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Induction and activation of human Th_{17} by targeting antigens to dendritic cells via Dectin-1

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Abstract

Recent compelling evidence indicates that Th_{17} confer host immunity against a variety of microbes, including extracellular and intracellular pathogens. Therefore, understanding mechanisms for the induction and activation of antigen-specific Th_{17} is important for the rational design of vaccines against pathogens. To study this, we employed an *in vitro* system in which influenza HA1 was delivered to dendritic cells (DCs) via Dectin-1 using anti-hDectin-1-HA1 recombinant fusion proteins. We found that healthy individuals maintained broad ranges of HA1-specific memory Th_{17} that were efficiently activated by DCs targeted with anti-hDectin-1-HA1. Nonetheless, these DCs were not able to induce significant level of HA1-specific Th_{17} response even in the presence of Th_{17}-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 were essential for naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17}. This process was dependent on MyD88, but not IRAK1/4. Thus, interruptions in STAT3 or MyD88 signaling led to substantially diminished HA1-specific Th_{17} induction. Taken together, the de novo generation of pathogen-specific human Th_{17} requires complex but complementary actions of multiple signals. Data from this study will help us design new and effective vaccine strategy that can promote Th_{17}-mediated immunity against microbial pathogens.

Keywords

Dendritic cell; T cell; Dectin-1; Th_{17}; IL-17; Vaccine; Immunity

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[2]Abbreviations used in this paper: mAb, monoclonal Ab; DCs, dendritic cells; PBMCs, peripheral blood mononuclear cells; HA, hemagglutinin; MYD88, myeloid differentiation primary response gene (88); IRAK, Interleukin-1 receptor-associated kinase; STAT, Signal transducer and activator of transcription; TCR, T cell receptor; MHC, Major histocompatibility complex; LPS, lipopolysaccharide; TLR, Toll-like receptor; PRR, Pattern-recognition receptor.

Disclosures

The authors have no conflicting financial interests.

Introduction

IL-17-producing $CD4^+$ T cells (Th_{17}) have been broadly linked to inflammatory diseases (1). However, recent compelling evidence indicates that Th_{17} also play an important role against both extracellular and intracellular microbial pathogens, including bacteria, fungi, parasites and viruses (2-13). Furthermore, the immunity conferred by Th_{17} is associated with improved survival of cancer patients (14, 15). Accordingly, Th_{17}-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 antigen-specific Th_{17} in the context of T cell receptor (TCR) ligation by peptides and major histocompatibility complexes (MHCs).

The induction of Th_{17} has been mainly studied in the context of inflammatory cytokine milieu. In mice, TGFβ (18, 19), IL-6 (19), IL-1β (20), IL-21 (21), IL-23 (22), and IL-9 (23) contribute to Th_{17} induction. In humans, IL-1β with IL-6 was initially reported to induce Th_{17} differentiation, and this was inhibited by TGFβ and IL-12 (9). TGFβ was also reported to be required for Th_{17} development (24), but Yang *et al*. (25) demonstrated that human Th_{17} could be developed in the presence of TGFβ and IL-21, but not TGFβ and IL-6. In contrast, Volpe *et al.* (26) showed that pro-inflammatory cytokines were all required and acted synergistically to generate human Th_{17}. These series of findings suggest that each of these cytokines might contribute to Th_{17} 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 Th_{17} differentiation (27). Unlike in mice, however, our understanding of the induction and activation of antigen-specific Th_{17} in humans is still limited. This is mainly due to limitations of reliable experimental systems as well as difficulties in the assessment of antigen-specific T cell responses after *in vitro* priming of T cells particularly when the frequency of antigen-specific T cells is low. Thus, previous studies (9, 24-27) employed polyclonal T cell activators, such as anti-CD3/CD28 antibodies and phorbol 12-myristate 13-acetate (PMA), to prime and/or reactivate T cells to assess the magnitude and quality of T cell responses. Although these studies led to great progresses in our understanding of human Th_{17} especially in the context of inflammatory diseases, biology of T cells primed and/or re-activated with such polyclonal activators may not always represent the biology of T cells primed and/or re-activated with MHC II/peptide complexes presented by antigen presenting cells (APCs). Therefore, it is valuable to study the induction and activation of antigen-specific human Th_{17} in the context of T cell receptor (TCR) ligation by the complexes of MHC II and antigen-derived peptides presented by APCs.

