JVI Journals.ASM.org

Crucial Roles of Interleukin-7 in the Development of T Follicular Helper Cells and in the Induction of Humoral Immunity

Yong Bok Seo,[a] Se Jin Im,[a] Hong Namkoong,[a] Sae Won Kim,[a] Young Woo Choi,[b] Moon Cheol Kang,[b] Hye Seong Lim,[c] Hyun Tak Jin,[c] Se Hwan Yang,[c] Mi La Cho,[e] You-Me Kim,[a,b] Seung-Woo Lee,[a,b] Young Ki Choi,[d] Charles D. Surh,[a,b] Young Chul Sung[a,b,c]

Department of Life Sciences[a] and Intergrative Biosciences and Biotechnology,[b] Pohang University of Science and Technology (POSTECH), Pohang, Gyungbuk, Republic of Korea; Research Institute, Genexine Co. Ltd., Seongnam, Republic of Korea[c]; College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju, Republic of Korea[d]; Rheumatism Research Center, Catholic Institutes of Medical Science, Seoul, Republic of Korea[e]

ABSTRACT

T follicular helper (Tfh) cells are specialized providers of cognate B cell help, which is important in promoting the induction of high-affinity antibody production in germinal centers (GCs). Interleukin-6 (IL-6) and IL-21 have been known to play important roles in Tfh cell differentiation. Here, we demonstrate that IL-7 plays a pivotal role in Tfh generation and GC formation *in vivo*, as treatment with anti-IL-7 neutralizing antibody markedly impaired the development of Tfh cells and IgG responses. Moreover, codelivery of mouse Fc-fused IL-7 (IL-7-mFc) with a vaccine enhanced the generation of GC B cells as well as Tfh cells but not other lineages of T helper cells, including Th1, Th2, and Th17 cells. Interestingly, a 6-fold-lower dose of an influenza virus vaccine codelivered with Fc-fused IL-7 induced higher antigen-specific and cross-reactive IgG titers than the vaccine alone in both mice and monkeys and led to markedly enhanced protection against heterologous influenza virus challenge in mice. Enhanced generation of Tfh cells by IL-7-mFc treatment was not significantly affected by the neutralization of IL-6 and IL-21, indicating an independent role of IL-7 on Tfh differentiation. Thus, IL-7 holds promise as a critical cytokine for selectively inducing Tfh cell generation and enhancing protective IgG responses.

IMPORTANCE

Here, we demonstrate for the first time that codelivery of Fc-fused IL-7 significantly increased influenza virus vaccine-induced antibody responses, accompanied by robust expansion of Tfh cells and GC B cells as well as enhanced GC formation. Furthermore, IL-7-mFc induced earlier and cross-reactive IgG responses, leading to striking protection against heterologous influenza virus challenge. These results suggest that Fc-fused IL-7 could be used for inducing strong and cross-protective humoral immunity against highly mutable viruses, such as HIV and hepatitis C virus, as well as influenza viruses.

Interleukin-7 (IL-7) is a heavily glycosylated cytokine with a molecular mass of 25 kDa, and it is a member of the common γ-chain receptor (CD132) cytokine family. IL-7 is secreted mainly by nonhematopoietic cells, such as thymic and intestinal epithelial cells, bone marrow stromal cells, keratinocytes, and reticular cells (1, 2). The receptor for IL-7, comprised of IL-7 receptor alpha (IL-7Rα; CD127) and the common γ chain, is expressed on various immune cells, including immature B cells, early thymocyte progenitors, and most mature T lymphocytes. IL-7 plays a role in the development of T cells, B cells, certain subsets of NK cells, and dendritic cells (DCs), as well as in the homeostasis of naive and memory T cells. In addition, IL-7 is important for γδ T cell proliferation and NKT cell maintenance. However, the role of IL-7 in the differentiation of T follicular helper (Tfh) cells and the induction of humoral immunity remains unclear.

Tfh cells are the unique $CD4^+$ T helper (Th) cells that provide cognate help to B cells to induce high-affinity antibody production in germinal centers (GCs) (3). GCs are specialized structures that develop within B cell follicles of secondary lymphoid tissues. GCs support intense B cell clonal expansion, somatic hypermutation, selection of high-affinity B cells, and class switching of immunoglobulin genes. These B cells ultimately are differentiated into both memory B cells and long-lived plasma cells that secrete high-affinity antibodies (2, 4).

The differentiation of Tfh cells depends on the expression of a master transcriptional repressor, Bcl-6, which inhibits non-Tfh lineage differentiation (Th1, Th2, and Th17) by repressing Blimp-1 (5). Tfh cells express CXCR5 and a number of costimulatory molecules, such as ICOS and PD-1 (6). In addition, IL-6 and IL-21 have been shown to be important soluble regulators for the differentiation of Tfh cells (7–9). Recently, IL-27 was shown to increase the Tfh cell differentiation by upregulating IL-21 production (10). However, there have been conflicting reports that the absence of either IL-6 or IL-21 exhibits significant, moderate, or minimal effects on Tfh cell differentiation. The absence of both cytokines resulted in a more significant decrease in Tfh cell generation than each cytokine alone *in vivo* (11), indicating that IL-6 and IL-21 have redundant and/or additive functions in Tfh differentiation. It remains to be determined whether exogenous IL-6 or IL-21 induces the expansion of Tfh cells, as previous findings mentioned above were based on the experiments using knockout mice and/or blockade antibodies. Moreover, the generation of Tfh cells is not completely impaired even in the absence of IL-6 and IL-21 (11). These results indicate that these

Received 28 February 2014 **Accepted** 23 May 2014
Published ahead of print 4 June 2014
Editor: D. S. Lyles
Address correspondence to Young Chul Sung, ycsung@postech.ac.kr.
Y.B.S. and S.J.I. contributed equally to this work.

doi:10.1128/JVI.00534-14

two cytokines by themselves are insufficient for full Tfh differentiation. Therefore, the full commitment of Tfh differentiation may require additional unknown factors and/or sustained activation of multisignaling pathways.

This study demonstrated that endogenous IL-7 is necessary for GC formation as well as the generation of Tfh cells in response to T cell-dependent antigens. Moreover, exogenous IL-7 significantly enhanced Tfh cell differentiation and GC formation after immunization with a vaccine and led to the increased induction of total and cross-reactive IgG responses, which were further confirmed by the protection against a lethal heterologous influenza virus challenge.

MATERIALS AND METHODS

Animals. Female BALB/c mice, C57BL/6 mice, and DO11.10 T cell receptor (TCR) transgenic mice were purchased from The Jackson Laboratory (USA). CD90.1$^+$ Rag1$^{-/-}$ OT-II mice were obtained by breeding CD90.1$^+$ OT-II mice to mice in the Rag1$^{-/-}$ background. All mice were housed under specific-pathogen-free conditions in an approved animal facility at POSTECH Biotech Center. Male cynomolgus monkeys were supplied from National Primate Research Center (NPRC; South Korea). Monkey experiments were performed in accordance with the procedures outlined in the guide for the care and use of laboratory animals and approved by the NPRC.

Production and purification of Fc-fused IL-7 proteins. The codon-optimized human IL-7 gene was fused to mouse Fc (IL-7-mFc) (12) or human Fc (IL-7-hFc) (13), and encoding plasmids were stably transfected into Chinese hamster ovary (CHO) cell lines. Cells were cultured in EX-CELL CHO DHFR$^-$ animal-component-free medium (SAFC, USA), and the supernatants were harvested and filtrated with a vacuum filter (Corning, USA). Affinity chromatography using a Hitrap Protein-A FF affinity column (Amersham-Pharmacia, USA) and MabSelect Sure (GE Healthcare, Sweden) was performed for the purification of IL-7-mFc and IL-7-hFc protein, respectively, according to the manufacturer's instructions. The expression of IL-7-mFc and IL-7-hFc was confirmed by Western blotting using anti-mouse IgG/human IgG and anti-IL-7 antibodies and silver staining analysis (>95% purity), and their concentrations were determined by human IL-7 enzyme-linked immunosorbent assay (ELISA) (BD Biosciences, USA).

Immunization, virus infection, and adoptive cell transfer. Mice and monkeys were injected intramuscularly with a trivalent inactivated-influenza vaccine (TIV) consisting of influenza virus strains H1N1 A/New Caledonia/20/99, H3N2 A/Fujian/411/2002, and B/Shanghai/361/2002 (GreenCross, South Korea) with or without recombinant IL-7 (Shenandoah Biotechnology, USA), IL-7-mFc, or IL-7-hFc. For OVA immunization, mice were immunized intraperitoneally (i.p.) with alum (Pierce Biotechnology, USA) combined with NP-OVA (Biosearch Technologies, USA) and with or without IL-7-mFc. Sera were collected at the indicated time points for immunological analyses. At 8 days postinjection, the immunized mice were lightly anesthetized by a 200-μl i.p. injection of ketamine (100 mg/kg of body weight; Yuhan, South Korea) and xylazine hydrochloride (10 mg/kg of bodyweight; Bayer, Belgium) in phosphate-buffered saline (PBS) and challenged with 50 μl of 2×10^3 PFU PR8/H1N1 influenza virus via nostrils using a micropipette.

For the adoptive cell transfer, single-cell suspensions of CD90.1$^+$ Rag1$^{-/-}$ OT-II cells were prepared and injected (1×10^5 to 5×10^5 cells per mouse) intravenously into the mice. Intraperitoneal immunization was performed at 1 day after the transfer.

Antibody ELISA. TIV or OVA-specific IgG titers were determined as previously described (14). 96-Well immunoplates (Nunc, Denmark) were coated with 50 μl of TIV (0.5 μg/ml) or OVA (10 μg/ml) in PBS. Sera were serially diluted in 5% nonfat milk in 0.05% Tween 20-containing PBS (PBST). ELISA endpoint titers were expressed as the highest dilution that yielded an optical density greater than the means plus three times the standard deviations of an identically diluted negative-control sample. TIV-specific antibody ELISA was performed as previously described (15). Sera diluted at 1:50 ratio in 5% nonfat milk in PBST were used.

