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Induction and Activation of Human Th17 by Targeting Antigens to Dendritic Cells via Dectin-1

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Recent compelling evidence indicates that Th17 confer host immunity against a variety of microbes, including extracellular and intracellular pathogens. Therefore, understanding mechanisms for the induction and activation of Ag-specific Th17 is important for the rational design of vaccines against pathogens. To study this, we employed an in vitro system in which influenza hemagglutinin (HA) 1 was delivered to dendritic cells (DCs) via Dectin-1 using anti-human Dectin-1 (hDectin-1)–HA1 recombinant fusion proteins. We found that healthy individuals maintained broad ranges of HA1-specific memory Th17 that were efficiently activated by DCs targeted with anti-hDectin-1–HA1. Nonetheless, these DCs were not able to induce a significant level of HA1-specific Th17 responses even in the presence of the Th17-promoting cytokines IL-1β and IL-6. We further found that the induction of surface IL-1R1 expression by signals via TCRs and common γ-chain receptors was essential for naive CD4+ T cell differentiation into HA1-specific Th17. This process was dependent on MyD88, but not IL-1R–associated kinase 1/4. Thus, interruptions in STAT3 or MyD88 signaling led to substantially diminished HA1-specific Th17 induction. Taken together, the de novo generation of pathogen-specific human Th17 requires complex, but complementary, actions of multiple signals. Data from this study will help us design a new and effective vaccine strategy that can promote Th17-mediated immunity against microbial pathogens. The Journal of Immunology, 2014, 192: 5776–5788.

Interleukin17–producing CD4+ T cells (Th17) have been broadly linked to inflammatory diseases (1). However, recent compelling evidence indicates that Th17 also plays an important role against both extracellular and intracellular microbial pathogens, including bacteria, fungi, parasites, and viruses (2–13). Furthermore, the immunity conferred by Th17 is associated with improved survival of cancer patients (14, 15). Accordingly, Th17-mediated therapeutic immunity has also been demonstrated in murine cancer models (16, 17). Therefore, it is important to understand molecular and cellular mechanisms for the induction and activation of Ag-specific Th17 in the context of TCR ligation by peptides and MHCs.

The induction of Th17 has been mainly studied in the context of inflammatory cytokine milieu. In mice, TGF-β (18, 19), IL-6 (19), IL-1β (20), IL-21 (21), IL-23 (22), and IL-9 (23) contribute to Th17 induction. In humans, IL-1β with IL-6 was initially reported to induce Th17 differentiation, and this was inhibited by TGF-β and IL-12 (9). TGF-β was also reported to be required for Th17 development (24), but Yang et al. (25) demonstrated that human Th17 could be developed in the presence of TGF-β and IL-21, but not TGF-β and IL-6. In contrast, Volpe et al. (26) showed that proinflammatory cytokines were all required and acted synergistically to generate human Th17. These series of findings suggest that each of these cytokines might contribute to Th17 development at certain stages of human T cell differentiation, although a recent finding has shown that IL-1β is essential in Candida albicans–induced human Th17 differentiation (27). Unlike in mice, however, our understanding of the induction and activation of Ag-specific Th17 in humans is still limited. This is mainly due to limitations of reliable experimental systems as well as difficulties in the assessment of Ag-specific T cell responses after in vitro priming of T cells, particularly when the frequency of Ag-specific T cells is low. Thus, previous studies (9, 24–27) employed polyclonal T cell activators, such as anti-CD3/CD28 Abs and PMA, to prime and/or reactivate T cells to assess the magnitude and quality of T cell responses. Although these studies led to great progress in our understanding of human Th17, especially in the context of inflammatory diseases, biology of T cells primed and/or reactivated with such polyclonal activators may not always represent the biology of T cells primed and/or reactivated with MHC class II (MHC II)–peptide complexes presented by APCs. Therefore, it is valuable to study the induction and activation of Ag-specific human Th17 in the context of TCR ligation by the complexes of MHC II and Ag-derived peptides presented by APCs.

Dendritic cells (DCs) are major APCs that can induce and shape the types of T cell response during microbial infections. DCs express pattern recognition receptors (PRRs), including TLRs and C-type lectin receptors, which are linked to antimicrobial immunity through the sensing of pathogen-associated molecular patterns (28, 29). Of these PRRs, Dectin-1 is particularly relevant to the Th17-mediated immunity in both mice and humans (3, 7, 30, 31). We and others (32–34) have shown that DCs can take up protein Ags via Dectin-1 and present antigenic peptides to both CD4+ and CD8+ T cells. Thus, we established an in vitro system in which the
HA1 subunit from hemagglutinin (HA) of influenza virus (H1N1, PR8), as a model Ag, could be delivered to DCs via human Dectin-1 (hDectin-1) using recombinant proteins of an agonistic anti–hDectin-1 fused to HA1. This system allowed us to dissect the complex and dynamic processes of the generation of HAI-specific human Th17 in the context of TCR ligation with MHC II–peptide complexes presented by DCs. In addition, we demonstrated that Ag targeting to DCs via hDectin-1 along with TLR2 ligands could promote Ag-specific Th17 responses in human.

Materials and Methods

Cells and culture medium

Blood from healthy volunteers was acquired under a protocol approved by the Institutional Review Board of Baylor Research Institute. PBMCs of healthy volunteers were isolated by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). IFN-α–conditioned DCs (IFNDCs) were generated by cultivating monocytes from healthy donors in serum-free media (Cellgenix) supplemented with GM-CSF (100 ng/ml) and IFN-α (500 U/ml). The medium was replenished with cytokines on day 1. IFN-α and GM-CSF were from the pharmacy at the Baylor University Medical Center (Dallas, TX). Enrichment Kit (StemCell Technologies) (purity >99.0%). Culture medium consisted of RPMI 1640 (Life Technologies) supplemented with HEPS buffer, 2 mM l-glutamine, 1% nonessential amino acids, sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% normal human serum AB (Gencell).

