulting spots were counted on an ImmunoSpot reader (Cellular Technology).

Detection of cytokines in culture supernatants

Luminex analysis with a multiplex kit (Millipore) was used to detect levels of cytokines present in culture supernatants. Purified memory OT-II cells were stimulated in vitro with 10 ng/ml PMA and 50 ng/ml ionomycin. After

8 h, culture supernatants were harvested and analyzed using a Bio-Plex 200 system (Bio-Rad Laboratories).

Statistical analysis

Unpaired two-tailed Student *t* tests ($\propto = 0.05$) were used to assess whether the means of two normally distributed groups differed significantly. The Welch correction was applied when variances were found to differ. One-way ANOVA analysis with a Bonferroni multiple comparison posttest was employed to compare multiple means. A p value <0.05 was considered significant. The log-rank test was used to test for significant differences in Kaplan–Meier survival curves. All error bars represent the SD.

Results

IL-6 is required for survival following high-dose IAV priming

To study the role of IL-6 in heterosubtypic protection, we first primed WT BALB/c or corresponding *Il6*$^{-/-}$ mice with a low dose (500 EID_{50} = 0.1 LD_{50} for WT mice) of the highly pathogenic mouse-adapted H1N1 IAV strain A/PR8 (30) and followed the course of the 1° response. Only minimal differences in weight loss and recovery distinguished WT and *Il6*$^{-/-}$ mice (Fig. 1A) and all mice survived. Because higher doses of IAV generate stronger T cell memory (31), we next challenged mice with 5-fold more virus (2500 EID_{50} = 0.5 LD_{50} for WT mice). Both strains initially lost equivalent weight (Fig. 1B), but whereas virtually all WT mice recovered, none of the *Il6*$^{-/-}$ mice survived (Fig. 1C). Identical results were observed in WT and *Il6*$^{-/-}$ C57BL/6 mice (data not shown). The reduced resistance of *Il6*$^{-/-}$ mice to a 2500 EID_{50} dose of IAV correlated with increased viral titers detected at 9 dpi but not at earlier time points (Fig. 1D), consistent with observations of IL-6 deficiency leading to impaired viral clearance during 1° infection (12, 13). Histological analysis at 7 dpi revealed greater inflammation in WT lungs based on perivascular, peribronchial, intraepithelial, and alveolar changes (Fig. 1E, 1F). Taken together, these results confirm that IL-6 mediates proinflammatory and antiviral effects that are required for the resolution of higher dose 1° IAV infection (12).

IL-6 is required for optimal heterosubtypic immunity

We next tested the importance of IL-6 during heterosubtypic challenge of WT and *Il6*$^{-/-}$ mice primed with 500 EID_{50} of A/PR8 (H1N1), from which all mice recovered (Fig. 1A). Primed mice were challenged after at least 45 d with a supralethal (150 LD_{50}) dose of the H3N2 A/Phil IAV strain. Compared to WT mice, *Il6*$^{-/-}$ mice lost significantly more body weight and their recovery was delayed several days (Fig. 2A). Nevertheless, all primed *Il6*$^{-/-}$ mice survived the 150 LD_{50} challenge (Fig. 2B). Enhanced weight loss in the *Il6*$^{-/-}$ mice correlated with higher viral titers and delayed viral clearance versus primed WT mice (Fig. 2C); however, an impressive degree of viral control was nonetheless evident in primed *Il6*$^{-/-}$ mice compared with unprimed WT mice challenged with the same dose of A/Phil (Fig. 2C). These results demonstrate that IL-6 contributes significantly to optimal viral clearance and reduced morbidity following heterosubtypic IAV challenge. This could be due either to its actions during 1° or 2° responses, or both.

Recall CD4 T cell responses are compromised in Il6$^{-/-}$ *mice*

To test a role for IL-6 in a model more closely resembling vaccine-generated heterosubtypic immunity, we used the attenuated cold-adapted H3N2 IAV strain A/Alaska/77 (21) as a priming virus. WT or *Il6*$^{-/-}$ C57BL/6 mice were primed with two doses of 2500 $TCID_{50}$ A/Alaska/72 10 d apart, followed at least 45 d later by challenge with 20 LD_{50} A/PR8 as in our previous studies (21). All primed WT and *Il6*$^{-/-}$ mice survived the 20 LD_{50} A/PR8 challenge whereas unprimed mice succumbed by 10 dpi (Fig. 3A, left panel), indicating induction of substantial heterosubtypic immunity by the attenuated IAV strain. However, primed *Il6*$^{-/-}$ mice lost significantly more weight and their recovery was delayed versus primed WT mice (Fig. 3A, right panel), consistent with results observed in the live nonattenuated heterosubtypic model summarized in Fig. 2.

Heterosubtypic immunity is largely dependent on memory T cell responses directed against epitopes of internal proteins that are shared between the priming and challenge virus (32). Therefore, we used ELISPOT analysis at 5 dpi, the peak of 2° T cell responses in this model (21), to enumerate virus-specific IL-2– and IFN-γ–producing T cells. Immunodominant MHC class I–restricted peptides derived from the nucleoprotein ($NP_{366–374}$) or RNA polymerase proteins ($PA_{224–233}$ and $PB1_{703–711}$) that are highly conserved between A/Alaska and A/PR8 (21) were used to detect CD8 T cell responses. IAV (PR8) lysate (33) was used to stimulate a broad spectrum of IAV-reactive CD4 T cells.

The number of IFN-γ–secreting T cells elicited by the immunodominant CD8 T cell epitopes was similar in WT and *Il6*$^{-/-}$ mice (Fig. 3B) as was the total number of activated $CD44^{hi}CD8^{+}$ T cells present in the lung (Fig. 3C). There was an indication that $PB1_{703–711}$ responses in the spleen may be somewhat dependent on IL-6, but the differences did not reach significance and were not present in the dLN or lung. Very few peptide-dependent IL-2 spots were detected from CD8 T cells (not shown). Thus, the generation of 2° IAV-specific CD8 T cell responses did not depend on the presence of IL-6 either during the 1° response or following 2° challenge.

In contrast, at 5 dpi, both IL-2 and IFN-γ spots generated in response to IAV lysate in the spleen, dLN, and lung (Fig. 3D, 3E), as well as the total number of activated $CD44^{hi}CD4^{+}$ T cells detected in the lung (Fig. 3F), were significantly reduced in *Il6*$^{-/-}$ mice, indicating by inference an impaired recall CD4 T cell response. To verify that the CD4 T cell response is impaired, the frequency and number of IAV-specific $CD4^{+}$ T cells capable of dual IFN-γ/IL-2 production at 5 dpi was assessed in the spleen, dLN, and lung by intracellular cytokine staining. When compared with WT hosts, significant reductions in the frequency (Fig. 3G, 3H), expressed as a ratio of the WT response, and number of dual IFN-γ/IL-2 cytokine producers (Fig. 3I) were observed in *Il6*$^{-/-}$ mice. Virtually no IAV lysate– or MHC class I peptide–dependent spots were detected in unprimed mice challenged with A/Phil at this time point, consistent with previous studies (21). In total, these results suggest that compromised heterosubtypic protection observed in *Il6*$^{-/-}$ mice is likely to be largely due to the reduced efficacy of recall $CD4^{+}$ T cell responses.