For PR8/H1N1 (H1N1, A/Puerto Rico/8/34) virus-specific antibody ELISA, PR8/H1N1 viruses first were inactivated using formalin as previously described (16), and 50 μl of inactivated PR8/H1N1 virus (6×10^6 PFU/ml) was coated onto each well. Sera were serially diluted and endpoint titers were expressed as mentioned above.

HI assay. Hemagglutination inhibition (HI) assay was performed as described previously (17). Briefly, NC/H1N1 (H1N1, A/New Caledonia/20/99) was diluted to contain 4 hemagglutinating units in PBS. Diluted viruses were incubated with serial 2-fold dilutions of receptor-destroying enzyme-treated serum samples, starting with a 1:20 dilution at room temperature for 30 min. Antigen-antibody mixtures were tested for hemagglutinin (HA) activity by the addition of 0.5% chicken red blood cells to determine the HI titers. The results are presented as the geometric mean titers of positive sera (≥20).

Blockade or neutralizing antibody treatments. Anti-mouse CD4 antibody (GK1.5 clone) and anti-IL-7 antibody (M25 clone) were prepared from ascites, purified, and diluted in PBS. Anti-IL-6 antibody and anti-IL-21 antibody were purchased from Bioxcell (USA) and eBioscience (USA), respectively. Mice were i.p. injected with anti-CD4 antibodies (500 μg/mouse) 1 day prior to vaccination. For neutralization of cytokines, anti-IL-7 (1 mg/mouse) antibodies were i.p. injected three times a week. Anti-IL-6 antibodies were administered as previously described (11), and anti-IL-21 (100 μg/mouse) antibodies were i.p. injected twice a week. The effectiveness of CD4 depletion was confirmed using anti-mouse CD4 antibody (RM4-4 clone; eBioscience, USA) and anti-mouse CD3 antibody (eBioscience, USA) by flow cytometry.

Flow cytometry. Single-cell suspensions were obtained by gentle mechanical disruption of lymph nodes or spleens. Cells were stained in fluorescence-activated cell sorting (FACS) buffer (1% fetal bovine serum [FBS] in PBS) with the appropriate combination of the following monoclonal antibodies: gamma interferon (IFN-γ) (clone XMG1.2), IL-4 (clone 11B11), IL-17 (clone ebio17B7), CD90.1 (clone HIS51), PD-1 (clone RMP1-30), B220 (clone RA3-6B2), CD4 (clone RM4-5), CD3e (clone 500A2), biotin-conjugated Fas (clone Jo2), GL-7 (clone GL7), Bcl-6 (clone K112-91), purified CXCR5 (clone 2G8) in conjunction with streptavidin-allophycocyanin (APC), and biotin-conjugated anti-rat IgG. Tfh cell staining was performed as described previously (18). All samples were analyzed using a Gallios flow cytometer (Beckman Coulter, USA) and Flow Jo software (TreeStar, USA).

Intracellular cytokine staining. At 1 week postimmunization (wpi), lymph node cells or splenocytes were restimulated either for 24 h with 10 μg/ml TIV HA protein in the TIV model or for 6 h with 1 μM $OVA_{323-339}$ in the OT-II model, respectively, in the presence of GolgiStop (BD Bioscience, USA). Subsequently, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Bioscience, USA) and then stained with anti-IFN-γ, anti-IL-4, or anti-IL-17 antibody.

Immunohistochemical analysis. Freshly obtained spleens were embedded in optimal-cutting-temperature (OCT) compound and fixed in methanol. To detect GCs, 8-μm sections were stained with biotin-conjugated peanut agglutinin (PNA) overnight and further incubated with streptavidin-conjugated APC and fluorescein isothiocyanate (FITC)-conjugated B220 for 2 h. The sections were mounted with PermaFlour mounting medium (Thermo, United Kingdom) and analyzed with a confocal scanning laser microscope (Zeiss, Germany).

Quantitative real-time PCR. Transferred CD90.1$^+$ OT-II cells were enriched from the spleen of recipient mice using biotin anti-CD19, biotin anti-B220, biotin anti-CD11b, biotin anti-CD11c, biotin anti-CD24, biotin anti-CD8a (BioLegend, USA), and streptavidin microbeads (BD Biosciences, USA) and further sorted into CD90.1$^+$ CD4$^+$ CD44$^+$ cell populations by FACS. RNA was prepared using a ReliaPrep RNA cell Miniprep system (Promega, USA), reverse transcribed into cDNA, and analyzed using ViiA 7 (Applied Biosystems, USA).

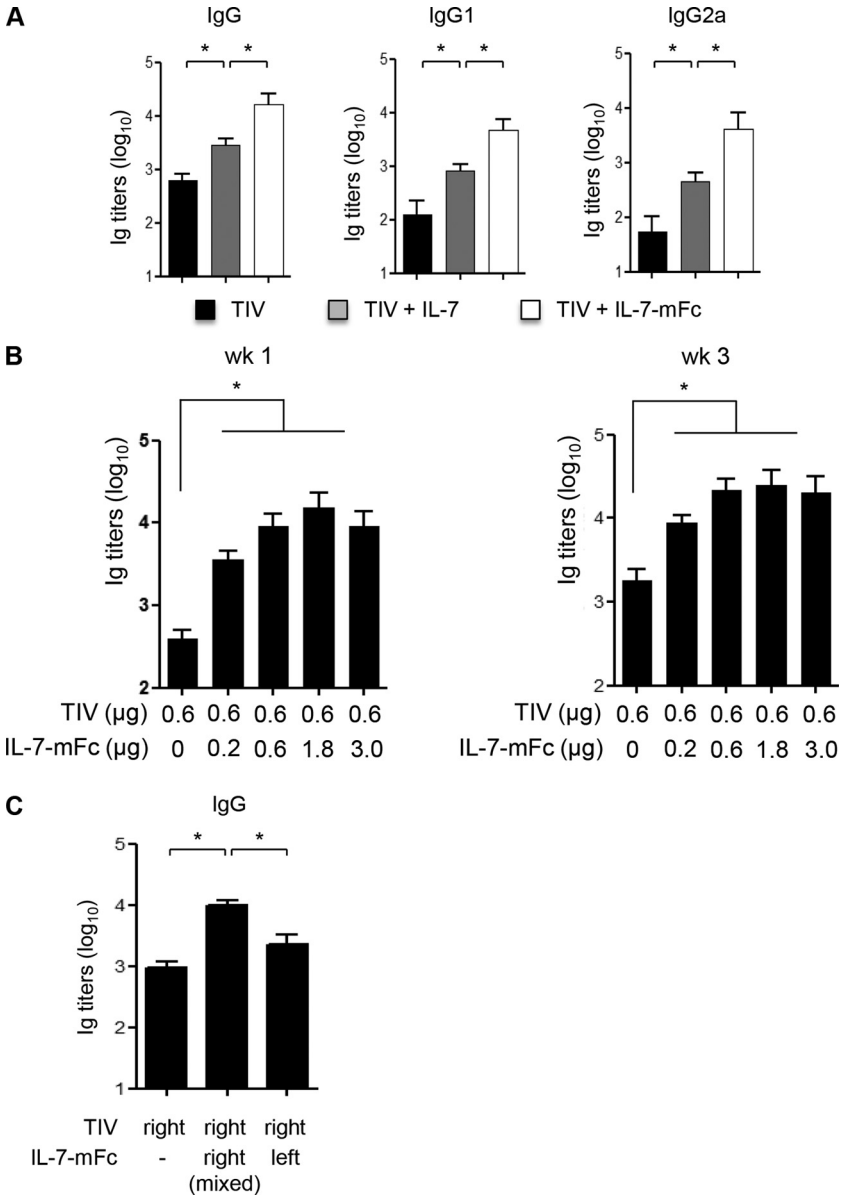

FIG 1 Augmented TIV-specific antibody responses by codelivery of IL-7 or IL-7-mFc. (A) BALB/c mice were immunized with 0.6 μg TIV alone or with either 1.8 μg IL-7 or 1.8 μg IL-7-mFc ($n = 8$/group). (B) BALB/c mice were immunized with 0.6 μg TIV alone or in combination with the indicated dose of IL-7-mFc ($n = 8$/group). (C) Mice were intramuscularly injected with 0.6 μg TIV into the right limb, and 1.8 μg IL-7-mFc was either added to the same injection as a mixture or administered into the opposite limb ($n = 8$/group). At 1 or 3 wpi, serum levels of IgG, IgG1, or IgG2a specific to TIV were determined individually by endpoint titration ELISA. Data, shown as means ± standard errors of the means (SEM), are representative of three independent experiments. TIV, trivalent inactivated influenza virus vaccine consisting of H1N1 A/New Caledonia/20/99, H3N2 A/Fujian/411/2002, and B/Shanghai/361/2002. *, $P < 0.05$ by Student's *t* test.

Statistical analysis. A two-tailed Student's *t* test was used to evaluate statistical differences between the experimental groups. Differences in survival rates between groups were determined using a log-rank test.

RESULTS

Effect of IL-7 or Fc-fused IL-7 cotreatment on the induction of IgG responses to TIV. To determine the role of IL-7 in IgG responses, we investigated whether the administration of exogenous IL-7 can enhance IgG responses induced by immunization with a trivalent inactivated influenza virus vaccine (TIV). When BALB/c mice were immunized with TIV alone or in combination with IL-7, a significant increase in total IgG as well as IgG1 and IgG2a titers was observed for codelivery of IL-7. Interestingly, the adjuvant effect of IL-7 was further improved by nonlytic mouse Fc fusion, presumably due to its prolonged half-life (Fig. 1A), which is in line with our previous report that codelivery of nonlytic Fc-fused IL-7 significantly increased both $CD4^+$ and $CD8^+$ T cell responses induced by DNA vaccine to a greater degree than did lytic Fc-fused IL-7 or unmodified IL-7 (12). Since the highest increase in the induction of the IgG response was observed in TIV plus IL-7-mFc at a 1:3 mass ratio (Fig. 1B), we used the same ratio for all of the following experiments.