Abs and reagents

Anti–CD4-allylphocyanin Cy7, anti–IFN-γ–PE Cy7, anti–CCR5–Pacific Blue, and anti–CCR6–Alexa Fluor 488 were purchased from Biodiagnost. Anti–IL-1R1–PE, anti–IL-6R–PerCP, anti–CCR9–PE, anti–CXCR3–FITC, anti–IL-25–p19, and control IgG were from R&D Systems. Neutralizing anti–IL-1 (α, β) Abs and reagents were also quantified through real-time RT-PCR using the LightCycler 480 (Roche Applied Science) with SYBR Green Master Mix (Roche). Expression levels of individual molecules were normalized to the amount of ACTB mRNA.

Statistical analysis

Statistical significance was determined using the Student t test, and significance was set at p < 0.05.

Results

Generation and characterization of recombinant protein of HAI fused to anti–hDectin-1 mAb

To deliver HAI to DCs via hDectin-1, a recombinant fusion protein (anti–hDectin-1–HA1) of an agonistic anti–hDectin-1 (34) and HA1 was made on a mouse V-region human IgG4s chimera with two site mutations (S228P and L235E) that further diminishes Ab binding to the FcR (36). IgG4-HA1 with the same mutations was made as a control. The putative structure of the recombinant fusion protein is presented in Supplemental Fig. 1. Recombinant proteins were expressed from stable Chinese hamster ovary cell lines, purified by protein A chromatography, and confirmed by the reduced SDS-PAGE gel analysis (Fig. 1A). Anti–hDectin-1–HA1, but not IgG4-HA1, specifically bound to 293F cells transfected with hDectin-1 (Fig. 1B). Neither anti-hDectin-1–HA1 nor IgG4-HA1 bound to DC activation

A total of 1 × 10^6 DCs was incubated with 1 μg/ml anti–hDectin-1–HA1 or 1 μg/ml IgG4-HA1 in the presence or absence of 40 ng/ml Pami(3) Cy5SK4(4) for 24 h. Cytofines in the culture supernatants were assessed by the BeadLyte cytokine assay kit (Upstate) as per the manufacturer’s protocol. IL-23 was measured using a human IL-23 ELISA kit (Biosciences).

DC–CD4+ T cell cocultures and Ag-specific CD4+ T cell responses

A total of 1 to 2 × 10^6 CFSE-labeled purified autologous CD4+ T cells were cocultured with 5 × 10^5 DCs loaded with indicated Abs, in the absence or presence of different TLR ligands. After 7 d, CD4+ T cell proliferation was measured by CFSE-dilution. In some experiments, anti–IL-23p19, anti–IL-6, anti–IL-6R, anti–IL-1B Abs, or control IgG (10 μg/ml) was added into the cocultures of DCs and CD4+ T cells. In some experiments, CD4+ T cells were incubated overnight with 50 U/ml IL-2 (Hoffmann-LaRoche), 50 ng/ml IL-7 (R&D Systems), 50 ng/ml IL-15 (PeproTech), and 50 ng/ml IL-21 (Invitrogen), and then naive CD4+ T cells were FACS sorted to obtain IL-1R1+ and IL-1R1- cells. For assessing Ag-specific CD4+ T cell responses, T cells were restimulated with 0.5–1 μM indicated peptides for 6 h in the presence of brefeldin A and stained with 7-aminomycin D, anti–CD4, anti–IFN-γ, and anti–IL-17 Abs labeled with fluorescent dyes. CD4+ T cells expressing intracellular IFN-γ and IL-17 were detected by flow cytometry (FACScanto; BD Biosciences). In separate experiments, CD4+ T cells were restimulated with indicated peptides for 48 h, and cytokines in the supernatants were assessed by the BeadLyte cytokine assay kit (Upstate) as per the manufacturer’s protocol.

Conventional and quantitative real-time RT-PCRs

Total RNA was isolated from CD4+ T cells using Ambion’s RNaseqeous kits (Life Technologies), and cDNA was synthesized with the Revert Aid H minus reverse transcriptase (Thermo). Conventional RT-PCR was performed using the primers TBSX21, RORC, GATA3, IL-1R1, IL-1B, and ACTB (β-actin) using the primers TBSX21 (forward, 5'-GAGGGGCGGCTTCCGCCAG-3'), reverse, 5'-TCCGCGGGCGTAGCTGAGA-3'); GATA3 (forward, 5'-AACTTGGGCACTCTCGAC-3'; reverse, 5'-TTGCAAGACGTTCCGATT-3'); RORC (forward, 5'-TCTGGAGCTGCTTTCATCA-3'; reverse, 5'-CCACGAGCCAGGGGCTA-3'); IL1B (forward, 5'-GCAAAGGTCCTCAG-CAGCGG-3'; reverse, 5'-GCTTGAGTCCGAGGATGC-3'); and ACTB (forward, 5'-CTGCGCTTTGCGAGTCGGCC-3'; reverse, 5'-GCTTGCGCTTCTCGGCACCAT-3'). Cq data were analyzed using the LightCycler 480 software (Roche Applied Science) with SYBR Green Master Mix. Expression levels of individual molecules were normalized to the amount of ACTB mRNA.
mock transfectants (data not shown). Anti–hDectin-1–HA1 (top panel, Fig. 1C) but not IgG4-HA1 (bottom panel, Fig. 1C) also bound to the surface of monocyte-derived IFNDCs in a dose-dependent manner. Taken together, HA1 could be efficiently delivered to DCs via hDectin-1 using anti–hDectin-1–HA1 fusion proteins.