Memory CD4 T cell generation is not impaired in Il6$^{-/-}$ *mice*

The decreased recall CD4 T cell responses in *Il6*$^{-/-}$ mice could reflect impaired memory cell generation after initial priming, reduced CD4 T cell memory maintenance, or a reduced 2° CD4 T cell response following rechallenge. To test these possibilities, we first enumerated memory CD4 T cells recognizing $NP_{311–325}$ in A/Alaska-primed WT and *Il6*$^{-/-}$ mice prior to heterosubtypic challenge using an MHC class II tetramer (34). The frequency and absolute number of $CD44^{high}tetramer^{+}$ cells detected in all organs tested at 45 dpi were equivalent in WT and *Il6*$^{-/-}$ mice (Fig. 4A, 4B). We also observed that the efficiency of memory formation from naive OT-II TCR Tg CD4 T cell donors transferred to WT or *Il6*$^{-/-}$ hosts that were subsequently challenged with A/PR8-OVA_{II} (Fig. 4C, 4D) was similar at 45 dpi. Furthermore, the IAV-primed OT-II cells in the spleen and dLN expressed similar levels of the IL-7R α-chain (CD127) (Fig. 4E), levels of which strongly correlate with the survival fitness of memory CD4 T cells (34). Interestingly, donor cells in the lung expressed reduced CD127 in *Il6*$^{-/-}$ hosts (Fig. 4E). Finally, cytokine production from restimulated memory OT-II cells isolated from WT or *Il6*$^{-/-}$ hosts, including the frequency of dual IFN-γ^{+}IL-2^{+}–producing cells that are often correlated with

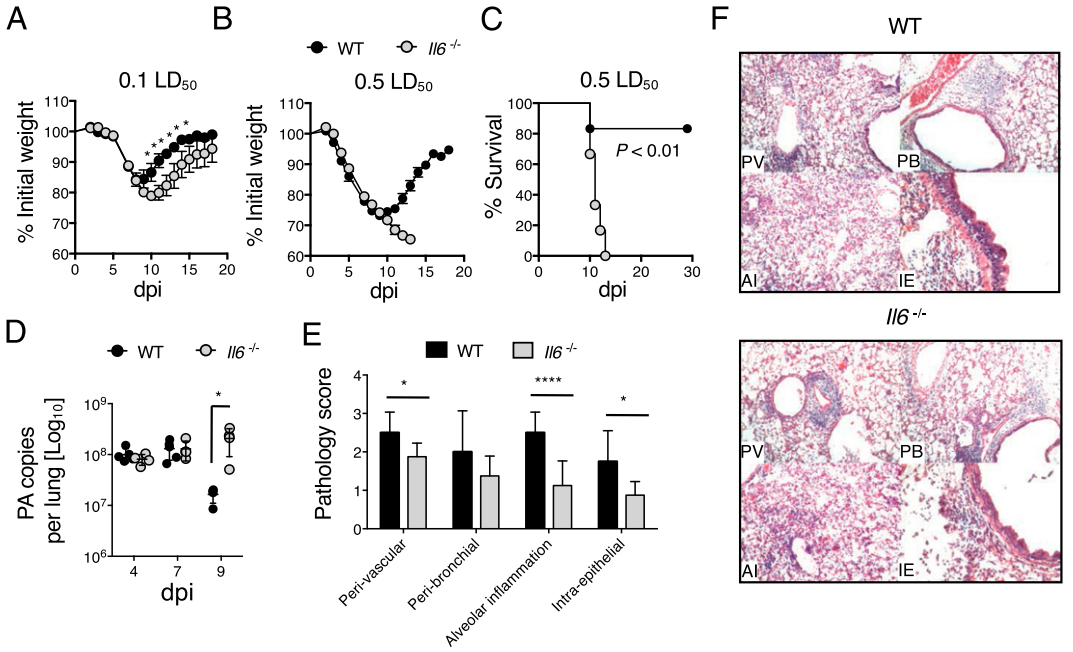

FIGURE 1. High priming doses of IAV are lethal in the absence of IL-6. WT (black symbols) or *Il6*$^{-/-}$ (gray symbols) mice on a BALB/c background were infected with a low dose (500 EID_{50} = 0.1 LD_{50} for WT mice) or high dose (2500 EID_{50} = 0.5 LD_{50}) of A/PR8 (H1N1). Weight loss following (**A**) low- and (**B**) high-dose challenge (n = 5 mice per group; one of five experiments). On the indicated days following 2500 EID_{50} challenge, (**C**) survival and (**D**) viral titers were assessed (n = 3–5 mice per group per d). H&E-stained lung sections were scored blindly for levels of histopathology at 7 dpi. Summary of analysis (n = 3–5 mice per group per d) (**E**) and representative H&E images at original magnification ×10 (**F**) for categories of inflammation scored in (E). *$p < 0.05$, ****$p < 0.0001$. AI, alveolar inflammation; IE, intraepithelial; PB, peribronchial; PV, perivascular.

superior protective function (35), was similar (Fig. 4F). Taken together, these results suggest that the absence of IL-6 expression does not significantly impact the generation or maintenance of functional memory CD4 T cells following IAV priming.

Memory CD4 T cell–mediated protection is compromised in the absence of IL-6

We next directly tested whether IL-6 expression impacts the protective potential of memory T cells responding against IAV. We employed an adoptive transfer model using well-characterized memory $CD8^+$ or $CD4^+$ T cell populations generated in vitro from naive TCR Tg precursors that we have shown protect against IAV and are equivalent to polyclonal in situ–generated IAV-specific memory cells in their response (22, 27, 36, 37). This experimental design allows us to focus on the protective capacity of the donor memory cells and eliminates contributions from host IAV-primed memory T and B cells, Ab, and IAV-primed lung environment that can all independently contribute to protection in IAV-primed animals (20).

We transferred 5 × 10^6 Tc1-polarized memory CD8 T cells, generated from HA (clone 4) TCR Tg cells, or Th1-polarized memory CD4 T cells, generated from HNT TCR Tg cells, to unprimed WT or *Il6*$^{-/-}$ hosts. This number of HA $CD8^+$ or HNT $CD4^+$ memory cells provides robust protection to unprimed mice against 10,000 EID_{50} (2 LD_{50}) A/PR8 challenge (27, 36). Memory CD8 T cells fully protected WT and *Il6*$^{-/-}$ hosts, with only slightly more weight loss observed in *Il6*$^{-/-}$ mice (Fig. 5A, 5B). In contrast, whereas memory CD4 T cells fully protected WT recipients, only about half of the *Il6*$^{-/-}$ recipients survived (Fig. 5C), with more weight loss and delayed time to recovery versus that observed in WT recipients (Fig. 5D). Transfer of 5 × 10^6 memory CD4 T cells did protect *Il6*$^{-/-}$ mice against a 4-fold lower 2500 EID_{50} A/PR8 challenge (still lethal to unprimed *Il6*$^{-/-}$ mice; see the open circles in Fig. 5E and as shown in Fig. 1). However, delayed recovery in *Il6*$^{-/-}$ versus WT hosts was still evident (Fig. 5E, 5F). These results confirm that memory $CD4^+$, but not $CD8^+$, T cell–mediated protection against IAV is dramatically reduced in the absence IL-6.