Moreover, the strongest IgG responses were induced by coimmunization of IL-7-mFc with TIV at the same site in a mixture

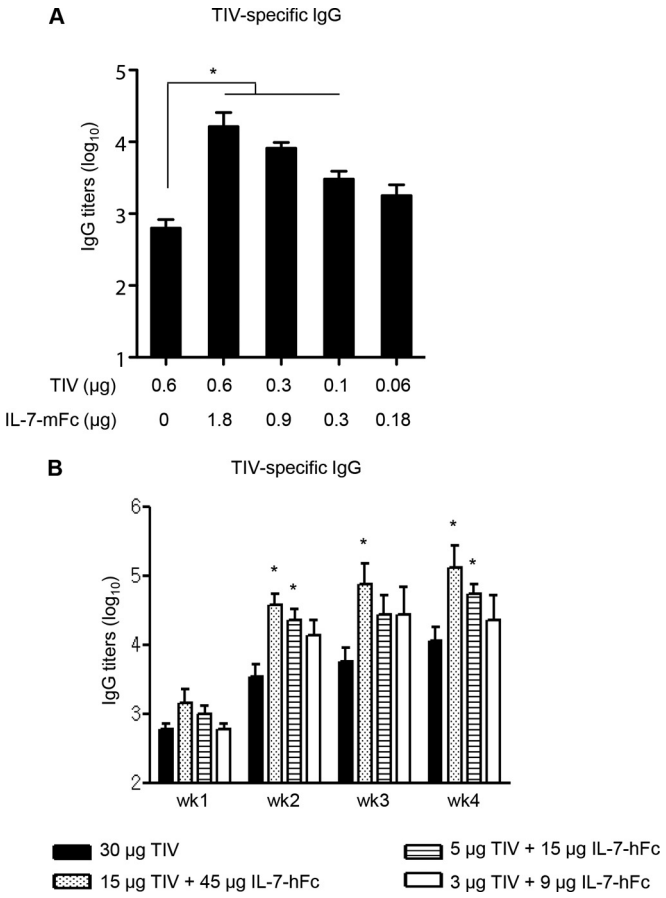

FIG 2 Dose-sparing effect by codelivery of Fc-fused IL-7. BALB/c mice (n = 8/group) (A) or cynomolgus monkeys (n = 4/group) (B) were immunized with the indicated doses of TIV in combination with IL-7-mFc or IL-7-hFc, respectively. Serum IgG responses specific to TIV protein were determined by endpoint titration ELISA at 1 wpi in mice or at 1 to 4 wpi in cynomolgus monkeys. Data, shown as means ± SEM, are representative of three independent mice experiments (A) or a single monkey experiment (B). *, $P < 0.05$ compared to the group treated only with TIV by Student's t test.

(Fig. 1C), indicating the importance of colocalization of the vaccine and adjuvant.

Dose-sparing effect of TIV by Fc-fused IL-7 cotreatment in mouse and monkey. To further evaluate the dose-sparing effect of TIV, mice were immunized with different doses of TIV plus IL-7-mFc in a mixture. As expected, a dose-dependent increase in IgG titers to TIV was observed (Fig. 2A). Interestingly, even with a 10-fold lower dose of TIV (0.06 μg), the addition of IL-7-mFc was able to elicit a 3-fold higher IgG titer than TIV (0.6 μg) alone. Moreover, a half dose of TIV (0.3 μg) with IL-7-mFc exhibited a detectable HI titer against NC/H1N1 in three out of four mice, whereas no HI response was observed with TIV (0.6 μg) alone (Table 1).

There are significant differences in the immune systems of mouse and human, including Ig subclasses, IgG receptors, and expression patterns of Th cytokines (19). While IL-7 is crucial for B cell development in mice, it is dispensable for human B cell development, at least during the neonatal stage (20). In this regard, we assessed the effect of human Fc-fused IL-7 (IL-7-hFc) (13) on the induction of IgG response in nonhuman primates. Cynomolgus monkeys were immunized with 30 μg of TIV alone or a lower titrating dose (15, 5, or 3 μg) of TIV with IL-7-hFc. As expected, we obtained similar results in mice; a 6-fold lower dose of TIV (5 μg) codelivered with IL-7-mFc increased the IgG titer to a significantly higher level than did TIV (30 μg) alone at 2 and 4 weeks postimmunization (Fig. 2B). Moreover, a 10-fold lower dose of TIV (3 μg) with IL-7-hFc exerted a higher seroprotective HI titer in more immunized monkeys than TIV (30 μg) alone (2/4 or 3/4 versus 1/4) (Table 1). Interestingly, detectable HI responses were observed among two out of four monkeys at 1 week postimmunization with a half dose of TIV (15 μg) plus IL-7-hFc, suggesting earlier induction of neutralizing IgG responses by codelivery of IL-7-hFc.

Enhanced cross-reactive IgG responses and protection by Fc-fused IL-7 cotreatment in mouse and monkey. To find out whether these adjuvant effects of Fc-fused IL-7 can induce cross-reactive IgG responses, we measured cross-reactive IgG titer using a different type of HA protein (PR8/H1N1) which exhibited 82% amino acid sequence homology with the NC/H1N1 HA protein included in TIV. Intriguingly, mice immunized with a 6-fold lower dose of TIV (0.1 μg) plus IL-7-mFc exhibited significantly higher cross-reactive IgG titers than mice treated with TIV alone (0.6 μg) (Fig. 3A). Similar to the results obtained in mice, lower doses of TIV (5 or 15 μg) plus IL-7-hFc induced higher cross-reactive IgG titers than did TIV alone (30 μg) in monkeys, although at a statistically insignificant level (n = 4) (Fig. 3B).

To further confirm the enhanced cross-reactive IgG titers by codelivery of IL-7-mFc, BALB/c mice were immunized with various doses of TIV with IL-7-mFc and then challenged with a lethal dose of heterologous virus (PR8/H1N1). Similar to naive mice, mice treated with IL-7-mFc alone suffered from rapid weight loss and died within 11 days postchallenge (dpc), indicating that IL-7-mFc alone did not provide any protection (Fig. 3C and D). Mice immunized with TIV alone appeared to lose weight and succumbed to the influenza virus, with only one out of eight (13%) mice surviving. In contrast, despite the initial weight loss detected during 8 dpc, mice immunized with TIV plus IL-7-mFc rapidly regained their weight and showed elongated survival in a dose-

TABLE 1 HI antibody titers against NC/H1N1 in the immunized mice and monkeys

TIV HA dose (μg) and animal group	IL-7-hFc dose (μg)	HI titer[a] (GMT[b]) against NC/H1N1 at wk: 1	2	3	4
Mouse					
0.6		<20 (0/4)			
0.6	1.8	33 (3/4)			
0.3	0.9	20 (3/4)			
0.1	0.3	<20 (0/4)			
0.06	0.18	<20 (0/4)			
Monkey					
30		0	20 (1/4)	20 (1/4)	20 (1/4)
15	45	20 (2/4)	213 (3/4)	213 (3/4)	320 (3/4)
5	15	0	53 (3/4)	53 (3/4)	53 (3/4)
3	9	0	80 (2/4)	80 (2/4)	60 (3/4)

[a] The geometric mean HI antibody titers against an NC/H1N1 virus (H1N1, A/New Caledonia/20/1999) are expressed as the reciprocal of the highest dilution of sera that inhibited hemagglutination at 3 wpi in mice and at 1 to 4 wpi in monkeys. The numbers of positive serum samples/total number of mouse or monkey serum samples are indicated in parentheses.
[b] GMT, geometric mean reciprocal endpoint titer.