**DCs loaded with anti–hDectin-1–HA1 can elicit HA1-specific Th17 responses**

Consistent with their binding to DCs (Fig. 1C), anti–hDectin-1–HA1–loaded IFNDCs induced greater proliferation of CD4+ T cells than did IgG4-HA1–loaded IFNDCs (Fig. 1D, top panel). Anti–hDectin-1 mAb alone or a combination of anti–hDectin-1 and IgG4-HA1 induced slightly enhanced CD4+ T cell proliferation compared with DCs alone (Fig. 1D, bottom panel). To test Ag specificity of the proliferating CD4+ T cells, T cells were re-stimulated with clusters of HA1 peptides (six peptides in each cluster; 17-mers overlapping by 11 aa) (Fig. 1E, top panel). Cluster 8 showed the greatest percentage of IFN-γ+CD4+ T cells. Individual peptides in cluster 8 were further tested in separate experiments (Fig. 1E, middle panel), and pep 43 and pep 45 resulted in substantially increased percentages of IFN-γ+CD4+ T cells. Pep 5 from cluster 1 and no peptide were negative controls. Experiments performed with blood mDCs showed similar results (Fig. 1E, bottom panel), although IFNDCs were more efficient than mDCs at eliciting CD4+ T cell responses. IFNDCs also resulted in a greater bystander proliferation of CD4+ T cells than...
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(Table continues)
did mDCs. We (35) and others (37) previously showed that DCs induced bystander T cell proliferation, as shown in Fig. 1D, and this bystander proliferation is further enhanced when the Ag-specific T cells are activated (35, 37).

Total CD4+ T cells cocultured with anti–hDectin-1–HA1–loaded IFNDCs (Fig. 1F, top panels) or mDCs (Fig. 1F, bottom panel) were restimulated with pep 43, pep 45, control pep 5, or no peptide for 48 h, and the amounts of IFN-γ, IL-13, and IL-17 in the supernatants were assessed. They secreted increased amount of IFN-γ and IL-13 in response to pep 43 and pep 45. In addition, they also secreted an increased amount of IL-17 in response to both pep 43 and pep 45 compared with control pep 5 or no peptide. Although the level of IL-17 was lower than those of IFN-γ and IL-13, our data suggested the presence of HAI-specific Th17 in the cultures. In separate experiments using cells from the same donor (donor 1), we were also able to detect HAI-specific IL-17–producing CD4+ T cells (Fig. 1G, left panel), although the frequency of IL-17+CD4+ T cells was far less than that of IFN-γ+CD4+ T cells (Fig. 1G, right panel) that are specific for HAI.

IL-23 secreted from IFNDCs (Fig. 1H) and mDCs (Fig. 1J) contributed to HAI-specific Th17 and Th1 responses (Fig. 1I, 1K), as blocking IL-23p19 during the cocultures of DCs and CD4+ T cells resulted in decreased IL-17– and slightly decreased IFN-γ–producing CD4+ T cell responses. DCs loaded with anti–hDectin-1 alone did not result in HAI-specific CD4+ T cell responses (data not shown).

Taken together, we concluded that anti–hDectin-1–HA1–loaded DCs can efficiently activate HAI-specific IFN-γ, IL-13, and IL-17–producing CD4+ T cells. HAI-derived peptides used throughout this study were characterized by performing the experiments in Fig. 1E, and their corresponding HLA class II types are summarized in Table I.

Anti–hDectin-1–HA1–loaded DCs expand memory Th17 but cannot efficiently induce HAI-specific Th17

To test whether anti–hDectin-1–HA1–loaded DCs could induce HAI-specific Th17, FACS-sorted naive and memory CD4+ T cells were cocultured with 1 μg/ml anti–hDectin-1–HA1–loaded IFNDCs for 7 d. CD4+ T cells were then restimulated with indicated peptides for 48 h, and the amounts of IFN-γ, IL-13, and IL-17 in the supernatants were assessed. Compared to unstimulated or control pep 5–stimulated CD4+ T cells, both naive (Fig. 2A, top left and middle panels) and memory CD4+ T cells (Fig. 2A, bottom left and middle panels) stimulated with pep 22 secreted significantly increased amounts of both IFN-γ and IL-13. Interestingly, however, an increased amount of IL-17 by pep 22 was observed only in the culture supernatant of memory, but not naive, CD4+ T cells. Data from experiments using cells from five healthy donors further demonstrated that IFNDCs loaded with anti–hDectin-1–HA1 could efficiently activate HAI-specific memory Th17 cells, but they could not efficiently prime HAI-specific Th17 responses (Fig. 2B). Similar observations were made from experiments using blood mDCs (Fig. 2C, 2D). Naive and memory CD4+ T cells were cocultured with 1 μg/ml anti–hDectin-1–HA1–loaded mDCs for 7 d. CD4+ T cells were then restimulated for 48 h with indicated peptides. Pep 45–stimulated naive T cells secreted increased amounts of both IFN-γ (Fig. 2C, top left panel) and IL-13 (Fig. 2C, top middle panel), but not IL-17 (Fig. 2C, top right panel), compared with naive T cells stimulated with control pep 5 or no peptide. Pep 45–stimulated memory T cells secreted increased amounts of all three cytokines, including IL-17, compared with memory T cells stimulated with control pep 5 or no peptide (Fig. 2C, bottom panel). Data from experiments using cells from three healthy donors (Fig. 2D) further showed that anti–hDectin-1–HA1–loaded mDCs efficiently activate HAI-specific memory Th17, but they are not efficient to induce naive CD4+ T cell differentiation into HAI-specific Th17.

Thus, we concluded that DCs loaded with anti–hDectin-1–HA1 can efficiently activate HAI-specific memory Th17, but cannot efficiently prime HAI-specific Th17 responses in vitro.