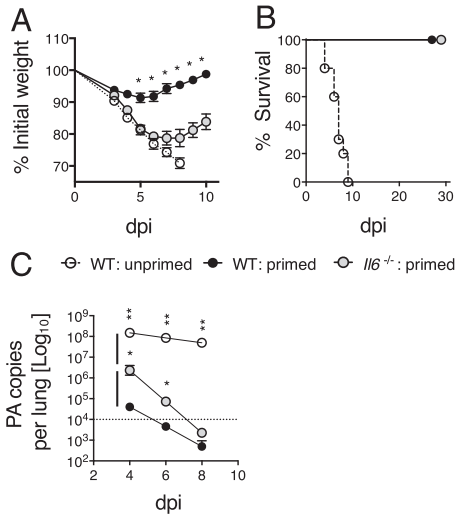

FIGURE 2. IL-6 is required for optimal heterosubtypic protection. WT or *Il6*$^{-/-}$ mice on a BALB/c background were primed with a low dose (500 EID_{50} = 0.1 LD_{50} for WT mice) of A/PR8 (H1N1). After 45 d mice were challenged with a 150 LD_{50} dose of A/Philippines (H3N2) virus and (**A**) morbidity and (**B**) survival were monitored (n = 5 mice per group; one of two experiments). Viral titers (**C**) from lungs of A/PR8-primed WT or *Il6*$^{-/-}$ mice (n = 4 mice per group) or unprimed WT control mice (n = 3) were assessed on the stated day after infection after 150 A/Phil challenge. Data are representative of one of two experiments. The dashed line in (C) represents sensitivity cutoff of the assay. *$p < 0.05$, **$p < 0.005$.

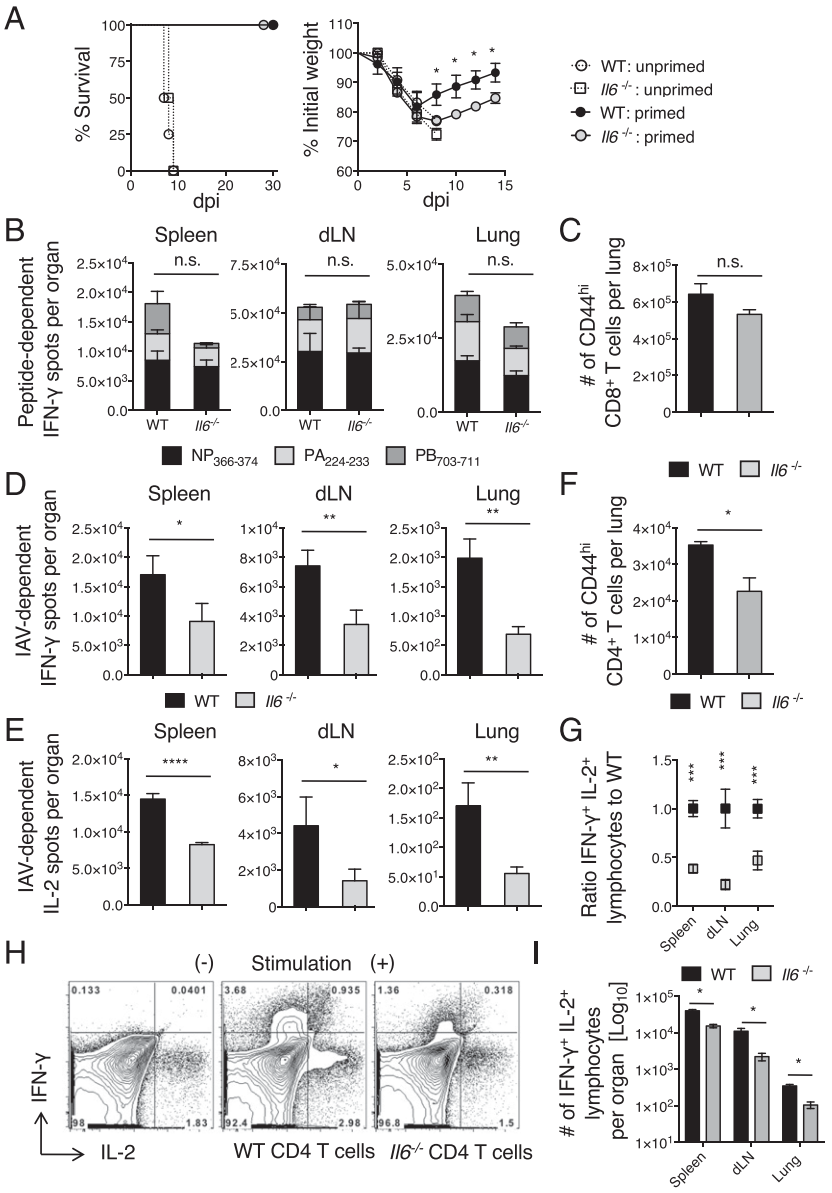

FIGURE 3. CD4 but not CD8 T cell cytokine responses are reduced during heterosubtypic challenge of mice primed with attenuated IAV. WT or *Il6*$^{-/-}$ C57BL/6 mice were primed with two doses of 2500 $TCID_{50}$ A/Alaska 10 d apart. At day 45 after priming, mice were challenged with 20 LD_{50} A/PR8. Survival and weight loss (**A**) of primed or unprimed control WT and *Il6*$^{-/-}$ mice ($n = 4$ mice per group; one of two experiments) are shown. IFN-γ responses elicited by immunodominant MHC class I–restricted peptides were enumerated by ELISPOT from cells isolated from stated organs 5 d after A/PR8 challenge primed WT or *Il6*$^{-/-}$ mice (**B**) and numbers of $CD44^{hi}$ CD8 T cells in the lung were determined by FACS (**C**) ($n = 3$ mice per group; one of two experiments). Similarly, IFN-γ (**D**) and IL-2 (**E**) responses were enumerated in stated organs by ELISPOT using IAV lysate to stimulate CD4 T cells ($n = 3$ mice per group; one of two experiments), and the number of $CD44^{hi}$ CD4 T cells in the lung was determined by FACS (**F**). Dual IFN-γ and IL-2 production by CD4 T cells in the stated organs in response to IAV lysate was determined by ICCS, and the frequency expressed as a ratio to the WT response (**G**), representative staining of spleen cells (**H**), and the enumeration of dual cytokine-producing cells (**I**) are shown. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$, $****p < 0.0001$.