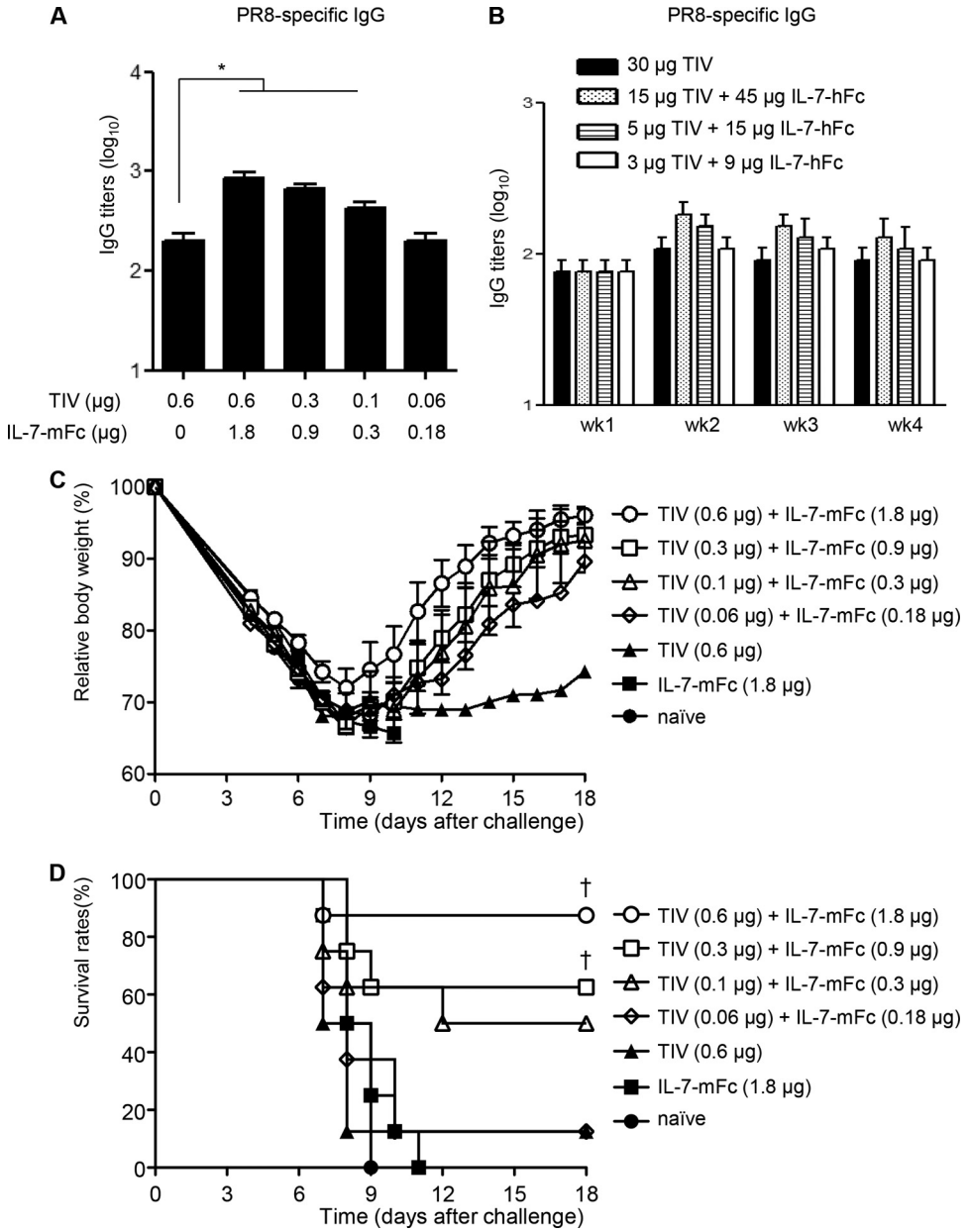

FIG 3 Induction of cross-reactive IgG responses and enhanced protective efficacy against heterologous influenza virus challenge by cotreatment of IL-7-mFc. (A and B) BALB/c mice (n = 8/group) (A) or cynomolgus monkeys (n = 4/group) (B) were immunized with the indicated doses of TIV in combination with IL-7-mFc or IL-7-hFc, respectively. Serum IgG responses specific to inactivated PR8/H1N1 virus particles were determined by endpoint titration ELISA at 1 wpi in mice or at 1 to 4 wpi in cynomolgus monkeys. (C and D) BALB/c mice were immunized with the indicated doses of TIV in the presence or absence of IL-7-mFc (n = 8/group). At 8 days after immunization, mice were challenged intranasally with a lethal dose of PR8/H1N1 virus (2×10^3 PFU). Mice were observed daily to monitor body weight (C) and survival rate (D). Data, shown as means ± SEM, are representative of three independent mice experiments (A) or a single monkey experiment (B). *, $P < 0.05$ compared to the TIV-only group by Student's t test. PR8/H1N1, H1N1 A/Puerto Rico/8/34. Data are representative of two independent experiments with similar results. †, $P < 0.05$ compared to the TIV-only group by a log-rank test.

dependent manner, resulting in an 88% (7/8) survival rate with the same dose of TIV (0.6 μg) plus IL-7-mFc (Fig. 3). Overall, it is likely that the survival rate correlated directly with the magnitude of the cross-reactive IgG responses induced by TIV with IL-7-mFc.

Enhanced expansion of Tfh cells and germinal-center B cells by IL-7-mFc cotreatment. Since it was reported that IgG responses could be induced without $CD4^+$ T cell help (21), we investigated whether $CD4^+$ T cells play a crucial role in the induction of IgG responses by TIV in the presence or absence of IL-7-mFc. Upon treatment with $CD4^+$ T cell-depleting antibodies, TIV-induced IgG responses were not generated at all, regardless of IL-7-mFc treatment, suggesting a crucial role of $CD4^+$ T cells in IgG induction (Fig. 4A).

To analyze which $CD4^+$ T cell subsets have an impact on the increased IgG responses, BALB/c mice were immunized with TIV alone or in combination with IL-7-mFc. Codelivery of IL-7-mFc with TIV did not significantly enhance TIV-specific Th1 ($CD4^+$ IFN-γ^+), Th2 ($CD4^+$ IL-4^+), and Th17 ($CD4^+$ IL-17^+) cells

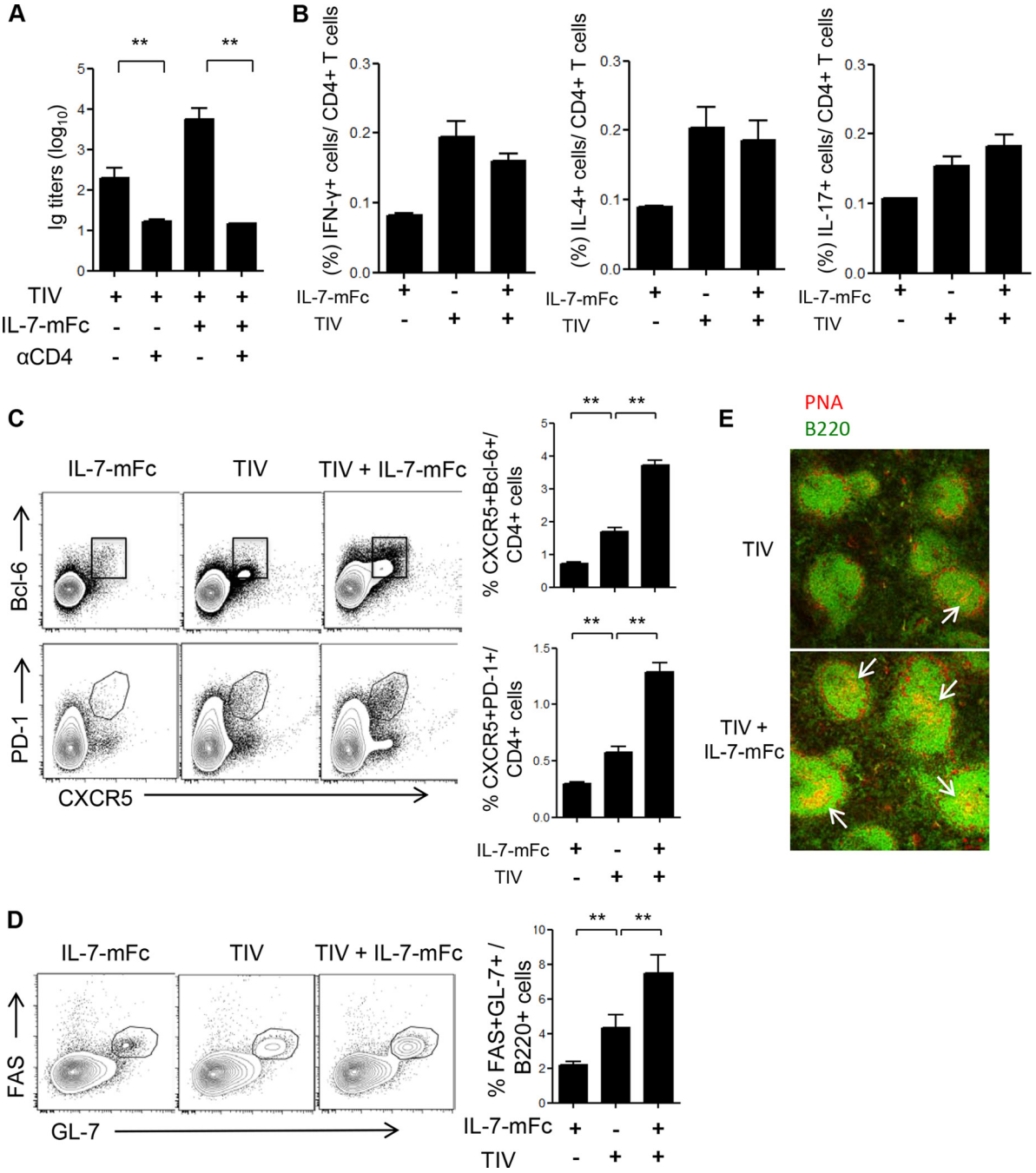

FIG 4 Expansion of Tfh cells and GC B cells, as well as GC formation by TIV cotreatment with IL-7-mFc. BALB/c mice were immunized with 0.6 μg TIV alone or in combination with IL-7-mFc. CD4-depleting antibody (α-CD4) was treated 1 day prior to immunization with the vaccine ($n = 6$/group). (A) At 1 wpi, HA-specific IgG responses were determined by endpoint titration ELISA. (B to E) BALB/c mice were immunized with 0.6 μg TIV with or without 1.8 μg IL-7-mFc ($n = 10$/group). (B) At 1 wpi, draining lymph node cells were stimulated with 10 μg/ml TIV HA for 24 h, and the percentage of Th1 (IFN-γ^+), Th2 (IL-4^+), or Th17 (IL-17^+) cells was measured by intracellular cytokine staining. Tfh cells (CXCR5$^+$ Bcl-6$^+$ and CXCR5$^+$ PD-1$^+$) among CD4$^+$ T cells (C) or germinal center B cells (FAS$^+$ GL-7$^+$) among B220$^+$ B cells (D) were analyzed by flow cytometry. (E) Representative images of PNA staining (red) for detecting PNA$^+$ GCs (depicted by arrows) in spleen by immunohistochemical analysis are shown. Data, shown as means ± SEM, are representative of two independent experiments. $P < 0.05$ (*) and $P < 0.01$ (**) by Student's t test.

compared to TIV alone (Fig. 4B). These results are inconsistent with a previous report that observed the facilitation of Th1 cell development by IL-7 treatment in the experimental autoimmune encephalomyelitis (EAE) model (22). The discrepancy may be due to the difference in experimental conditions (*in vitro* versus *in vivo*) and the nature of T cells stimulated by IL-7 (naive T cells versus Th1-polarized T cells). In contrast, codelivery of IL-7-mFc with TIV immunization markedly increased the frequency of Tfh cells (Fig. 4C), which were represented as CD4$^+$ CXCR5$^+$ Bcl6$^+$ or CD4$^+$ CXCR5$^+$ PD-1$^+$ T cells (3).