Phenotypes of HAI-specific Th17 are different from those of other Th17

HAI peptide-specific IFN-γ+CD4+ and IL-17+CD4+ T cells were further characterized by assessing the expression levels of chemokine receptors, β7 integrin, and CD161 (Fig. 3A). Compared to IFN-γ+CD4+ T cells (Fig. 3A, left panel) expressing CXCR3, IL-17+CD4+ T cells (Fig. 3A, right panel) expressed increased levels of CCR6 and CD161 (38, 39). Interestingly, fractions of the HAI-specific IL-17+CD4+ T cells also expressed a high level of CCR9, which could support the presence of Th17 in the gut mucosa (40). Fractions of both IFN-γ+CD4+ and IL-17+CD4+ T cells expressed β7 integrin. CCR5 was similarly expressed on both IFN-γ+CD4+ and IL-17+CD4+ T cells.

We also assessed the expression levels of these receptors on IFN-γ+CD4+ and IL-17+CD4+ T cells restimulated with PMA and ionomycin (Fig. 3B). Similar to the HAI-specific IFN-γ+CD4+ T cells (Fig. 3A, left panel), PMA/ionomycin-induced IFN-γ+ CD4+ T cells (Fig. 3B, left panel) expressed CCR6, CXCR3, β7 integrin, and CD161. Both pep 43– (Fig. 3A, right panel) and PMA/ionomycin-induced IL-17+CD4+ T cells (Fig. 3B, right panel) expressed similar levels of these receptors. However, PMA/ionomycin-induced IL-17+CD4+ T cells did not express CCR9 that was expressed on pep 43–induced IL-17+CD4+ T cells. Pep 43–induced IFN-γ+CD4+ and IL-17+CD4+ T cells also expressed increased levels of CCR5 compared with those induced with PMA/ionomycin.

In conclusion, these data demonstrated that phenotypes of HAI-specific human Th17 cells are not the same as those of other Th17 cells activated with polyclonal activators.

IL-1β and IL-6 are insufficient to induce naive CD4+ T cell differentiation into HAI-specific Th17

Both IL-1β with IL-6 have been known to contribute to the induction of human Th17 differentiation (9, 27). We have shown that anti–hDectin-1 mAb and TLR2 ligand synergistically act on DCs to secrete IL-6 and IL-1β (34). Synergistic actions of signals via Dectin-1 and TLR2 can also increase IL-23, but decrease IL-12 secretion from DCs (41), thereby favoring Th17 responses (9). Thus, we first tested whether DCs loaded with anti–hDectin-1-

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**Table I.** (Continued)

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Peptides were predicted by an algorithm found at: http://tools.immuneepitope.org/analyze/cgi-bin/mhc_II_binding.py.

ARB, average relative binding; NA, alleles not available in the algorithm.
HA1 plus TLR2 ligand could enhance HA1-specific Th17 responses (Fig. 4A). Total CD4+ T cells were cocultured for 7 d with IFNDCs loaded with anti–hDectin-1–HA1 in the presence or absence of different concentrations of Pam(3)CysSK(4). CD4+ T cells were then restimulated for 48 h with the indicated peptides, and cytokines in the supernatants were assessed. HA1-specific Th17 (Fig. 4A, right panel) and Th1 responses (Fig. 4A, left panel) were enhanced by Pam(3)CysSK(4). Of the concentrations tested, 40 ng/ml Pam(3)CysSK(4) resulted in the highest levels of IL-17 production. Accordingly, DCs loaded with 1 μg/ml anti–hDectin-1–HA1 plus 40 ng/ml Pam(3)CysSK(4) secreted increased amounts of both IL-1b (Fig. 4B, left panel) and IL-6 (Fig. 4B, right panel).

We next tested whether DCs loaded with anti–hDectin-1–HA1 plus 40 ng/ml Pam(3)CysSK(4) could induce HA1-specific Th17 responses (Fig. 4A). Total CD4+ T cells were cocultured for 7 d with IFNDCs or blood mDCs loaded with anti–Dectin-1–HA1 in the absence or presence of 40 ng/ml Pam(3)CysSK(4). Both IFNDCs (Fig. 4C) and mDCs (Fig. 4D) were able to activate HA1-specific memory Th17, and these Th17 responses were further enhanced by Pam(3)CysSK(4). However, neither IFNDCs nor blood mDCs induced HA1-specific Th17 even in the presence of Pam(3) CysSK(4). Thus, the HA1-specific Th17 responses observed in Fig. 4A were due to the activation of HA1-specific memory CD4+ T cells. TLR2 is known to be expressed in human T cells (42), but Pam(3)CysSK(4) alone did not induce CD4+ T cells to secrete significant amount of cytokines tested, although it slightly increased the amount of TNF-α secreted by anti-CD3/CD28–activated CD4+ T cells (data not shown). DCs washed after Pam(3)CysSK(4) treatment were still able to enhance anti–hDectin-1–HA1–loaded DC-mediated HA1-specific Th17 responses (data not shown). Therefore, the enhanced HA1-specific memory Th17 responses by

**FIGURE 2.** DCs loaded with anti–hDectin-1–HA1 activate memory Th17, but cannot efficiently induce HA1-specific Th17. FACS-sorted naive and memory CD4+ T cells were cocultured for 7 d with 1 μg/ml anti–hDectin-1–HA1–load IFNDCs. T cells were restimulated with 1 μM pep 43 (A) or PMA/ionomycin (B) for 6 h. Cells were stained for intracellular IFN-γ and IL-17 as well as surface receptors indicated. Four independent experiments using cells from two different healthy donors showed similar results.

**FIGURE 3.** Phenotypes of HA1-specific and PMA/ionomycin-activated Th17. Total CD4+ T cells were cocultured for 7 d with 1 μg/ml anti–hDectin-1–HA1–loaded IFNDCs. T cells were restimulated with 1 μM pep 43 (A) or PMA/ionomycin (B) for 6 h. Cells were stained for intracellular IFN-γ and IL-17 as well as surface receptors indicated. Four independent experiments using cells from two different healthy donors showed similar results.
Taken together, we concluded that DCs loaded with anti–hDectin-1–HA1 and TLR2 ligand could greatly promote HA1-specific memory Th17 responses. However, they were not efficient to induce naive CD4+ T cell differentiation into HA1-specific Th17 even in the presence of IL-1β and IL-6. These results suggested that naive CD4+ T cells require additional signals to differentiate into HA1-specific Th17.