Early host-derived IL-6 is required for optimal memory CD4 T cell–mediated protection

To confirm the importance of IL-6 removed acutely rather than IL-6 absent throughout host development, we treated WT recipients of memory CD4 T cells with IL-6–neutralizing or isotype control Ab from 0 to 8 dpi. The IL-6–neutralizing Ab treatment reduced the protective efficacy of transferred memory CD4 T cells (Fig. 5G, black versus gray-filled circles) to an extent similar to that observed in *Il6*$^{-/-}$ hosts. This result also indicates that host-derived IL-6 plays a key role in maximizing memory CD4 T cell–mediated protection, as similar impaired protection was seen following transfer of WT memory $CD4^+$ T cells to *Il6*$^{-/-}$ mice, where donor cell–derived IL-6 could potentially impact the outcome, and in WT hosts treated with IL-6–neutralizing Ab, where all IL-6 (donor and host-derived) is subject to blocking.

To determine when IL-6 signals are needed, we treated recipients with IL-6–neutralizing Ab from 0 to 8 dpi, or only at early (0 and 2 dpi) or later time points (6 and 8 dpi). Early blockade of IL-6 impaired survival (Fig. 5G) and increased morbidity (Fig. 5H) as dramatically as did treatment throughout (0–8 dpi). In contrast, late administration of IL-6–neutralizing Ab had no impact (Fig. 5G, 5H). This finding indicates that IL-6 is needed only during the first few days after challenge to support optimal protective memory CD4 T cell responses.

Impaired B cell and neutrophil responses do not underlie reduced CD4 T cell protection

The reduced protective efficacy of memory CD4 T cells in the absence of IL-6 could be due to impaired responses of memory CD4 T cells themselves and/or to IL-6–dependent effects on other cell types known to contribute to IAV clearance. Diminished B cell Ab production has been implicated in the enhanced susceptibility of *Il6*$^{-/-}$ mice to 1° IAV challenge (11). To test whether IL-6 is needed for memory CD4 T cell protection through a B cell–dependent mechanism, we used J_HD mice that lack B cells as hosts. J_HD recipients of memory CD4 T cells were treated with IL-6–neutralizing or control Ab following a lethal A/PR8 challenge for this strain (2500 EID_{50}) (36). As expected from our earlier studies, memory CD4 T cells were able to transfer protection to the B cell–deficient J_HD hosts (36), but this protection was abrogated by IL-6–neutralizing Ab treatment (Supplemental Fig. 1A, 1B), strongly suggesting that the requirement for IL-6 in memory CD4 T cell–mediated protection is independent of any action, direct or indirect, on B cells.

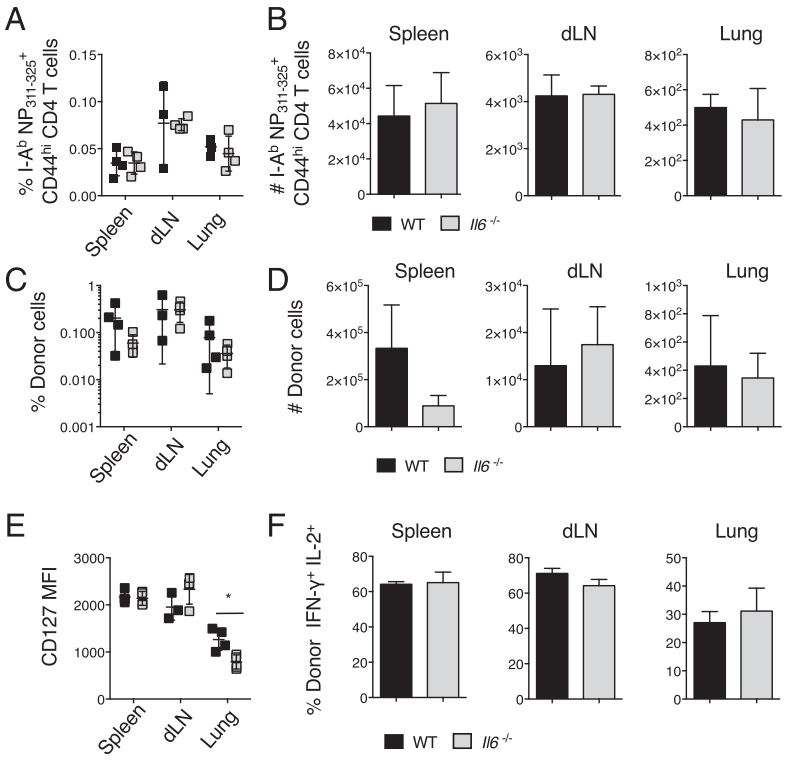

FIGURE 4. Memory CD4 T cell formation following IAV priming is not impaired in the absence of IL-6. WT or *Il6*$^{-/-}$ C57BL/6 mice were primed with A/Alaska as described in Fig. 3. The (**A**) percentage and (**B**) absolute number of $NP_{311–325}$ tetramer^{+}CD44^{high} CD4 T cells was enumerated at 45 dpi (n = 4 mice per group; one of two similar experiments). In separate experiments, WT or *Il6*$^{-/-}$ mice receiving 1×10^6 Thy-disparate naive OT-II donor cells were primed with 0.1 LD_{50} A/PR8-OVA_{II}. At 45 dpi, the (**C**) percentage and (**D**) absolute number of donor cells detected in stated organs were determined (n = 4 mice per group; one of two experiments). The mean fluorescence intensity (MFI) of CD127 on donor cells was determined by FACS (**E**). The percentage of donor cells coproducing IFN-γ and IL-2 was determined by intracellular cytokine staining following ex vivo stimulation (**F**). *$p < 0.05$.

Reduced neutrophil responses have also been suggested to underlie the enhanced susceptibility of *Il6*$^{-/-}$ mice to 1° IAV infection (12). To test the importance of neutrophils in contributing to memory CD4 T cell–mediated protection, we thoroughly depleted neutrophils in recipients of memory CD4 T cells prior to lethal A/PR8 challenge. Protection was robust in both WT and J_HD hosts regardless of whether neutrophils were present (Supplemental Fig. 1C–F), indicating that any defects in neutrophil responses are not responsible for the reduced protective efficacy of memory CD4 T cells responding against IAV in the absence of IL-6.

Early memory CD4 T cell responses are not impacted by IL-6

Because IL-6 is required only within the first few days after infection for optimal memory CD4 T cell–mediated protection, we asked whether memory CD4 T cell activation is compromised in *Il6*$^{-/-}$ hosts following IAV challenge, as has been suggested previously (38). Donor memory CD4 T cells were CFSE labeled and transferred to WT or *Il6*$^{-/-}$ mice followed by challenge with 10,000 EID_{50} A/PR8. No differences in CD69 expression (Fig. 6A), division profile based on analysis of CFSE dilution (Fig. 6B), or donor cell number were detected between WT and *Il6*$^{-/-}$ hosts at 4 dpi in the spleen or dLN (Fig. 6C).