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 toll-like receptors (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 Th_{17}-mediated immunity in both mice and humans (3, 7, 30, 31). We and others have shown that DCs can take up protein antigens via Dectin-1 and present antigenic peptides to both $CD4^+$ and $CD8^+$ T cells (32-34). Thus, we established an *in vitro* system in which HA1 subunit from hemagglutinin (HA) of influenza virus (H1N1, PR8), as a model antigen, could be delivered to DCs via hDectin-1 using recombinant proteins of an

agonistic anti-hDectin-1 fused to HA1. This system allowed us for the first time to dissect the complex and dynamic processes of the generation of HA1-specific human Th_{17} in the context of TCR ligation with MHC II/peptide complexes presented by DCs. In addition, we demonstrated that antigen targeting to DCs via hDectin-1 along with TLR2 ligands could promote antigen-specific Th_{17} responses in human.

Materials and methods

Cells and culture medium

Blood from healthy volunteers were acquired under a protocol approved by the Institutional Review Board (IRB) of Baylor Research Institute (BRI). Peripheral blood mononuclear cells (PBMCs) of healthy volunteers were isolated by density gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare, Sweden). IFNDCs were generated by culturing monocytes from healthy donors in serum free media (Cellgenix, Germany) supplemented with GM-CSF (100 ng/ml) and IFNα (500 units/ml). The medium was replenished with cytokines on day 1. IFNα and GM-CSF were from the Pharmacy at the Baylor University Medical Center (Dallas, TX). Autologous $CD4^+$ T cells were purified using EasySep Human $CD4^+$ T Cell Enrichment Kit (StemCell Technologies, Canada). Naïve ($CD45RA^+CD45RO^-CCR7^+$), memory ($CD45RA^-CD45RO^+$) $CD4^+$ T cells, and mDCs ($Lin^-HLA\text{-}DR^+CD11c^+CD123^-$) were sorted by FACS Aria (BD Biosciences, CA) (purity>99.0%). Culture medium consisted of RPMI 1640 (GIBCO, NY) supplemented with HEPES buffer, 2 mM L-glutamine, 1% nonessential amino-acids, sodium pyvurate, 50 units/ml penicillin, 50 μg/ml streptomycin and 10% normal human serum AB (GemCell, TX).

Antibodies and reagents

Anti-CD4-APC Cy7, anti-IFNγ–PE Cy7, anti-CCR5-pacific blue and anti-CCR6-Alexa Fluor 488 were purchased from Biolegend (CA). Anti-IL-1R1-PE, anti-IL-6R-PerCP, anti-CCR9-PE, anti-CXCR3-FITC, anti-IL-23p19 and control IgG were from R&D Systems (MN). Neutralizing anti-IL-1β and anti-IL-6/IL-6R were made in house. Anti-CD45RA-FITC, anti-CD45RO-PE, anti-integrin β7-PE, and anti-CD161-PE were purchased from BD Biosciences. Anti-IL-17-APC (eBioscience, CA) and anti-human IgG-PE (Jackson ImmunoResearch Laboratories, PA) were used. GolgiPlug was purchased from BD Pharmingen (CA). CFSE (Molecular probes, Oregon) was used for measuring $CD4^+$ T cell proliferation. TLR ligands, including Pam(3)CysSK(4) were purchased from Invivogen (Oregon). Jak2 inhibitor (1,2,3,4,5,6-Hexabromocyclohexane), Jak3 inhibitor (4-(4′-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline), pan Jak inhibitor (2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one), STAT5 inhibitor (Pimozide) and IRAK-1/4 inhibitor (N-(2-Morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole) were purchased from EMD chemicals (PA). STAT3 inhibitor (Stattic) was from Sigma-Aldrich (MO). MyD88 homodimerization inhibitory peptide (Imgenex, CA) was used. Anti-CD3/CD28 microbeads were purchased from Miltenyi Biotec (Germany).

Cloning and purification of chimeric recombinant mAbs fused to HA1

The Flu HA1 fragment containing nt 52-993 of Influenza A virus (A/Puerto Rico/8/34/ Mount Sinai(H1N1)) hemagglutinin (gi|21693168|gb|AF389118.1|) (with A619C and C959T changes) was engineered with a flanking NheI site followed by a flexible sequence (GATACAACAGAACCTGCAACACCTACAACACCTGTAACAACA) at the 5-prime end and 6 X His tag coding sequence followed by stop codon and NotI site at the 3-prime end (35). The NheI and NotI enzyme-digested fragment was fused downstream of and in-frame with the heavy chain coding region at the NheI site by NheI-NotI. Stable CHO-S cells were grown in GlutaMAX and HT media (Life Technologies, NY) and recombinant proteins were purified by protein A colum chromatography. Purified proteins were confirmed by reduced-SDS gel analysis.