Naive CD4+ T cells express IL-6R, but not IL-1R1, that is inducible by synergistic actions of signals via common γ-chain receptors and TCRs

Despite IL-1β and IL-6 secretion by DCs after stimulation with anti–hDectin-1–HA1 and TLR2 ligand, there was no significant enhancement of the induction of HA1-specific Th17 (Fig. 4C, 4D). We therefore investigated the expression levels of cytokine receptors on the surface of naive and memory CD4+ T cells in peripheral blood of healthy donors. Both naive and memory CD4+ T cells expressed similar levels of surface IL-6R (Fig. 5A, left panel). However, surface IL-1R1 was detected only on memory CD4+ T cells (Fig. 5A, right panel). The presence of IL-6 in the culture medium (Fig. 4B) and the constitutive expression of IL-6R on naive CD4+ T cell (Fig. 5A, top left panel), but no significant level of HA1-specific Th17 response (Fig. 4C, 4D), suggested that IL-6 is not the key factor that turns on the program for the differentiation of naive CD4+ T cells into HA1-derived peptide-specific Th17. Thus, we hypothesized that the induction of surface IL-1R1 expression on naive CD4+ T cells could be a prerequisite for the enhanced de novo induction of Ag-specific Th17.

Induction of IL-1R1 expression on the surface of naive CD4+ T cells is known to be very modest and takes 5 to 6 d after TCR stimulation in the presence of IL-2 (43). Naive CD4+ T cells from cord blood started to express IL-1R1 on 6 d of culture in the presence of TGF-β, IL-7, and IL-15 (44). Therefore, we first sought cytokine signaling that could rapidly induce IL-1R1 expression on the surface of naive CD4+ T cells. Fig. 5B showed that naive CD4+ T cells from the peripheral blood of healthy donors expressed IL-1R1, although it was not detected on the cell surface (Fig. 5A). IL-1R1 expression was upregulated within 18 h by treating naive CD4+ T cells with the combination of IL-2, IL-7, IL-15, and IL-21 (Fig. 5B). In contrast to previously published data (44), naive CD4+ T cells treated with the combination of common γ-chain cytokines also expressed surface IL-1R1 within 36 h (Fig. 5C), and this was consistent with the mRNA expression levels (Fig. 5B). The individual cytokines showed variable effects on IL-1R1 expression (Fig. 5B), but they were not able to induce surface IL-1R1 expression on naive CD4+ T cells (data not shown).

This was further confirmed by the data showing that Jak3 and Pan-Jak inhibitors, but not Jak2 inhibitor, suppressed common γ-chain cytokine-induced IL-1R1 upregulation (Fig. 5D). Accordingly, STAT3 inhibitor resulted in decreased expression of IL-1R1 (Fig. 5E). These data indicated that IL-2, IL-7, IL-15, and IL-21 act synergistically to induce surface IL-1R1 expression on naive CD4+ T cells.

TCR ligation by peptide–MHC complexes is an inevitable process for the induction of antigenic peptide-specific T cell responses. Thus, we tested whether TCR signaling contributed to the IL-1R1 expression in naive CD4+ T cells (Fig. 5F, 5G). FACs-sorted naive CD4+ T cells were cocultured for 18 h with IFNDCs alone, anti–hDectin-1–HA1–loaded IFNDCs, and HA1 peptide-loaded IFNDCs in the presence or absence of the combination of common γ-chain cytokines (Fig. 5F). DCs loaded with either anti–hDectin-1–HA1 or HA1 peptide slightly, but significantly (p = 0.0107 and p = 0.0239 for anti–Dectin-HA1 and pep 43–loaded IFNDCs, respectively), increased the expression of...
**FIGURE 5.** Naive CD4+ T cells do not express IL-1R1 that is inducible by synergistic actions of signals from TCRs and common \( \gamma \)-chain receptors. (A) IL-1R1 and IL-6R expression on naive and memory CD4+ T cells in the peripheral blood. CD4+ T cells from six healthy donors showed similar results. (B) Real-time (bottom panel) and conventional (top panel) RT-PCR analysis of IL-1R1 expression in naive CD4+ T cells treated for 18 h with indicated cytokines. (C) Surface IL-1R1 expression on naive CD4+ T cells treated for 36 h with the combination of the cytokines in (B). (D) and (E) Real-time RT-PCR analysis of IL-1R1 expression in naive CD4+ T cells treated with the combination of the cytokines in (B) in the presence or absence of indicated inhibitors. Data are pooled from three independent experiments. The \( p \) values were acquired by Student \( t \) test. (F and G) Real-time (bottom panel) and conventional (top panel) RT-PCR analysis of IL-1R1 expression in naive CD4+ T cells cocultured overnight with IFNDCs alone, IFNDCs loaded with HA1 peptide (pep 43, 1 \( \mu \)M), or IFNDCs loaded with 1 \( \mu \)g/ml anti-hDectin-1–HA1 in the presence or absence of the combination of common \( \gamma \)-chain cytokines (F) or different amounts of anti-CD3/CD28-coated microbeads (G). (F) Error bars indicate mean ± SD of duplicate assay of two independent experiments. In (B), (C), and (G), error bars indicate mean ± SD of triplicate assay, and three independent experiments showed similar results. The \( p \) values were acquired by Student \( t \) test.

**IL-1R1** in naive CD4+ T cells. However, the combination of common \( \gamma \)-chain cytokines was more efficient than the Ag-loaded DCs at inducing IL-1R1 expression in naive CD4+ T cells. This was not surprising, because the frequency of HA1 Ag-specific CD4+ T cells in the naive pool is low. In contrast, the combination of common \( \gamma \)-chain cytokines acts on majority of the naive T cells in the cultures.