Memory CD4 T cells recognizing IAV Ag on MHC class II^{+} APCs in the lung drive potent innate immune activation, resulting in reduced viral titers by 4 dpi (22, 39). We tested a role for IL-6 in orchestrating this early memory CD4 T cell–mediated viral control by assessing A/PR8 titers in WT or *Il6*$^{-/-}$ recipients of memory CD4 T cells at 4 dpi. Both WT and *Il6*$^{-/-}$ recipients displayed comparable and significant viral control at 4 dpi compared with control mice not receiving donor cells (Fig. 6D). These results indicate that neither the initial activation of, nor initial viral control mediated by, memory CD4 T cells is compromised in the absence of IL-6.

2° CD4 T cell effector number and function are reduced in the absence of IL-6

We next asked whether IL-6 impacts the magnitude and/or function of 2° CD4 T cell effectors present at 7 dpi, as this late phase of the recall response is ultimately critical for memory CD4 T cell–mediated protection (37). We transferred donor memory cells to WT mice, challenged with A/PR8, and treated recipients with IL-6–neutralizing or control Ab from 0 to 6 dpi. We used 2500 EID_{50} rather than 10,000 EID_{50}, as the onset of death of some mice challenged with the higher dose when treated with IL-6–neutralizing Ab could complicate analysis. At 7 dpi, the peak of 2° effector response in this model (37), the number of donor cells detected in the spleen and dLN of mice treated with IL-6–neutralizing or isotype Ab was similar (Fig. 7A). However, the number of donor cells in the lung was significantly reduced by IL-6–neutralizing Ab treatment (Fig. 7A), indicating that IL-6 is critical for maximal 2° effector accumulation at the site of infection where many of the key effector functions of CD4 T cells are thought to be mediated (40).

We analyzed 2° effector function to look for additional effects of IL-6. Strikingly, neutralization of IL-6 reduced the frequency of cells producing IFN-γ together with IL-2 in all organs tested (Fig. 7B), and the mean fluorescence intensity of IFN-γ^{+} cells was also reduced (Fig. 7C). Only very low numbers of IL-10^{+} or IL-17^{+} donor cells were detected, consistent with previous studies (37), and these were not impacted by Ab treatment (not shown). Most importantly, impaired viral clearance by adoptively transferred memory CD4 T cells at 7 dpi was observed in hosts treated with IL-6–neutralizing Ab (Fig. 7D).

To confirm that the absence of IL-6 signals only during heterosubtypic challenge also reduces dual IFN-γ^{+}/IL-2^{+} 2° effector responses from polyclonal CD4 T cells, we challenged WT A/Alaska/6/77-primed mice with PR8 as in Fig. 3, treated mice with IL-6–neutralizing or isotype control Abs on days 0, 2, and 4 after PR8 challenge, and assessed the cytokine response of $NP_{311–325}$ tetramer^{+} CD4 T cells (Fig. 7E). In comparison with isotype-treated mice, reduced frequencies and total numbers of dual IFN-γ^{+}/IL-2^{+} $NP_{311–325}^{+}$ cells were detected at 5 dpi in mice treated with IL-6–neutralizing Abs (Fig. 7F–H). These findings are consistent with the impaired cytokine production of *Il6*$^{-/-}$ versus WT mice detected in response to IAV lysate stimulation of polyclonal CD4 T cells

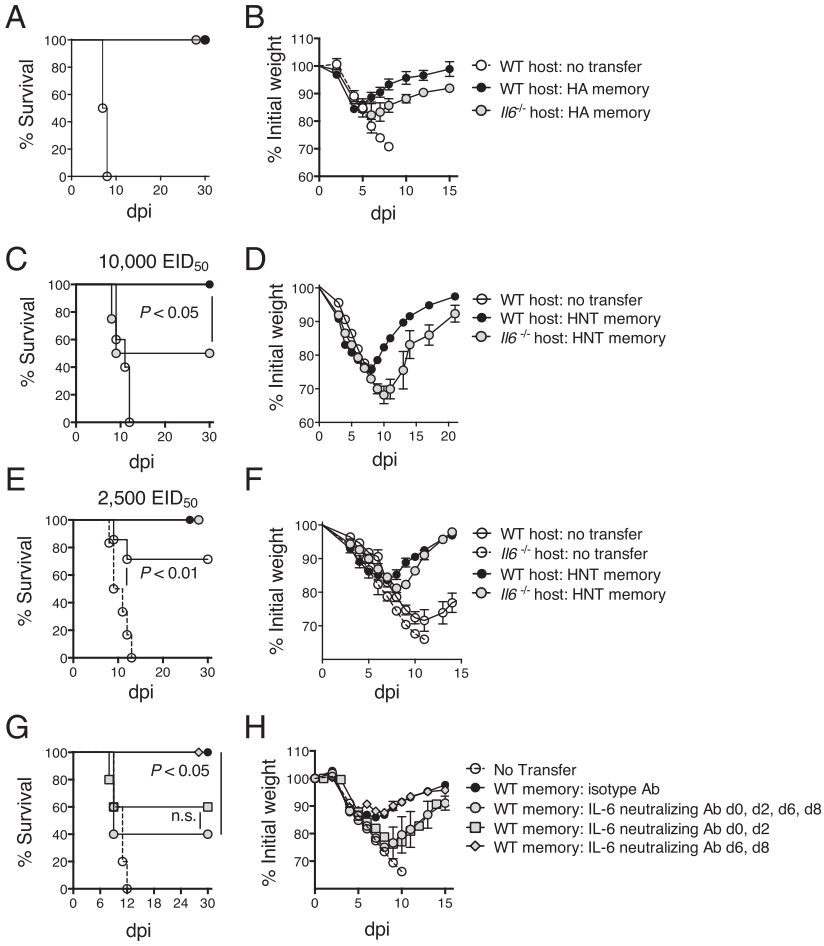

FIGURE 5. Protection mediated by memory $CD4^+$, but not $CD8^+$, T cells is compromised in the absence of IL-6. Memory CD8 T cells (5×10^6) generated from HA TCR Tg cells were transferred to naive WT or $Il6^{-/-}$ BALB/c mice. Mice were subsequently infected with 10,000 EID_{50} A/PR8 and their (**A**) survival and (**B**) weight loss were monitored ($n = 10$ mice per group) and compared with mice not receiving donor memory cells ($n = 3$ mice). WT or $Il6^{-/-}$ BALB/c mice that received 5×10^6 memory CD4 T cells generated from HNT TCR Tg cells were challenged with 10,000 EID_{50} A/PR8 and (**C**) survival and (**D**) weight loss were monitored. Analysis was also done on mice challenged with 2500 EID_{50} A/PR8 (**E** and **F**) ($n = 10$ mice per group). In separate experiments, WT mice receiving 5×10^6 memory HNT CD4 T cells were treated with 0.5 mg of IL-6–neutralizing Ab on the stated days after 10,000 EID_{50} A/PR8 challenge. Control mice were treated with isotype control Ab at 0, 2, 4, 6, and 8 dpi. Survival (**G**) and weight loss (**H**) are depicted for five mice per group (one of three experiments).

responding to heterosubtypic challenge summarized in Fig. 3. In total, these results strongly support the hypothesis that early IL-6 signals to memory CD4 T cells play a key role in maximizing the antiviral capacity of late-acting 2° CD4 T cell effectors.