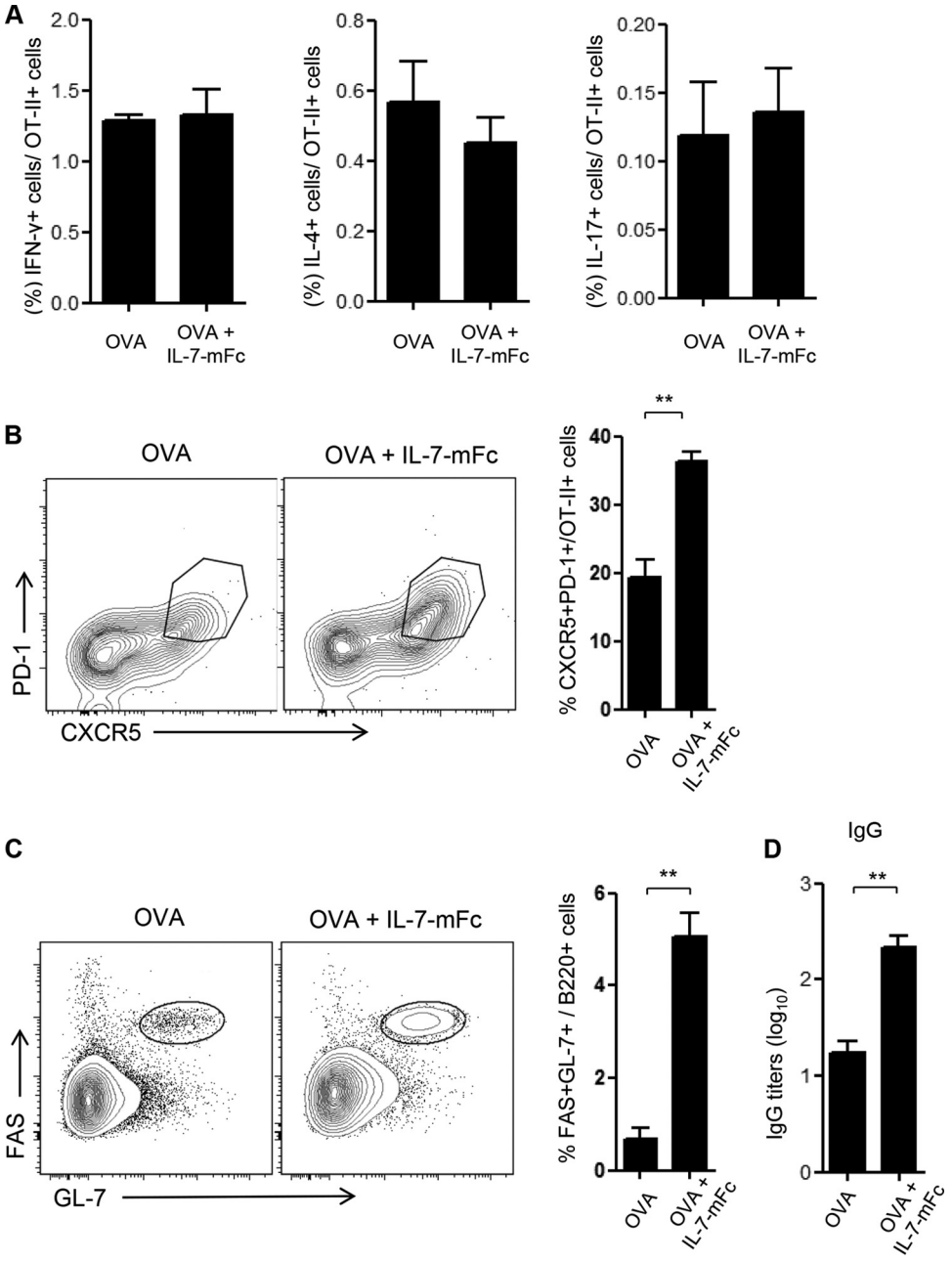

FIG 5 Effect of IL-7-mFc cotreatment on the generation of Th subsets in OT-II transfer model. C57BL/6 mice were intravenously injected with OT-II cells and then immunized with 10 μg OVA protein in the presence or absence of 30 μg IL-7-mFc ($n = 5$/group). At 1 wpi, splenocytes were isolated and restimulated for 6 h with 1 μM $OVA_{232-339}$ peptide. (A) Gated $CD4^+$ T cells were analyzed by intracellular cytokine staining for IFN-γ, IL-4, or IL-17 production. The percentage of Tfh cells in OT-II cells (B) and GC B cells in $B220^+$ cells (C) was determined as previously described. (D) Serum levels of OVA-specific IgG were determined by endpoint titration ELISA. Data, shown as means ± SEM, are representative of two independent experiments. **, $P < 0.01$ by Student's *t* test.

Consistent with the enhanced frequency of Tfh cells, codelivery of IL-7-mFc exhibited a significantly increased frequency of GC B cells ($B220^+$ Fas^+ $GL\text{-}7^+$) compared to TIV alone (Fig. 4D). GCs are inducible lymphoid microenvironments located within the B cell follicle (23), and PNA was widely used to probe GCs (24, 25). Thus, the architectures that are stained positively for both B220 and PNA ($B220^+$ PNA^+) represent GCs, and their frequency is markedly increased in IL-7-mFc-cotreated mice compared to levels for the control group. (Fig. 4E). To further investigate whether IL-7-mFc-mediated expansion of Tfh cells is antigen specific, OVA-specific $CD4^+$ T cells were adoptively transferred into C57BL/6 mice and then immunized with OVA with or without IL-7-mFc. Similar to the results obtained in the TIV model, the number of non-Tfh $CD4^+$-lineage subsets was not increased by IL-7-mFc cotreatment, whereas the frequency of OVA-specific Tfh cells and GC B cells as well as the level of IgG titers to OVA antigen were significantly increased (Fig. 5). These results indicate that the effect of exogenous IL-7-mFc on the differentiation of Tfh cells is independent of immunogen type (TIV or OVA), experimental model (with or without T cell transfer), and animal strain (BALB/c or C57BL/6).

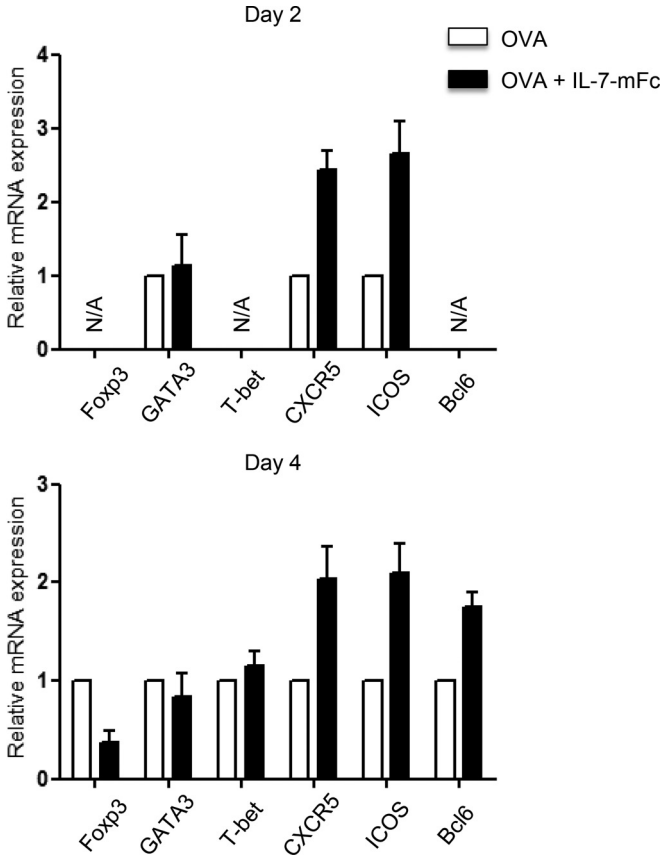

FIG 6 Comparison of mRNA expression levels between antigen-only and IL-7-mFc cotreatment. C57BL/6 mice were transferred with naive OT-II cells (5×10^5) and then immunized with 10 μg OVA (with alum) in the presence or absence of 30 μg IL-7-mFc ($n = 4$/group). The transferred OT-II cells were sorted from spleen of the immunized mice on day 2 or day 4 after immunization. mRNA levels of the indicated molecules were determined by quantitative real-time PCR. Data are representative of three independent experiments. NA, data not available owing to the failure of detecting the mRNA level of either group.

When we investigated the expression of Tfh cell-related genes, the levels of CXCR5 and ICOS were increased at days 2 and 4 and Bcl6 expression was enhanced at day 4. In contrast, transcriptional factors of non-Tfh $CD4^+$ lineage subsets, such as Foxp3, GATA3, and T-bet, were not affected (Fig. 6).

Effect of IL-7 on the development of GC B cells and Tfh cells is independent of IL-6 and IL-21. Since IL-6 and IL-21 are well known as crucial factors for Tfh cell differentiation, we investigated whether enhanced Tfh cell generation by IL-7 treatment was mediated by IL-6 and/or IL-21. Consistent with a previous report (11), the generation of Tfh cells (Fig. 7A) and GC B cells (Fig. 7B) induced by TIV immunization was significantly, but not completely, impaired by cotreatment of anti-IL-6/IL-21 antibodies. However, the suppressive effects of IL-6/IL-21 blockades was not observed in the presence of IL-7-mFc with TIV (Fig. 7A and B), suggesting that exogenous IL-7 could compensate for the neutralization of IL-6 and IL-21 and/or enhance Tfh cell differentiation in an IL-6- and IL-21-independent manner.