To further explore the roles of signaling via TCR in the induction of IL-1R1 expression, naive CD4+ T cells were cultured with different ratios of anti-CD3/CD28–coated beads. Fig. 5G shows that anti-CD3/CD28 microbeads increased IL-1R1 expression, and this increase was anti-CD3/CD28 dose dependent. Thus, naive CD4+ T cells treated with the beads at 1:2 ratio expressed higher levels of IL-1R1 than those treated with the beads at 1:8 ratio, demonstrating that IL-1R1 expression is positively correlated to the strength of TCR signaling. Furthermore, anti-CD3/CD28–induced upregulation of IL-1R1 expression was enhanced by the combination of common \( \gamma \)-chain cytokines, showing a synergistic effect of signals from common \( \gamma \)-chain receptors and TCRs on naive CD4+ T cells to upregulate IL-1R1 expression.

Taken together, naive CD4+ T cells express IL-6R, but not IL-1R1, on their surface. Nevertheless, IL-1R1 expression can be rapidly induced by the synergistic actions of signals via common \( \gamma \)-chain receptors and TCRs.

**IL-1R1**, but not **IL-1R1**, naive CD4+ T cells differentiate into HA1-specific Th17

To test whether anti–hDectin-1–HA1–loaded DCs were able to induce IL-1R1+ naive CD4+ T cell differentiation into HA1-specific Th17, IL-1R1–, and IL-1R1+, naive CD4+ T cells were cocultured with anti–hDectin-1–HA1–loaded IFNDCs. After 7 d, both IL-1R1– and IL-1R1+ naive CD4+ T cells secreted IFN-\( \gamma \) and IL-13 in response to pep 43 during 48-h restimulation (Fig. 6A, left and middle panels). HA1-specific Th17 responses were observed only in the cultures of IL-1R1+ naive CD4+ T cells (Fig. 6A, right panel). We were not able to detect HA1-specific IL-17+CD4+ T cells by FACS. However, we observed that a fraction of IL-1R1+ naive CD4+ T cells cocultured with DCs expressed intracellular IL-17 in response to PMA/ionomycin (Fig. 6A). Blocking IL-1\( \beta \) during the cocultures of IL-1R1+ naive CD4+ T cells and DCs substantially impaired HA1-specific Th17 responses (Fig. 6B, right panel). Thus, the surface IL-1R1 induction on naive CD4+ T cells by signals via both TCR and common \( \gamma \)-chain receptors is a crucial for the enhanced induction of HA1-specific human Th17.

Blocking IL-6 also greatly decreased the induction of HA1-specific Th17 from IL-1R1+ naive CD4+ T cells (Fig. 6B, right panel). Thus, STAT3 inhibitors, which block IL-6–mediated signals, also decreased RORC expression in the IL-1R1+ naive CD4+ T cells on day 5 of the cocultures with anti–hDectin-1–HA1–loaded DCs (Fig. 6C). Fig. 6C also demonstrated that IL-1R1–induced RORC expression is dependent on MyD88, but not IRAK1 or IRAK4.

Taken together, we concluded that both IL-1\( \beta \) and IL-6 contribute to the induction of HA1-specific Th17. However, surface IL-1R1 expression on naive CD4+ T cells is essential for the enhanced induction of HA1-specific Th17. Surface IL-1R1+ naive CD4+ T cells did not efficiently differentiate into HA1-specific Th17 (Figs. 2, 6A).
IL-6 contributes to the induction of HA1-specific Th17 through IL-1β induction in TCR-activated T cells

IL-6 has been known to contribute to Th17 induction in both mice (19) and humans (9). However, IL-6 can contribute to the induction of HA1-specific Th17 only when naive CD4+ T cells express IL-1R1 (Fig. 6B), although IL-6R is expressed on the surface of naive CD4+ T cells (Fig. 5A, top left panel). To further investigate the roles of IL-6 in the induction of Th17, both IL-1R1− and IL-1R1+ naive CD4+ T cells were incubated with anti-CD3/CD28-coated microbeads at a 1:40 ratio in the absence or presence of IL-1β, IL-6, or both IL-1β and IL-6 (Fig. 6D). Anti-CD3/CD28-coated microbeads at 1:40 did not upregulate IL-1R1 expression in naive CD4+ T cells (data not shown). After 5 d, the expression levels of RORC, TBX21, and GATA3 in the two groups of naive CD4+ T cells were assessed. Consistent with the data in Fig. 6A, IL-1R1− naive CD4+ T cells expressed both TBX21 and GATA3, but not RORC, in any tested condition. The expression levels of TBX21 and GATA3 in IL-1R1− CD4+ T cells treated with either IL-6 alone or in combination with IL-1β were variable in different experiments (data not shown). However, IL-1R1+ naive CD4+ T cells expressed RORC in the presence of either IL-1β or IL-6 alone. IL-1β plus IL-6 induced the greatest level of RORC expression. In line with the data in Fig. 6B (right panel) and 6C, IL-6 alone could also induce RORC expression in IL-1R1+ naive CD4+ T cells (Fig. 6D, right panel). Indeed, IL-6 was more efficient than IL-1β at inducing RORC expression in IL-1R1+ naive CD4+ T cells. Therefore, we hypothesized that IL-6 could induce these naive CD4+ T cells to express IL-1β followed by the induction of RORC expression and thus could amplify IL-1β–induced RORC expression. As shown in Fig. 6E, exogenous IL-6 was able to induce IL-1R1− and particularly IL-1R1+ naive CD4+ T cells to upregulate IL-1β expression. Furthermore, blocking IL-1β led to decreased IL-6–mediated RORC expression in IL-1R1+ naive CD4+ T cells (Fig. 6F).

Taken together, IL-1R1− naive CD4+ T cells were able to differentiate into HA1-derived peptide-specific Th17 when they were
activated via both TCRs and IL-1R1. IL-6 could argument Th17 responses by inducing IL-1β and thus enhance RORC expression in the TCR-activated IL-1R1+ naive CD4+ T cells.