In contrast to the dramatic impact on 2° CD4 T cell effectors, IL-6 blockade did not impact the magnitude of, or IFN-γ or granzyme B expression by, 2° CD8 T cell effectors derived from memory TCR Tg HA cells (Supplemental Fig. 2). This is in agreement with similar CD8 T cell responses in WT and $Il6^{-/-}$ mice during heterosubtypic challenge (Fig. 3). IL-6 blockade also did not alter 1° CD4 T cell effector responses generated from naive HNT TCR Tg donor cells, consistent with previous studies (12) (data not shown). Taken together, these results thus support a unique role for IL-6 in selectively programming the maximal efficacy of memory CD4 T cell responses against IAV.

Direct IL-6 signals to memory CD4 T cells maximize 2° effector responses

IL-6 could signal memory CD4 T cells directly or it could exert indirect IL-6–dependent positive effects on the 2° CD4 T cell effector response by signaling other adaptive or innate immune populations, including APCs or $Foxp3^+$ regulatory T cells (Tregs) (41). To determine whether direct IL-6 signals to IAV-specific memory CD4 T cells are necessary to enhance 2° effector responses, we generated memory CD4 T cells from OT-II cells lacking expression of the IL-6R α-chain ($Il6ra^{-/-}$) and from OT-II cells obtained from $Il6ra^{+/+}$ littermates. The spectrum and level of cytokines detected upon restimulation of the in vitro–generated $Il6ra^{-/-}$ and $Il6ra^{+/+}$ memory CD4 T cells was similar (Fig. 8A), as was the yield of cells recovered (not shown). This indicates that direct IL-6 signals to unprimed CD4 T cells are not required for the generation of functional Th1-like memory populations in vitro, which agrees with the in vivo studies summarized in Fig. 4. However, when transferred to WT C57BL/6 hosts and then challenged with A/PR8-OVA_{II}, the 2° CD4 T cell effector response in the lung generated from $Il6ra^{-/-}$ donor cells was severely impaired in terms of absolute number and cytokine production compared with 2° effectors generated from $Il6ra^{+/+}$ donor cells (Fig. 8B–D).

To confirm that IL-6 has a direct role on memory CD4 T cells responding to IAV, we cotransferred WT (Thy1.1/Thy1.2) and $Il6ra^{-/-}$ (Thy1.2) OT-II memory cells to WT C57BL/6.Thy1.1 hosts. The memory cells from $Il6ra^{-/-}$ donors displayed impaired 2° effector accumulation (Fig. 8E, 8F) and cytokine production (data not shown). As both donor populations are responding in the presence of the same endogenous $Foxp3^+$ Treg population, and the $Il6ra^{-/-}$ and $Il6ra^{+/+}$ 2° effectors contain minimal but comparable frequencies of $CD25^+Foxp3^+$ cells (Fig. 8G, 8H), these results further reveal the importance of IL-6 signals directly received by memory CD4 T cells that program maximal protective 2° CD4 T cell effector responses during heterosubtypic IAV challenge.

Discussion

Optimal clearance of heterosubtypic IAV infection requires a complex interplay of multiple elements of the innate and adaptive arms of the immune system that is incompletely understood. Memory CD4 T cells provide strong protection against experimental IAV infection (36, 42–45), and increased numbers of multifunctional IAV-specific memory CD4 T cells correlate with

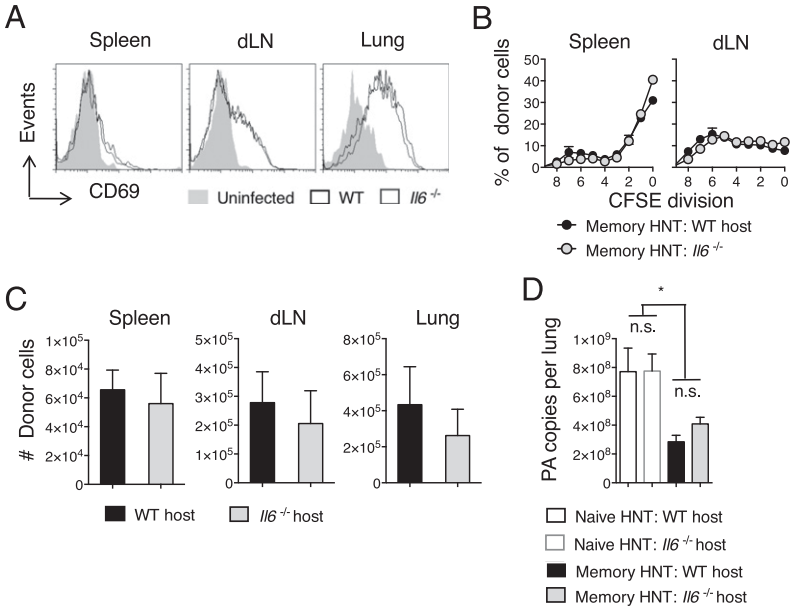

FIGURE 6. Initial activation of memory CD4 T cells and early viral control are not impacted by IL-6. Unprimed Thy-disparate WT or $Il6^{-/-}$ mice received 1×10^6 CFSE-labeled HNT memory CD4 cells and were challenge with 10,000 EID_{50} A/PR8. Representative (**A**) donor CD69 expression, (**B**) proliferation profile determined by loss of CFSE label, and (**C**) cell number at 4 dpi were assessed in stated organs ($n = 3$ mice per group). Results from one of three similar experiments are shown. In separate experiments, (**D**) pulmonary viral titers were determined at 4 dpi from lungs of WT BALB/c or $Il6^{-/-}$ mice receiving 5×10^6 memory HNT cells or not and challenged with 10,000 EID_{50} A/PR8 ($n = 4$ per group). Results are representative of two separate experiments. $^*p < 0.05$.

improved outcomes in human studies (46, 47). We have reported that late-acting 2° effectors, which peak at ~7 dpi, are critical for complete memory CD4 T cell–mediated protection against IAV (21). Uncovering signals that maximize this 2° effector response could lead to strategies to improve vaccine-induced T cell protection against IAV and other pathogens against which neutralizing Ab alone cannot confer long-term immunity. We recently found that autocrine IL-2 maximizes the magnitude of 1° but not 2° CD4 T cell effector populations in the lung (34), suggesting that unique cytokine signals other than IL-2 might act to specifically regulate 2° effector accumulation at the site of infection. In this study, we show that direct IL-6 signals to memory CD4 T cells delivered early after IAV challenge support maximal 2° effector responses in terms of magnitude, function, and protective capacity.