To investigate whether endogenous IL-7 as well as IL-6 and IL-21 could regulate Tfh cell differentiation and GC B cell formation, anti-IL-6, anti-IL-7, or anti-IL-21 neutralizing antibodies, either alone or in combination, were administered during immunization with TIV. As expected, cotreatment of IL-6 or IL-21 blockade significantly decreased the frequency of Tfh cells. Interestingly, cotreatment of IL-7 blockade antibody exhibited a significant decrease in the frequency of Tfh cells, which was further decreased by combined treatment of IL-6 and IL-21 blockades (Fig. 8A). Moreover, the frequency and number of GC B cells as well as TIV-specific IgG responses were significantly reduced by IL-7 blockade (Fig. 8B and C). Taken together, IL-7 as a distinct cytokine, independent of IL-6 and IL-21, plays a critical role in inducing the generation of Tfh cells and GC B cells on immunization under physiological conditions.

DISCUSSION

Considering that pandemic influenza viruses occur suddenly and spread rapidly, it is important to generate sufficient amounts of influenza virus vaccines capable of inducing protective antibody responses as early as possible in order to limit viral transmission. Here, we demonstrated that codelivery of Fc-fused IL-7 significantly enhances not only TIV-induced IgG titer but also early neutralizing IgG responses. Moreover, IL-7 cotreatment exhibits prominent dose-sparing benefits of TIV in monkeys as well as in mice. Enhanced cross-reactive IgG responses also were confirmed by increased protection against lethal doses of heterologous influenza virus in mice. It was reported that cross-reactive antibody responses are critical factors for the protective immunity against highly variable pathogens, such as HIV and influenza virus (26). It is possible that the induction of cross-reactive IgG responses observed in this study are due to enhanced T cell receptor (TCR) repertoire diversity (27) as well as increased frequency of Tfh cells by IL-7 cotreatment during immunization. Cross-reactive epitopes are well conserved among strains of highly mutable viruses but generally are subdominant for T cell activation (28). IL-7R signaling could activate subdominant epitope-specific T cells by lowering the threshold of T cell activation, leading to the expansion of the T cell pool that can recognize diverse T cell epitopes (29). Within a pool of increased T cells, exogenous IL-7 may further promote the differentiation of T cells into functional Tfh cells, followed by increased GC formation. During the GC reaction, B cells undergo clonal expansion with somatic hypermutation of the immunoglobulin gene and affinity maturation by migrating back and forth between dark and light zones of the GC (23), resulting in an increase of the B cell receptor repertoire. Moreover, the increased numbers of Tfh cells could activate B cells presenting subdominant T cell epitopes via T cell-B cell cognate interaction. As a result, activated B cells recognizing conserved B cell epitopes could differentiate into plasma cells to produce cross-reactive antibodies. It was reported that Tfh cells are important for the increase of total antibody responses by providing help to B cells and maintaining the GC reaction (30). Indeed, the development of Tfh and GC B cells, as well as IgG responses, was markedly impaired by cotreatment of IL-7 blockade during immunization with TIV, suggesting a critical role of endogenous IL-7 in Tfh cell generation as well as the induction of IgG responses.

In this study, we demonstrated that codelivery of exogenous IL-7 with antigen considerably promoted the differentiation of naive T cells into Tfh cells. Although the precise mechanisms by which IL-7 upregulates Tfh cell generation remain to be studied, several possibilities could be envisaged based on previous reports. Given that the effect of IL-7 has been determined in an IL-7R-dependent manner, IL-7R-expressing cells participating in the Tfh

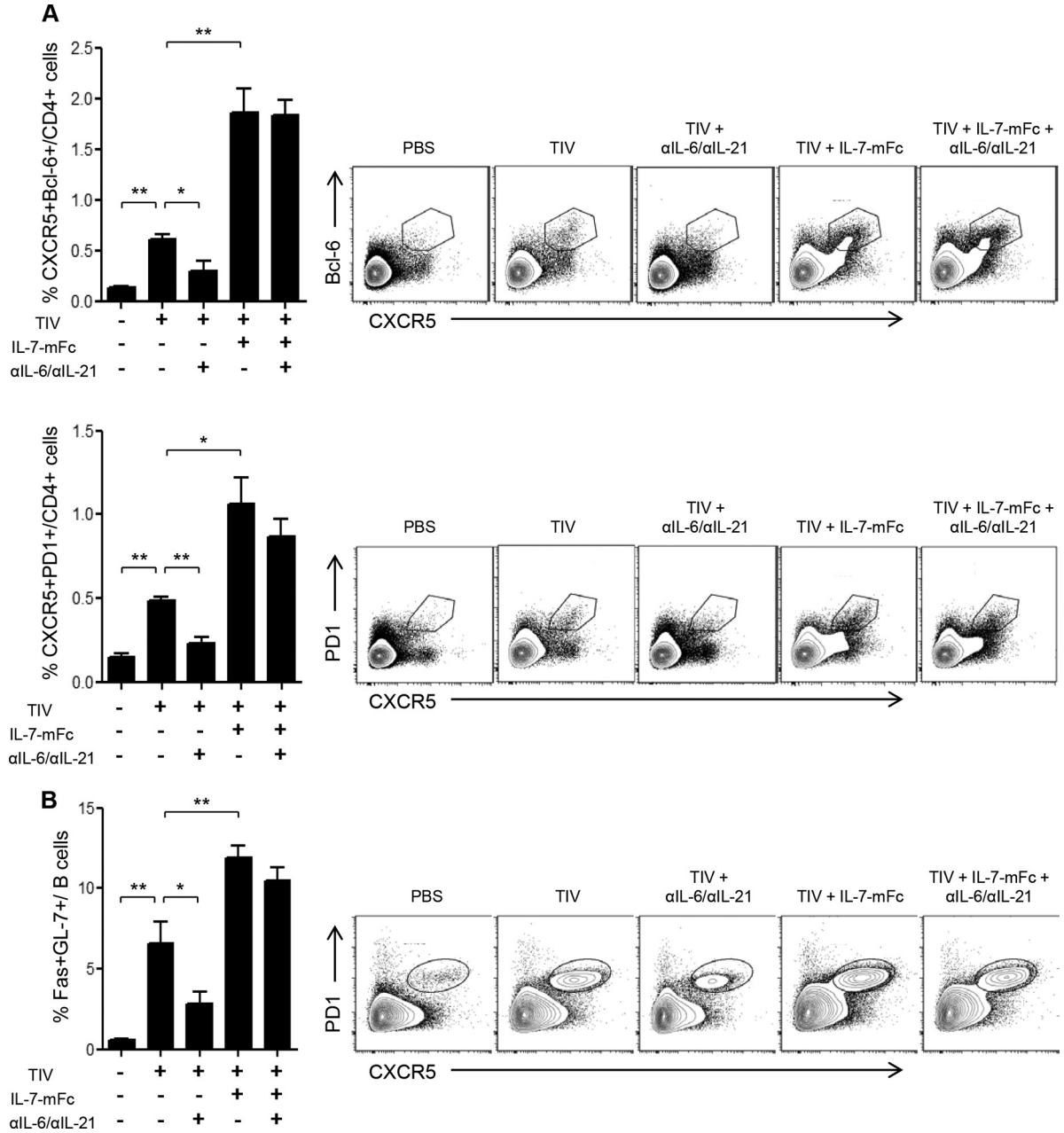

FIG 7 Effect of the IL-6/IL-21 blockade on the generation of Tfh cells and GC B cells by IL-7-mFc cotreatment. BALB/c mice were immunized with 10 μg TIV with or without 30 μg IL-7-mFc. In addition, the combination of anti-IL-6 and anti-IL-21 neutralizing antibodies was injected into the immunized mice as described in Materials and Methods ($n = 5$/group). At 1 wpi, draining lymph node cells were analyzed to evaluate the percentage of Tfh cells (A) and GC B cells (B). Data, shown as means ± SEM, are representative of two independent experiments. $P < 0.05$ (*) and $P < 0.01$ (**) by Student's t test.

cell development process might be prime candidates, such as $CD4^+$ T cells and dendritic cells (DCs). Since $CD4^+$ T cells themselves express high levels of IL-7R, direct action of IL-7 on a $CD4^+$ T cell might be the most effective way to promote Tfh cells. Thus, it is likely that IL-7 could directly augment several T-cell-intrinsic factors, such as surface molecules (ICOS and CD40L) (18, 31, 32), signaling factors (STAT-1, STAT-3, and STAT-4) (8, 33, 34), and transcriptional factors (Bcl6) (35), in $CD4^+$ T cells, all of which may play indispensable roles in Tfh cell development. Correspondently, we observed a significant increase in ICOS expression on transferred OVA-specific $CD4^+$ T cells upon cotreatment with OVA and IL-7-mFc (data not shown). Moreover, when sorted naive $CD4^+$ T cells were treated with IL-7-mFc *in vitro*, increased activation of STAT1, STAT3, and STAT5 was observed by immunoblot assay (data not shown), yielding results consistent with those of previous reports that showed T cell stimulation by IL-7 (36–38). This is particularly interesting because the activation of STAT1 and STAT3 signaling pathways has been known to drive Tfh cell development (33, 39). Alternatively, IL-7-mFc treatment may induce activation of DCs and subsequent secretion of a wide range of cytokines that could drive Tfh cell development. A proper activation of DCs is important in the process of Tfh cell differen-