Peptides

Overlapping 17-mer peptides (staggered by 11 amino acids) spanning the entire HA1 subunits of hemagglutinin (HA) (A/PR/8/34 H1N1) were purchased from Biosynthesis (TX).

Binding of recombinant fusion proteins to hDectin-1 and hDEC205

293F cells transfected with a full-length hDectin-1 or hDEC205 and IFNDCs were incubated with different concentrations of recombinant fusion proteins (anti-hDectin-1-HA1, anti-hDEC205-HA1, and IgG4-HA1) for 25 min on ice. Cells were then washed twice with 2% FCS in phosphate-buffered saline (PBS), and then stained with anti-human IgG-PE for 20 min. Cells were analyzed by flow cytometry.

DC activation

1×10^5 DCs were incubated with 1 μg/ml anti-hDectin-1-HA1 or 1 μg/ml IgG4-HA1 in the presence or absence of 40 ng/ml Pam(3)CysSK(4) for 24h. Cytokines in the culture supernatants were assessed by the BeadLyte cytokine assay kit (Upstate, MA) as per the manufacturer's protocol. IL-23 was measured using a human IL-23 ELISA kit (eBiosciences, CA).

DC/$CD4^+$ T cell co-cultures and antigen-specific $CD4^+$ T cell responses

$1\text{-}2\times10^5$ CFSE-labeled purified autologous $CD4^+$ T cells were co-cultured with 5×10^3 DCs loaded with indicated antibodies, in the absence or presence of different TLR ligands. After 7 days, $CD4^+$ T cell proliferation was measured by CFSE-dilution. In some experiments, anti-IL-23p19, anti-IL-6 and anti-IL-6R, anti-IL-1β antibodies or control IgG (10 μg/ml) were added into the co-cultures of DCs and $CD4^+$ T cells. In some experiments, $CD4^+$ T cells were incubated overnight with 50 units/ml IL-2 (Hoffmann-LaRoche, NJ), 50 units/ml IL-7 (R&D Systems, MN), 50 ng/ml IL-15 (Peprotech, NY) and 50 ng/ml IL-21 (Invitrogen, NY) and then naïve $CD4^+$ T cells were FACS-sorted to obtain IL-$1R1^-$ and IL-$1R1^+$ cells. For assessing antigen-specific $CD4^+$ T cell responses, T cells were restimulated with 0.5-1 μM indicated peptides for 6h in the presence of Brefeldin A, and stained with 7-AAD, anti-CD4, anti-IFNγ and anti-IL-17 antibodies labeled with fluorescent dyes. $CD4^+$ T cells expressing intracellular IFNγ and IL-17 were detected by flow cytometry (FACS Canto,

BD). In separate experiments, $CD4^+$ T cells were restimulated with indicated peptides for 48h, and cytokines in the supernatants were assessed by the BeadLyte cytokine assay kit (Upstate, MA) as per the manufacturer's protocol.

Conventional and quantitative Real-time RT-PCRs

Total RNA was isolated from $CD4^+$ T cells using Ambion's RNAqueous kits (Life Technologies) and cDNA was synthesized with Reverse Transcription System (Promega, CA). Conventional RT-PCR was performed for *TBX21, RORC, GATA3, IL-1R1, IL-1B* and *ACTB* (β-actin) using the primers, *TBX21* (forward, 5′-GAGGGGCGGGTCCTCGACGG-3′; reverse, 5′-TCGCGGCGGGTAGGCGTAGG-3′), *GATA3* (forward, 5′-AACTGTGGGGCAACCTCGAC-3′; reverse, 5′-TTGCAGACAGGGTCCCCATT-3′), *RORC* (forward, 5′-TCTGGAGCTGGCCTTTCATCATCA-3′; reverse, 5′-TCTGCTCACTTCCAAAGAGCTGGT-3′), *IL-1R1* (forward, 5′-CCACAGCCCAAGGGCGGGGCTA-3′; reverse, 5′-CTGCAGCACCTCTCAGGAGAGCCGC-3′), *IL1B* (forward, 5′-GCAAGGGCTTCAGGCAGGCCG-3′; reverse, 5′-GCTGTGAGTCCCGGAGCGTGC-3′) and *ACTB* (forward, 5′-CTCGCCTTTGCCGATCCGCCGC-3′; reverse, 5′-GCTCTGGGCCTCGTCGCCCACAT-3′). *IL1B, RORC, IL-1R1* and *ACTB* were also quantified through real-time RT-PCR using the Lightcycler 480 machine (Roche Applied Bioscience, NJ) using 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's t-test and significance was set at $p<0.05$.