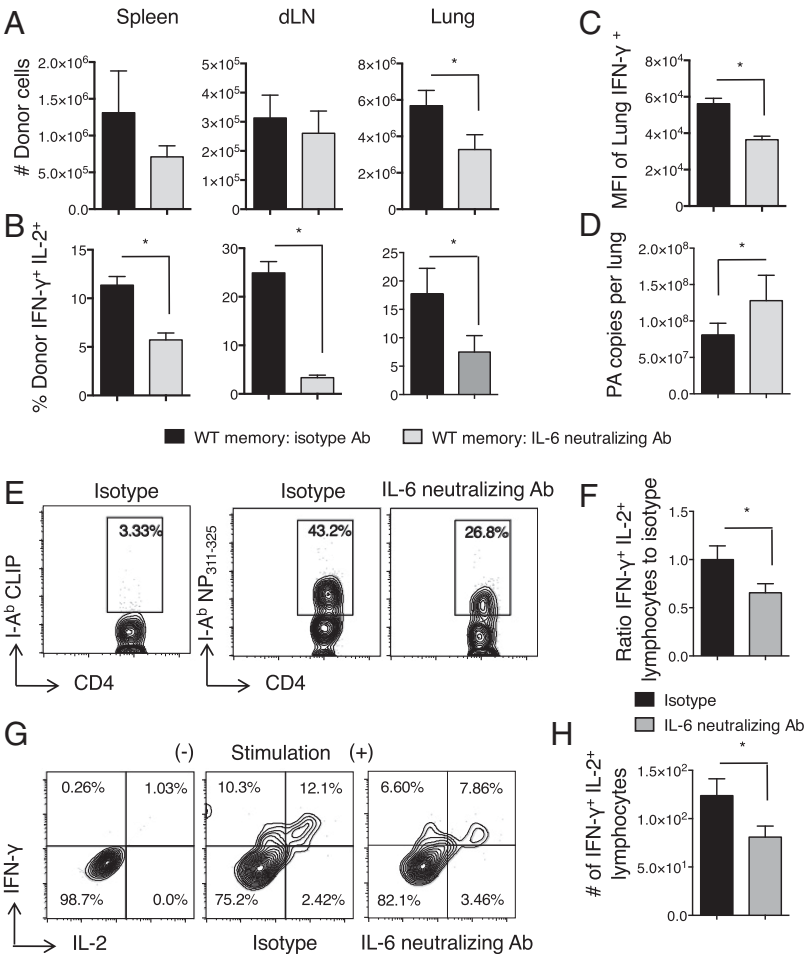

FIGURE 7. 2° CD4 T cell effector number and function are reduced in the absence of IL-6. HNT memory cells (1×10^6) were transferred to naive WT BALB/c mice subsequently infected with 2500 EID_{50} A/PR8 and treated with 0.5 mg of isotype or IL-6–neutralizing Ab at 0, 2, 4, and 6 dpi. Donor cells were enumerated at 7 dpi (**A**). Dual IFN-γ^+/IL-2^+ cytokine-producing cells (**B**) and mean fluorescence intensity (MFI) of IFN-γ signal (**C**) were assessed at 7 dpi by ICCS ($n = 4$ mice per group; representative of three similar experiments). Viral titers (**D**) were determined at 7 dpi in mice receiving donor HNT memory cells followed by 2500 EID_{50} A/PR8 challenge ($n = 4$ mice per group; one of two experiments). WT C57BL/6 mice were primed with A/Alaska, challenged with A/PR8 as described in Fig. 3, and treated with isotype or IL-6–neutralizing Ab as described above. At 5 dpi, $NP_{311–325}$ tetramer$^+$ CD4 T cells detected in the lung (**E**) were assessed for dual IFN-γ^+/IL-2^+ cytokine-producing cells. The frequency (**F** and **G**) and number (**H**) are shown ($n = 4$ mice per group; one of two similar experiments). $^*p < 0.05$.