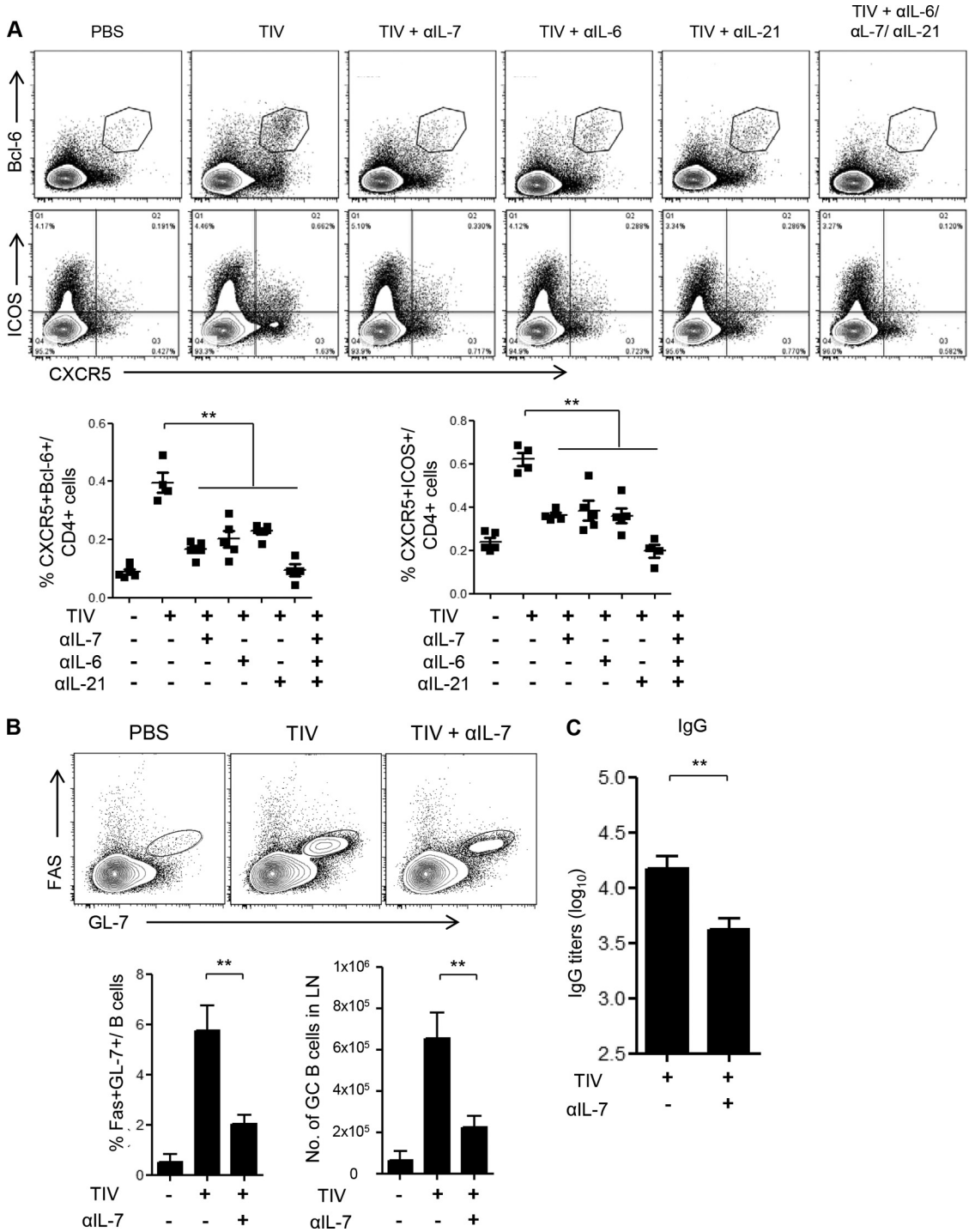

FIG 8 Effects of the IL-7 blockade on Tfh cell and GC B cell development during TIV immunization. BALB/c mice first were immunized with 10 μg TIV and then treated with anti-IL-6, anti-IL-7, or anti-IL-21 neutralizing antibody, either alone or in combination, as described in Materials and Methods ($n = 5$/group). At 1 wpi, draining lymph node cells were analyzed to determine the frequency of Tfh cells (A) and the frequency and the number of GC B cells (B). (C) At 1 wpi, serum levels of IgG to TIV were determined by endpoint titration ELISA. Data, shown as means ± SEM, are representative of two independent experiments. **, $P < 0.01$ by Student's *t* test.

tiation, as cognate interactions between DCs and naive $CD4^+$ T cells not only provide necessary and sufficient signals to induce initial commitment of Tfh cell differentiation (40) but also promote preferential induction of Tfh cell formation through their sustained interaction (41, 42). Prior studies have shown that multiple DC subsets that express IL-7R, such as miDC, cDC, and pDC (43–45), could secrete cytokines required for the induction of Tfh cell differentiation. For instance, it was previously shown that activated cDCs produce a substantial amount of IL-6 in response to CD40 stimulation through the activation of type I interferon sig-

naling (46, 47). Similarly, pDCs are known to produce IL-6 upon CD40 stimulation as well as tumor growth factor beta treatment (48, 49). More importantly, activated human DCs could play a role in the induction of IL-21-secreting Tfh-like cells via IL-12-mediated STAT4 activation (50). Therefore, although the cytokine secretion profiles of various DC subsets upon exogenous IL-7 treatment remain to be determined, the activation of DCs by IL-7 also may produce helper molecules that could skew the differentiation pathway of T cells toward Tfh cells. In sum, it is likely that IL-7 induces Tfh cell differentiation by upregulating the expression of Tfh cell-inducing molecules on $CD4^+$ T cells directly or by stimulating the secretion of cytokines from multiple DC subsets, which would be considered an indirect effect. Future studies using the elegant conditional knockout mice that abolish IL-7R-mediated signaling in $CD4^+$ T cells only during the T cell differentiation period or in DCs would be appropriate to elucidate clear mechanisms of IL-7 in Tfh cell development.

ACKNOWLEDGMENTS

We thank Jonathan Sprent for reviewing the manuscript and providing valuable comments and Kwan Seok Lee for his devoted animal care.

This study was supported by a grant of the Korean Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (HI12C1664), the Ministry of Knowledge Economy (MKE; 1415126993), through the fostering project of the Osong Academy-Industry Convergence (BAIO). This research was supported by BK21 Plus (10Z20130012243), funded by the Ministry of Education, South Korea, and by grants from Genexine Co. Ltd., South Korea.

REFERENCES

1. **Goodwin RG, Lupton S, Schmierer A, Hjerrild KJ, Jerzy R, Clevenger W, Gillis S, Cosman D, Namen AE.** 1989. Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells. Proc. Natl. Acad. Sci. U. S. A. **86:**302–306. http://dx.doi.org/10.1073/pnas.86.1.302.
2. **Allen CD, Okada T, Tang HL, Cyster JG.** 2007. Imaging of germinal center selection events during affinity maturation. Science **315:**528–531. http://dx.doi.org/10.1126/science.1136736.
3. **Crotty S.** 2011. Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. **29:**621–663. http://dx.doi.org/10.1146/annurev-immunol-031210-101400.
4. **Cyster JG.** 2010. Shining a light on germinal center B cells. Cell **143:**503–505. http://dx.doi.org/10.1016/j.cell.2010.10.036.
5. **Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, Dent AL, Craft J, Crotty S.** 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. Science **325:**1006–1010. http://dx.doi.org/10.1126/science.1175870.
6. **Ma CS, Deenick EK, Batten M, Tangye SG.** 2012. The origins, function, and regulation of T follicular helper cells. J. Exp. Med. **209:**1241–1253. http://dx.doi.org/10.1084/jem.20120994.
7. **Harker JA, Lewis GM, Mack L, Zuniga EI.** 2011. Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. Science **334:**825–829. http://dx.doi.org/10.1126/science.1208421.
8. **Nurieva RI, Chung Y, Hwang D, Yang XO, Kang HS, Ma L, Wang YH, Watowich SS, Jetten AM, Tian Q, Dong C.** 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. Immunity **29:**138–149. http://dx.doi.org/10.1016/j.immuni.2008.05.009.
9. **Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C.** 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity **29:**127–137. http://dx.doi.org/10.1016/j.immuni.2008.06.001.
10. **Batten M, Ramamoorthi N, Kljavin NM, Ma CS, Cox JH, Dengler HS, Danilenko DM, Caplazi P, Wong M, Fulcher DA, Cook MC, King C, Tangye SG, de Sauvage FJ, Ghilardi N.** 2010. IL-27 supports germinal center function by enhancing IL-21 production and the function of T follicular helper cells. J. Exp. Med. **207:**2895–2906. http://dx.doi.org/10.1084/jem.20100064.
11. **Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, Yusuf I, Crotty S.** 2011. IL-21 and IL-6 are critical for different aspects of B cell immunity and redundantly induce optimal follicular helper CD4 T cell (Tfh) differentiation. PLoS One **6:**e17739. http://dx.doi.org/10.1371/journal.pone.0017739.
12. **Nam HJ, Song MY, Choi DH, Yang SH, Jin HT, Sung YC.** 2010. Marked enhancement of antigen-specific T-cell responses by IL-7-fused nonlytic, but not lytic, Fc as a genetic adjuvant. Eur. J. Immunol. **40:**351–358. http://dx.doi.org/10.1002/eji.200939271.
13. **Im SJ, Yang SI, Yang SH, Choi DH, Choi SY, Kim HS, Jang do, Jin SKS, Chung YK, Kim SH, Paik SH, Park YC, Chung MK, Kim YB, Han KH, Choi KY, Sung YC.** 2011. Natural form of noncytolytic flexible human Fc as a long-acting carrier of agonistic ligand, erythropoietin. PLoS One **6:**e24574. http://dx.doi.org/10.1371/journal.pone.0024574.
14. **Park KS, Lee J, Ahn SS, Byun YH, Seong BL, Baek YH, Song MS, Choi YK, Na YJ, Hwang I, Sung YC, Lee CG.** 2009. Mucosal immunity induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2 avian influenza virus challenge. Virology **395:**182–189. http://dx.doi.org/10.1016/j.virol.2009.09.018.
15. **Sin JI, Sung JH, Suh YS, Lee AH, Chung JH, Sung YC.** 1997. Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of coinjection with a granulocyte-macrophage colony stimulating factor gene. Vaccine **15:**1827–1833. http://dx.doi.org/10.1016/S0264-410X(97)88856-1.
16. **Jonges M, Liu WM, van der Vries E, Jacobi R, Pronk I, Boog C, Koopmans M, Meijer A, Soethout E.** 2010. Influenza virus inactivation for studies of antigenicity and phenotypic neuraminidase inhibitor resistance profiling. J. Clin. Microbiol. **48:**928–940. http://dx.doi.org/10.1128/JCM.02045-09.
17. **Lee JH, Pascua PN, Song MS, Baek YH, Kim CJ, Choi HW, Sung MH, Webby RJ, Webster RG, Poo H, Choi YK.** 2009. Isolation and genetic characterization of H5N2 influenza viruses from pigs in Korea. J. Virol. **83:**4205–4215. http://dx.doi.org/10.1128/JVI.02403-09.
18. **Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, Lao C, Crotty S.** 2011. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity **34:**932–946. http://dx.doi.org/10.1016/j.immuni.2011.03.023.
19. **Mestas J, Hughes CC.** 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. **172:**2731–2738. http://dx.doi.org/10.4049/jimmunol.172.5.2731.
20. **Prieyl JA, LeBien TW.** 1996. Interleukin 7 independent development of human B cells. Proc. Natl. Acad. Sci. U. S. A. **93:**10348–10353. http://dx.doi.org/10.1073/pnas.93.19.10348.
21. **Lee BO, Rangel-Moreno J, Moyron-Quiroz JE, Hartson L, Makris M, Sprague F, Lund FE, Randall TD.** 2005. CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection. J. Immunol. **175:**5827–5838. http://dx.doi.org/10.4049/jimmunol.175.9.5827.
22. **Lee LF, Axtell R, Tu GH, Logronio K, Dilley J, Yu J, Rickert M, Han B, Evering W, Walker MG, Shi J, de Jong BA, Killestein J, Polman CH, Steinman L, Lin JC.** 2011. IL-7 promotes T(H)1 development and serum IL-7 predicts clinical response to interferon-beta in multiple sclerosis. Sci. Transl, Med. 3:93ra68. http://dx.doi.org/10.1126/scitranslmed.3002400.
23. **Victora GD, Nussenzweig MC.** 2012. Germinal centers. Annu. Rev. Immunol. **30:**429–457. http://dx.doi.org/10.1146/annurev-immunol-020711-075032.
24. **Coico RF, Bhogal BS, Thorbecke GJ.** 1983. Relationship of germinal centers in lymphoid tissue to immunologic memory. VI. Transfer of B cell memory with lymph node cells fractionated according to their receptors for peanut agglutinin. J. Immunol. **131:**2254–2257.
25. **Ko HJ, Yang H, Yang JY, Seo SU, Chang SY, Seong JK, Kweon MN.** 2012. Expansion of Tfh-like cells during chronic Salmonella exposure mediates the generation of autoimmune hypergammaglobulinemia in MyD88-deficient mice. Eur. J. Immunol. **42:**618–628. http://dx.doi.org/10.1002/eji.201141748.
26. **Corti D, Lanzavecchia A.** 2013. Broadly neutralizing antiviral antibodies. Annu. Rev. Immunol. **31:**705–742. http://dx.doi.org/10.1146/annurev-immunol-032712-095916.
27. **Sportes C, Hakim FT, Memon SA, Zhang H, Chua KS, Brown MR, Fleisher TA, Krumlauf MC, Babb RR, Chow CK, Fry TJ, Engels J, Buffet**