Results

Generation and characterization of recombinant protein of HA1 fused to anti-hDectin-1 mAb

To deliver HA1 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 variable region-human IgG4κ chimera with two site mutations (S228P and L235E) that further diminishes antibody binding to the Fc receptor (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 CHO cell lines, purified by Protein A chromatography, and confirmed by the reduced SDS-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 mock transfectants (data not shown). Anti-hDectin-1-HA1 (upper panel in Fig. 1C) but not IgG4-HA1 (lower panel in 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 Th_{17} responses

Consistent with their bindings 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, upper panel). Anti-hDectin-1 mAb alone or combination of anti-hDectin-1 and IgG4-HA1 induced slightly enhanced $CD4^+$ T cell proliferation compared to DCs alone (Fig. 1D, lower panel). To test antigen specificity of the proliferating $CD4^+$ T cells, T cells were restimulated with clusters of HA1 peptides (6 peptides in each cluster; 17-mers overlapping by 11 amino acids) (Fig. 1E, upper panels). Cluster 8 showed the greatest percentage of $IFN\gamma^+CD4^+$ T cells. Individual peptides in cluster 8 were further tested in separate experiments (Fig. 1E, middle panels), and pep 43 and pep 45 resulted in substantially increased percentage of $IFN\gamma^+CD4^+$ T cells. Pep 5 from cluster 1 and no peptide were negative controls. Experiments performed with blood myeloid DCs (mDCs) showed similar results (Fig. 1E, lower panels), although IFNDCs were more efficient than mDCs at eliciting $CD4^+$ T cell responses. IFNDCs also resulted in a greater by-stander proliferation of $CD4^+$ T cells than did mDCs. We (35) and others (37) previously showed that DCs induced by-stander T cell proliferation, as shown in Fig. 1D, and this by-stander proliferation is further enhanced when the antigen-specific T cells are activated (35, 37).

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

IL-23 secreted from IFNDCs (Fig. 1H) and mDCs (Fig. 1J) contributed to HA1-specific Th_{17} and Th_1 responses (Fig. 1I, 1K), as blocking IL-23p19 during the co-cultures of DCs and $CD4^+$ T cells resulted in decreased IL-17- and slightly decreased IFNγ-producing $CD4^+$ T cell responses. DCs loaded with anti-hDectin-1 alone did not result in HA1-specific $CD4^+$ T cell responses (data not shown).

Taken together, we concluded that anti-hDectin-1-HA1-loaded DCs can efficiently activate HA1-specific IFNγ-, IL-13-, and IL-17-producing $CD4^+$ T cells. HA1-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 1.

Anti-hDectin-1-HA1-loaded DCs expand memory Th_{17} but cannot efficiently induce HA1-specific Th_{17}

To test whether anti-hDectin-1-HA1-loaded DCs could induce HA1-specific Th_{17}, FACS-sorted naïve and memory $CD4^+$ T cells were co-cultured with 1 μg/ml anti-hDectin-1-HA1-

loaded IFNDCs for 7 days. $CD4^+$ T cells were then restimulated with indicated peptides for 48h and the amounts of IFNγ, IL-13, and IL-17 in the supernatants were assessed. Compared to unstimulated or control pep 5-stimulated $CD4^+$ T cells, both naïve (Fig. 2A, upper left and middle) and memory $CD4^+$ T cells (lower left and middle) stimulated with pep 22 secreted significantly increased amounts of both IFNγ and IL-13. Interestingly, however, increased amount of IL-17 by pep 22 was observed only in the culture supernatant of memory, but not naïve $CD4^+$ T cells. Data from experiments using cells from five healthy donors further demonstrated that IFNDCs loaded with anti-hDectin-1-HA1 could efficiently activate HA1-specific memory Th_{17} cells, but they could not efficiently prime HA1-specific Th_{17} responses (Fig. 2B). Similar observations were made from experiments using blood mDCs (Fig. 2C, 2D). Naïve and memory $CD4^+$ T cells were co-cultured with 1 μg/ml anti-hDectin-1-HA1-loaded mDCs for 7 days. $CD4^+$ T cells were then restimulated for 48h with indicated peptides. Pep 45-stimulated naïve T cells secreted increased amounts of both IFNγ (Fig. 2C, upper left panel) and IL-13 (Fig. 2C, upper middle panel), but not IL-17 (Fig. 2C, upper right panel), compared to naïve T cells stimulated with control pep 5 or no peptide. Pep 45-stimulated memory T cells secreted increased amounts of the all three cytokines, including IL-17, compared to memory T cells stimulated with control pep 5 or no peptide (Fig. 2C, lower panel). Data from experiments using cells from 3 healthy donors (Fig. 2D) further showed that anti-hDectin-1-HA1-loaded mDCs efficiently activate HA1-specific memory Th_{17}, but they are not efficient to induce naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17}. Thus, we concluded that DCs loaded with anti-hDectin-1-HA1 can efficiently activate HA1-specific memory Th_{17}, but cannot efficiently prime HA1-specific Th_{17} responses *in vitro.*