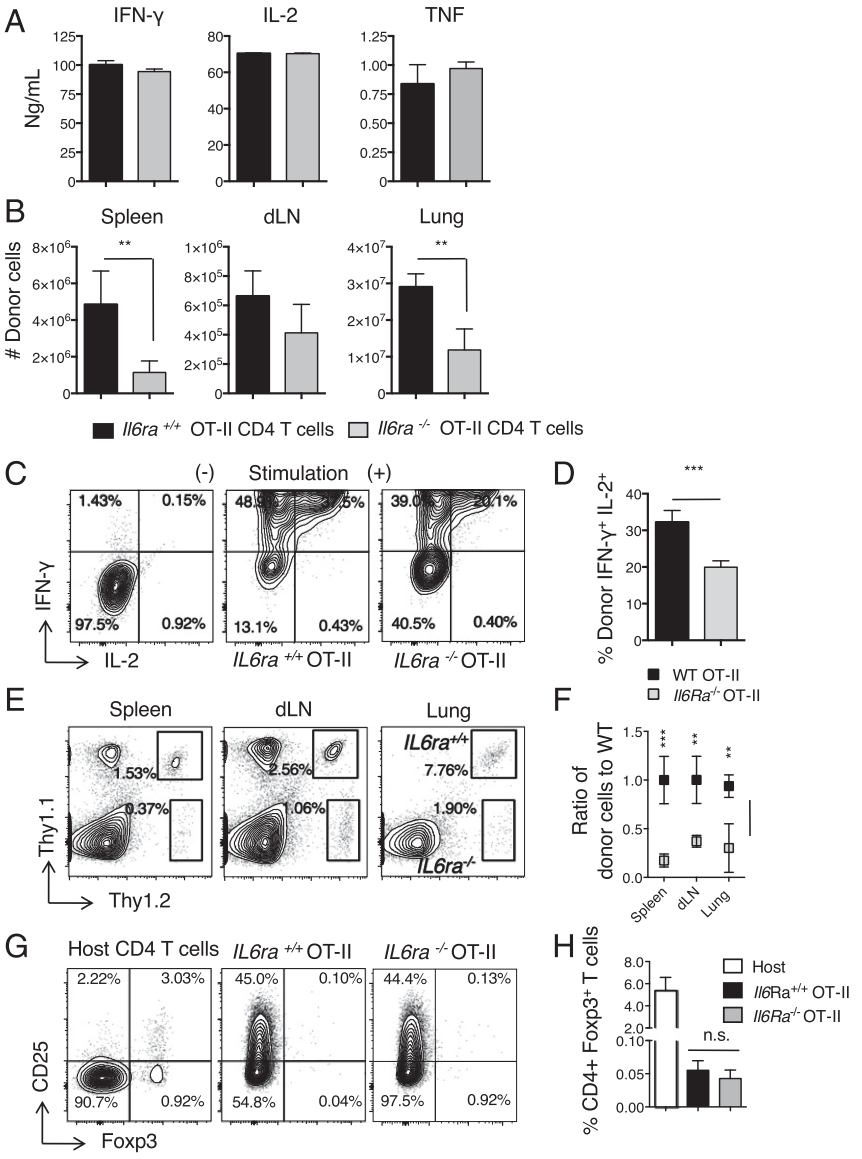

FIGURE 8. Direct IL-6 signals to memory CD4 T cells maximize 2° effector responses. Memory populations were generated in vitro from *IL6ra*$^{+/+}$ and *Il6ra*$^{-/-}$ OT-II cells. Cytokine production (**A**) from an equal number of *IL6ra*$^{+/+}$ and *Il6ra*$^{-/-}$ memory cells was determined by Luminex analysis of supernatants from triplicate wells 8 h after stimulation. Results are representative of three separate experiments. *IL6ra*$^{+/+}$ or *Il6ra*$^{-/-}$ OT-II memory cells (1×10^6) were transferred to different Thy-disparate hosts followed by infection with 0.1 LD_{50} A/PR8-OVA_{II}. At 7 dpi, the total number of donor cells (**B**) and IFN-γ$^+$ and IL-2$^+$ cytokine production in the lung were determined from four mice per group (one of two separate experiments). Representative ICCS staining of lung cells in the absence and presence of stimulation is shown in (**C**), and the percentage of dual IFN-γ$^+$IL-2$^+$ donor cells is shown in (**D**). In separate experiments, WT. Thy1.2/Thy1.1 or *Il6ra*$^{-/-}$.Thy1.2 OT-II memory cells (1×10^6) were cotransferred to the same WT C57BL/6.Thy1.1 host followed by infection with 0.1 LD_{50} A/PR8-OVA_{II}. At 7 dpi, the total frequency of donor cells (**E**), the ratio of *Il6ra*$^{-/-}$ to WT OT-II memory cells (**F**), and the frequency of CD25$^+$Foxp3$^+$CD4$^+$ T cells lymphocytes from the host or donor populations (**G** and **H**) were determined from four mice per group (one of two separate experiments). $^{**}p < 0.005$, $^{***}p < 0.001$.

Through analysis of endogenous T cells in IAV-primed animals and adoptive transfer models, we find that IL-6 impacts memory CD4 T cell recall more than 2° CD8 T cell recall. This suggests that the amount of IL-6 produced early on during heterosubtypic challenge may be key in determining the relative contribution of IAV-specific memory CD4$^+$ versus CD8$^+$ T cells to protection. Such a mechanism could, at least in part, underlie observations of multiple seemingly distinct forms of heterosubtypic immunity observed in different experimental systems that rely more or less on either memory CD4$^+$ or CD8$^+$ T responses (20). The level of IL-6 produced following heterosubtypic challenge may be impacted by several variables, including the dose and strain of viruses used, the duration between priming and recall challenge, and the age, strain, or sex of the animals analyzed. We speculate that other innate cytokines produced upon IAV infection may also have a selective regulatory role on the 2° CD8 T cell effector response during heterosubtypic challenge. In support of this hypothesis, memory CD8 T cells deficient for expression of type I IFN receptor have been shown to be less efficient at promoting IAV clearance than are WT memory CD8 T cells of the same specificity (48). The ability of early innate inflammatory signals to selectively impact the protective capacity of recall CD4$^+$ versus CD8$^+$ T cell responses suggests that there is an important initial therapeutic window during which delivery of targeted mediators may significantly improve vaccine-induced, or adoptively transferred, memory T cell responses.

The increased magnitude of 2° effector responses following early IL-6 signaling to memory CD4 T cells is consistent with the known pro–survival activity of the cytokine (49–51). Previously, we found that IL-6 produced during cognate interactions between CD4 T cells and dendritic cells in vitro enhanced expression of the antiapoptotic molecule Bcl2 in the responding T cells (52). We did not observe changes in Bcl2 expression by 2° effectors responding against IAV in the presence or absence of IL-6 (data not shown), but this does not rule out that regulation by IL-6 of other pro- or antiapoptotic molecules plays a role in maximizing the recall CD4 T cell response. Alternatively, or in addition to an antiapoptotic effect, IL-6 may optimize 2° effector infiltration and/or retention in the lung either by impacting chemokine gradients or chemokine receptor expression on the 2° effector cells (53). Recent work also suggests that IL-6 can impact mitochondrial respiration in effector T cells, thereby enhancing cytokine production (54). Finally, IL-6 may regulate elements of the autophagy pathway (55), which has recently been shown to play an important role in effector CD4 T cell survival in vivo (56). Detailed further studies are needed to discriminate the importance of each of these potential mechanisms

in contributing to the role of IL-6 in maximizing protective 2° CD4 T cell effector responses against IAV.

Although not required for memory CD4 T cell generation, we show in the present study that IL-6 signals are needed for optimal recall of memory CD4 T cells. This is in agreement with recent observations of Nish et al. (57); however, in contrast to their findings, we did not observe impaired 1° effector function in the absence of IL-6 in our studies. This may be due to differences in the experimental system employed, as IAV, a rapidly replicating pathogen, likely generates a more robust and sustained inflammatory response than do the immunizations and models of autoimmunity used by Nish et al. (57). One or more IAV-induced inflammatory factors may compensate in the absence of IL-6 to allow for adequate 1° effector responses in the studies summarized here. Interestingly, impaired recall CD4 T cell responses against IAV in *Il6*$^{-/-}$ mice have also been attributed to IL-6 being required to reduce Treg–mediated suppression (38). Our results clearly implicate direct IL-6 signals to memory CD4 T cells as critical for maximizing 2° effector responses, but they do not formally rule out an impact of IL-6 on Treg-mediated suppression in addition to its direct impact on memory CD4 T cells. However, we note that recall CD8 T cell responses were not impaired in the absence of IL-6 in our studies, whereas a recent report found dramatic regulation of the magnitude of 2° CD8 T cell responses by Tregs in IAV-primed mice (58).

The importance of adjuvants in stimulating innate inflammatory responses to improve vaccine-induced generation of memory T and B cell subsets is well known. The role innate signals play in recall responses of primed lymphocytes is much less clear. The therapeutic manipulation of IL-6, and likely other inflammatory mediators, may offer a means to maximize or tailor vaccine-induced T cell responses against pathogens and cancers. Our findings also highlight broad differences in how IL-6 affects the outcome of 1° versus 2° IAV challenge. *Il6*$^{-/-}$ mice primed with cold-adapted IAV survive doses of heterosubtypic virus at least an order or magnitude greater than is lethal to unprimed mice, albeit with more morbidity and delayed viral clearance compared with IAV-primed WT mice. It is most likely that heterosubtypic immunity remains strong in primed *Il6*$^{-/-}$ mice due to IAV-specific memory CD8 T cell responses that are largely unaffected by the absence of IL-6 and are able to potently clear IAV through multiple distinct mechanisms (27). These studies further support the overall concept that vaccines able to generate strong IAV-specific memory CD4$^+$ (46), as well as CD8$^+$ (59), T cells and neutralizing Ab should together provide the strongest protection against IAV infection, including in situations where innate immune responses may be compromised (60).

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Disclosures

The authors have no financial conflicts of interest.

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