R, Morre M, Amato RJ, Venzon DJ, Korngold R, Pecora A, Gress RE, Mackall CL. 2008. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. J. Exp. Med. **205:**1701–1714. http://dx.doi.org/10.1084/jem.20071681.

28. **Fomsgaard A, Karlsson I, Gram G, Schou C, Tang S, Bang P, Kromann I, Andersen P, Andreasen LV.** 2011. Development and preclinical safety evaluation of a new therapeutic HIV-1 vaccine based on 18 T-cell minimal epitope peptides applying a novel cationic adjuvant CAF01. Vaccine **29:** 7067–7074. http://dx.doi.org/10.1016/j.vaccine.2011.07.025.
29. **Melchionda F, Fry TJ, Milliron MJ, McKirdy MA, Tagaya Y, Mackall CL.** 2005. Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool. J. Clin. Investig. **115:** 1177–1187. http://dx.doi.org/10.1172/JCI23134.
30. **Deenick EK, Ma CS, Brink R, Tangye SG.** 2011. Regulation of T follicular helper cell formation and function by antigen presenting cells. Curr. Opin. Immunol. **23:**111–118. http://dx.doi.org/10.1016/j.coi.2010.10.007.
31. **Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, Durandy A, Baumann U, Schlesier M, Welcher AA, Peter HH, Warnatz K.** 2006. ICOS deficiency is associated with a severe reduction of $CXCR5^+$ CD4 germinal center Th cells. J. Immunol. **177:**4927–4932. http://dx.doi.org/10.4049/jimmunol.177.7.4927.
32. **Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, Ma J, Tezuka K, Yagita H, Okumura K.** 2005. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J. Immunol. **175:**2340–2348. http://dx.doi.org/10.4049/jimmunol.175.4.2340.
33. **Choi YS, Eto D, Yang JA, Lao C, Crotty S.** 2013. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. J. Immunol. **190:**3049–3053. http://dx.doi.org/10.4049/jimmunol.1203032.
34. **Nakayamada S, Kanno Y, Takahashi H, Jankovic D, Lu KT, Johnson TA, Sun HW, Vahedi G, Hakim O, Handon R, Schwartzberg PL, Hager GL, O'Shea JJ.** 2011. Early Th1 cell differentiation is marked by a Tfh cell-like transition. Immunity **35:**919–931. http://dx.doi.org/10.1016/j.immuni.2011.11.012.
35. **Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C.** 2009. Bcl6 mediates the development of T follicular helper cells. Science **325:**1001–1005. http://dx.doi.org/10.1126/science.1176676.
36. **Hotson AN, Hardy JW, Hale MB, Contag CH, Nolan GP.** 2009. The T cell STAT signaling network is reprogrammed within hours of bacteremia via secondary signals. J. Immunol. **182:**7558–7568. http://dx.doi.org/10.4049/jimmunol.0803666.
37. **Lu N, Wang YH, Arima K, Hanabuchi S, Liu YJ.** 2009. TSLP and IL-7 use two different mechanisms to regulate human CD4+ T cell homeostasis. J. Exp. Med. **206:**2111–2119. http://dx.doi.org/10.1084/jem.20090153.
38. **Michel ML, Pang DJ, Haque SF, Potocnik AJ, Pennington DJ, Hayday AC.** 2012. Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. Proc. Natl. Acad. Sci. U. S. A. **109:** 17549–17554. http://dx.doi.org/10.1073/pnas.1204327109.
39. **Lu KT, Kanno Y, Cannons JL, Handon R, Bible P, Elkahloun AG, Anderson SM, Wei L, Sun H, O'Shea JJ, Schwartzberg PL.** 2011. Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. Immunity **35:**622–632. http://dx.doi.org/10.1016/j.immuni.2011.07.015.
40. **Goenka R, Barnett LG, Silver JS, O'Neill PJ, Hunter CA, Cancro MP, Laufer TM.** 2011. Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation. J. Immunol. **187:**1091–1095. http://dx.doi.org/10.4049/jimmunol.1100853.
41. **Deenick EK, Chan A, Ma CS, Gatto D, Schwartzberg PL, Brink R, Tangye SG.** 2010. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. Immunity **33:**241–253. http://dx.doi.org/10.1016/j.immuni.2010.07.015.
42. **Fazilleau N, McHeyzer-Williams LJ, Rosen H, McHeyzer-Williams MG.** 2009. The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. **10:**375–384. http://dx.doi.org/10.1038/ni.1704.
43. **Rochman Y, Spolski R, Leonard WJ.** 2009. New insights into the regulation of T cells by gamma(c) family cytokines. Nat. Rev. Immunol. **9:**480–490. http://dx.doi.org/10.1038/nri2580.
44. **Varas A, Vicente A, Sacedon R, Zapata AG.** 1998. Interleukin-7 influences the development of thymic dendritic cells. Blood **92:**93–100.
45. **Vogt TK, Link A, Perrin J, Finke D, Luther SA.** 2009. Novel function for interleukin-7 in dendritic cell development. Blood **113:**3961–3968. http://dx.doi.org/10.1182/blood-2008-08-176321.
46. **Ferrer IR, Liu D, Pinelli DF, Koehn BH, Stempora LL, Ford ML.** 2012. CD40/CD154 blockade inhibits dendritic cell expression of inflammatory cytokines but not costimulatory molecules. J. Immunol. **189:**4387–4395. http://dx.doi.org/10.4049/jimmunol.1201757.
47. **Cucak H, Yrlid U, Reizis B, Kalinke U, Johansson-Lindbom B.** 2009. Type I interferon signaling in dendritic cells stimulates the development of lymph-node-resident T follicular helper cells. Immunity **31:**491–501. http://dx.doi.org/10.1016/j.immuni.2009.07.005.
48. **Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J.** 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity **19:**225–234. http://dx.doi.org/10.1016/S1074-7613(03)00208-5.
49. **Bonnefoy F, Couturier M, Clauzon A, Remy-Martin JP, Gaugler B, Tiberghien P, Chen W, Saas P, Perruche S.** 2011. TGF-beta-exposed plasmacytoid dendritic cells participate in Th17 commitment. J. Immunol. **186:**6157–6164. http://dx.doi.org/10.4049/jimmunol.1002497.
50. **Schmitt N, Morita R, Bourdery L, Bentebibel SE, Zurawski SM, Banchereau J, Ueno H.** 2009. Human dendritic cells induce the differentiation of interleukin-21-producing T follicular helper-like cells through interleukin-12. Immunity **31:**158–169. http://dx.doi.org/10.1016/j.immuni.2009.04.016.