Targeting Ags to DCs via Dectin-1 can elicit Ag-specific human Th17 responses that can be further promoted by TLR2 and TLR4 ligands

Ag targeting to DCs via Dectin-1 using recombinant fusion protein of an agonistic anti–hDectin-1 mAb and Ag could elicit Ag-specific Th17 responses. IL-23 secreted from anti–hDectin-1–HA1–loaded DCs contributed to HA1-specific Th17 responses (Fig. 1H, 1I). In addition, anti–hDectin-1–HA1 and TLR2 ligands synergized to activate DCs to secrete IL-1β and IL-6, resulting in the enhanced HA1-specific Th17 responses (Fig. 4A, 4B).

We further tested the effects of other TLR ligands on the magnitude of HA1-specific Th17 responses elicited by DCs loaded with anti–hDectin-1–HA1 (Fig. 7A). Compared to other TLR ligands tested, Pam(3)CysSK(4) was the most efficient at enhancing the HA1-specific Th17 responses, as it synergizes with anti–hDectin-1–HA1 to induce increased amounts of both IL-1β and IL-6 secretion from DCs (Fig. 4B). The enhanced HA1-specific Th17 responses by the combination of anti–hDectin-1–HA1 and TLR2 ligands were further confirmed (Fig. 7B, 7C).

Total CD4+ T cells from eight healthy donors were cocultured for 7 d with DCs loaded with 1 µg/ml anti–hDectin-1–HA1 in the absence or presence of TLR2 ligands, Pam(3)CysSK(4) (Fig. 7B), and Porphyromonas gingivalis LPS (Fig. 7C). Consistent with the data in Figs. 4B and 7A, both Pam(3)CysSK(4) and P. gingivalis LPS promoted HA1-specific Th17 responses elicited by anti–hDectin-1–HA1–loaded DCs. Escherichia coli LPS was also able to enhance HA1-specific Th17 responses elicited by anti–hDectin-1–HA1–loaded DCs. Escherichia coli LPS was also able to enhance HA1-specific memory Th17, although the magnitudes of Th17 responses in the healthy individuals were variable. In addition, a vaccine model made of an agonistic anti–hDectin-1 and Ags along with TLR2 or TLR4 ligands could offer great potential in promoting Th17-mediated host immunity against infections.

FIGURE 7. Healthy individuals maintain influenza HA1-specific Th17 that can be promoted by the combination of anti–hDectin-1–HA1 and TLR2 ligands. (A) Total CD4+ T cells were cocultured for 7 d with IFNDCs loaded with 1 µg/ml anti–hDectin-1–HA1 in the presence or absence of indicated TLR ligands [40 ng/ml Pam(3)CysSK(4), 1 µg/ml poly IC, 50 ng/ml flagellin, and 500 ng/ml R848]. T cells were then restimulated for 48 h with 1 µM of indicated peptides. Cytokines in the culture supernatants were assessed. Two independent experiments showed similar results. (B and C) Total CD4+ T cells from healthy donors were cocultured for 7 d with 1 µg/ml anti–hDectin-1–HA1–loaded IFNDCs in the presence or absence of 40 ng/ml Pam(3)CysSK(4) (B) or P. gingivalis LPS (C). T cells were then restimulated with 1 µM indicated peptides. Cytokines in the culture supernatants were assessed. (D) Total CD4+ T cells were cocultured for 7 d with IFNDCs (top panels) or mDCs (bottom panels) loaded with 1 µg/ml anti–hDectin-1–HA1 in the presence or absence of P. gingivalis LPS (40 ng/ml) or E. coli LPS (100 ng/ml). T cells were then restimulated for 48 h with 1 µM of indicated peptides. Cytokines in the culture supernatants were assessed. Two independent experiments showed similar results. In (A)–(D), error bars indicate mean ± SD of triplicate assay.

Poly IC, polyinosinic-polycytidylic acid.
Data in Fig. 8 further support our notion that targeting Ags to DCs via dectin-1 using recombinant fusion protein of an agonistic anti–hDectin-1 mAb and Ag is an efficient strategy to elicit Ag-specific Th17 responses. Anti–hDEC205-HA1 fusion protein was made (Fig. 8A). Anti–hDEC205-HA1 (Fig. 8B, bottom panel) but not IgG4-HA1 (Fig. 8B, top panel) bound to the surface of monocyte-derived IFNDCs in a dose-dependent manner. IFNDCs loaded with anti–hDEC205-HA1 resulted in HA1-specific Th1, Th2, and Th17 responses (Fig. 8C). More importantly, HA1-specific Th17 responses elicited with anti–DEC205-HA1 were further enhanced by anti–Dectin-1 mAb (Fig. 8C, left panel). Anti–Dectin-1 mAb did not increase HA1-specific IFN-γ/CD4+ T cell responses (Fig. 8C, middle panel). We next tested whether anti–hDEC205-HA1 and Pam(3)CysSK(4) synergized to promote HA1-specific CD4+ T cell responses (Fig. 8D). Only the higher concentration (200 ng/ml) of Pam(3)CysSK(4) could enhance HA1-specific Th1, Th2, and Th17 responses. These data showed that TLR2 signaling does not synergize with DEC205 to promote HA1-specific Th17 responses.

**FIGURE 8.** HA1-specific Th17 responses elicited by anti–hDEC205-HA1 can be enhanced by the activation of DCs via Dectin-1 but not TLR2. (A) Reduced SDS-PAGE analysis of anti–hDEC205 mAb (lane 1) and anti–hDEC205-HA1 (lane 2). (B) Binding of anti–hDEC205-HA1 and IgG4-HA1 to IFNDCs. (C) Total CD4+ T cells were cocultured for 7 d with IFNDCs loaded with 1 μg/ml anti–hDEC205-HA1 in the presence 5 μg/ml control IgG or anti–Dectin-1 mAb. T cells were then restimulated for 48 h with 1 μM of pep 43 or control pep 5. Cytokines in the culture supernatants were assessed. Values acquired with control pep 5 were subtracted. Two independent experiments showed similar results. (D) Effects of different concentrations of Pam(3)CysSK(4) on HA1-specific CD4+ T cell responses were assessed. Ctrl, control.