Phenotypes of HA1-specific Th_{17} are different from those of other Th_{17}

HA1 peptide-specific $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells were further characterized by assessing the expression levels of chemokine receptors, β7 integrin, and CD161 (Fig. 3A). Compared to $IFN\gamma^+CD4^+$ T cells (Fig. 3A, left panel) expressing CXCR3, $IL\text{-}17^+CD4^+$ T cells (Fig. 3A, right panel) expressed increased levels of CCR6 and CD161 (38, 39). Interestingly, fractions of the HA1-specific $IL\text{-}17^+CD4^+$ T cells also expressed a high level of CCR9, which could support the presence of Th_{17} in the gut mucosa (40). Fractions of $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells expressed β7 integrin. CCR5 was similarly expressed on both $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells.

We also assessed the expression levels of these receptors on $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells restimulated with PMA and ionomycin (Fig. 3B). Similar to the HA1-specific $IFN\gamma^+CD4^+$ T cells (Fig. 3A, left panel), PMA/ionomycin-induced $IFN\gamma^+CD4^+$T cells (Fig. 3B, left panel) expressed CCR6, CXCR3, β7-integrin, and CD161. Both pep 43- (Fig. 3A, right panel) and PMA/ionomycin-induced $IL\text{-}17^+CD4^+$ T cells (Fig. 3B, right panel) also expressed similar levels of these receptors. However, PMA/ionomycin-induced $IL\text{-}17^+CD4^+$ T cells did not express CCR9 that was expressed on pep 43-induced $IL\text{-}17^+CD4^+$ T cells. Pep 43-induced $IFN\gamma^+CD4^+$ and $IL\text{-}17^+CD4^+$ T cells also expressed increased levels of CCR5 compared to those induced with PMA/ionomycin.

In conclusion, these data demonstrated that phenotypes of HA1-specific human Th_{17} cells are not the same to those of other Th_{17} cells activated with polyclonal activators.

IL-1β and IL-6 are insufficient to induce naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17}

Both IL-1β with IL-6 have been known to contribute to the induction of human Th_{17} 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 Th_{17} responses (9). Thus, we first tested whether DCs loaded with anti-hDectin-1-HA1 plus TLR2 ligand could enhance HA1-specific Th_{17} responses (Fig. 4A). Total $CD4^+$ T cells were co-cultured for 7 days 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 48h with the indicated peptides and cytokines in the supernatants were assessed. HA1-specific Th_{17} (Fig. 4A, right panel) and Th_1 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-1β (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 Th_{17} (Fig. 4C, 4D). FACS-sorted naïve ($CD45RA^+CD45RO^-CCR7^+$) and memory $CD4^+$ T cells ($CD45RA^-CD45RO^+$) were co-cultured with either 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 Th_{17}, and these Th_{17} responses were further enhanced by Pam(3)CysSK(4). However, neither IFNDCs nor blood mDCs induced HA1-specific Th_{17} even in the presence of Pam(3)CysSK(4). Thus, the HA1-specific Th_{17} 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 TNFα 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 Th_{17} responses (data not shown). Therefore, the enhanced HA1-specific memory Th_{17} responses by the combination of anti-hDectin-1-HA1 and Pam(3)CysSK(4) were due to the activation of DCs via Dectin-1 and TLR2.

Taken together, we concluded that DCs loaded with anti-hDectin-1-HA1 and TLR2 ligand could greatly promote HA1-specific memory Th_{17} responses. However, they were not efficient to induce naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17} even in the presence of IL-1β and IL-6. These results suggested that naïve $CD4^+$ T cells require additional signals to differentiate into HA1-specific Th_{17}.