**Discussion**

This study dissected the pathways for the de novo generation of protein Ag-derived peptide-specific human Th17 in the context of TCR ligation by MHC II–peptide complexes presented by DCs. It appears that the induction of protein Ag-specific Th17 requires more than the actions of previously known Th17-promoting cytokines, but rather occurs through the complementary actions of signals delivered from both innate and adaptive immune cells. Notably, synergistic actions of signals via both TCR and common γ-chain receptors play pivotal roles in programming naïve CD4+ T cells to respond to IL-1β followed by the induction of RORC expression. It was also important to note that phenotypes of Th17 activated with HA1 peptides and those activated with polyclonal activators are not the same. Finally, this study demonstrates that targeting Ags to DCs via Dectin-1 using an agonistic anti–hDectin-1 mAb, particularly with TLR2 ligands, can efficiently promote Ag-specific Th17 in which Ag-specific memory T cells have already been established.

It has been known that the immune system can favor Th17 responses to certain microbial pathogens, including fungi and bacteria, and this relies mainly on recognition of such pathogens via PRRs, particularly Dectin-1, followed by the induction of Th17-promoting cytokines from APCs (3, 45). However, Th17 are now considered as important immune arms against both extracellular and intracellular pathogens (2–11, 13, 30) as well as cancers (14–17). The assumption that human Th17 are mainly effector T cells with a short lifespan, as they are often found in peripheral tissues and organs (46), had raised a question as to the value of such vaccine-induced Th17-mediated immunity. However, recent studies have shown that human Th17 consists of long lived-effector memory cells (47, 48) and thus can contribute to long-lasting immunity. We also found that healthy individuals maintained influenza HA1-specific memory Th17, although the levels of Th17 among donors varied. Such memory Th17 could contribute to the protective immunity against influenza infections, presumably by enhancing CD8+ T cell and Ab responses (6, 13, 16, 49). More importantly, those Th17 responses can be greatly enhanced by the vaccine model, recombinant fusion protein of anti–hDectin-1 and Ags along with TLR2 and TLR4 ligands.

The mechanisms of human Th17 differentiation remained obscure, but previous studies have revealed several key cytokines that promote Th17 differentiation mainly in the context of inflammatory diseases. Data from this study are in agreement with the previous data, showing that IL-1β/IL-1R and IL-6 play key roles in human (9) and mouse Th17 differentiation (19, 20). The major role of IL-23, expanding memory Th17 (1, 50), is recapitulated by our data. We could detect active forms of TGF-β in the cocultures of DCs and T cells (data not shown). This suggests that TGF-β may not be a key cytokine that determines the generation of Ag-specific human Th17, whereas the induction of IL-1R1 expression and signals by IL-1β play key roles. It is important to note that experimental systems used in many of the previous studies were designed to test Th17 differentiation mainly in the context of inflammatory diseases. Thus, T cells were activated with polyclonal stimuli in polarized conditions made with exogenous cytokines or neutralizing Abs specific for targeted cytokines.

We have shown that the induction of Ag-specific Th17 requires more than the actions of currently known Th17-promoting cytokines. Foremost, the induction of surface IL-1R1 expression was the key step for naïve CD4+ T cell differentiation into pathogen-specific Th17. A rapid induction of surface IL-1R1 expression on naïve CD4+ T cells is directed by the synergistic actions of signals via TCRs and common γ-chain receptors.
In support of the important roles of common γ-chain cytokines, inhibitors of STAT3 abolished the upregulation of IL-1R1 expression. Although IL-2 and STAT5 showed suppressive functions in Th17 development (51), our data indicated that these could also contribute to the generation of Ag-specific Th17 by enhancing the expression of IL-1R1. Furthermore, IL-2 supports T cell proliferation. The contribution of STAT5 in the upregulation of IL-1R1 expression could not be measured because STAT5 inhibitor (55) suppressed the phosphorylation of STAT1,-3, and -5, as measured by phospho–flow cytometry (data not shown). IL-21 also uses the common γ-chain receptors and thus can contribute to the generation of pathogen-specific Th17 by promoting IL-1R1 expression. The roles of other common γ-chain cytokines, including IL-9 (23) as well as IL-7 and IL-15 (53), in Th17 responses have also been reported.

Although IL-1β/IL-1R1 was the key axis for the induction of RORC, IL-6–mediated STAT3 activation was required for efficient generation of pathogen-specific human Th17. This was further supported by the fact that mutations in STAT1 or STAT3 resulted in the deficiency of Th17-mediated immunity in patients with fungal infections (12, 54). Interestingly, however, IL-6 promotes the induction of Th17 only when naive CD4+ T cells express IL-1R1. Further experiments show that IL-6 contributes to the IL-1β-induced RORC expression by enhancing IL-1β expression in TCR-activated IL-1R1+ naive CD4+ T cells. The role of IL-1β in the induction of RORC expression through the action of MyD88 is supported by the previous studies, showing that MyD88 deficiency results in the lack of Th17-mediated immunity against chlamydial infection (55, 56). Moreover, defective IL-1R/MyD88 signaling is associated with impaired Th17 responses (57).

In conclusion, this study revealed the complex but complementary mechanisms for the induction of protein Ag-specific human Th17 in the context of TCR ligation by MHC–peptide complexes. Although there could be alternative pathways for the generation of Ag-specific human Th17, the pathway characterized with DCs, major immune inducers and modulators, could represent the major one. In addition, this study provides a rational strategy that can potentially enhance Th17-mediated host immunity against infections and certain types of cancers in humans.

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Disclosures

The authors have no financial conflicts of interest.

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