Naïve 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 Th_{17} (Fig. 4C, 4D). We therefore investigated the expression levels of cytokine receptors on the surface of naïve and memory $CD4^+$ T cells in peripheral blood of healthy donors. Both naïve 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 naïve $CD4^+$ T cell (Fig. 5A, top left panel), but no significant level of HA1-specific Th_{17} response (Fig. 4C, 4D) suggested that IL-6 is not the key factor that turns on the program for the differentiation of naïve $CD4^+$ T cells into HA1-derived peptide-specific Th_{17}. Thus, we hypothesized that the induction of surface IL-1R1 expression on naïve $CD4^+$ T cells could be a prerequisite for the enhanced de novo induction of antigen-specific Th_{17}.

Induction of IL-1R1 expression on the surface of naïve $CD4^+$ T cells is known to be very modest and takes 5-6 days after TCR stimulation in the presence of IL-2 (43). Naïve $CD4^+$ T cells from cord blood started to express IL-1R1 on 6 days of culture in the presence of TGFβ, IL-7 and IL-15 (44). Therefore, we first sought cytokine signaling that could rapidly induce IL-1R1 expression on the surface of naïve $CD4^+$ T cells. Fig. 5B showed that naïve $CD4^+$ 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 18h by treating naïve $CD4^+$ T cells with the combination of IL-2, IL-7, IL-15, and IL-21 (Fig. 5B). In contrast to the previously published data (44), naïve $CD4^+$ T cells treated with the combination of common γ-chain cytokines also expressed surface IL-1R1 within 36h (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 naïve $CD4^+$ T cells (data not shown). This was further confirmed by the data showing that Janus kinase 3 (Jak 3) and pan-Jak inhibitors but not Jak 2 inhibitor suppressed the 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 naïve $CD4^+$ 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 naïve $CD4^+$ T cells (Fig. 5F, 5G). FACS-sorted naïve $CD4^+$ T cells were co-cultured for 18h 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 pep43 loaded IFNDCs, respectively) increased the expression of *IL-1R1* in naïve $CD4^+$ T cells. However, the combination of common γ-chain cytokines was more efficient than the antigen-loaded DCs at inducing *IL-1R1* expression in naïve $CD4^+$ T cells. This was not surprising because the frequency of HA1 antigen-specific $CD4^+$ T cells in the naïve pool

is low. In contrast, the combination of common γ-chain cytokines acts on majority of the naïve T cells in the cultures.

To further explore the roles of signaling via TCR in the induction of *IL-1R1* expression, naïve $CD4^+$ 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, naïve $CD4^+$ 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 γ-chain cytokines, showing a synergistic effect of signals from common γ-chain receptors and TCRs on naïve $CD4^+$ T cells to upregulate IL-1R1 expression.

Taken together, naïve 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 γ-chain receptors and TCRs.

IL-1R1$^+$, but not IL-1R1$^-$, naïve CD4$^+$ T cells differentiate into HA1-specific Th$_{17}$

To test whether anti-hDectin-1-HA1-loaded DCs were able to induce IL-1R1$^+$ naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17}, IL-1R1$^-$ and IL-1R1$^+$ naïve $CD4^+$ T cells were co-cultured with anti-hDectin-1-HA1-loaded IFNDCs. After 7 days, both IL-1R1$^-$ and IL-1R1$^+$ naïve $CD4^+$ T cells secreted IFNγ and IL-13 in response to pep 43 during 48h restimulation (Fig. 6A, left and middle panels). HA1-specific Th_{17} responses were observed only in the cultures of IL-1R1$^+$ naïve $CD4^+$ 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 fraction of IL-1R1$^+$ naïve $CD4^+$ T cells co-cultured with DCs expressed intracellular IL-17 in response to PMA/ionomycin (Fig. 6A). Blocking IL-1β during the co-cultures of IL-1R1$^+$ naïve $CD4^+$ T cells and DCs substantially impaired HA1-specific Th_{17} responses (Fig. 6B, right panel). Thus, the surface IL-1R1 induction on naïve $CD4^+$ T cells by signals via both TCR and common γ-chain receptors is a crucial for the enhanced induction of HA1-specific human Th_{17}.

Blocking IL-6 also greatly decreased the induction of HA1-specific Th_{17} from IL-1R1$^+$ naïve $CD4^+$ T cells (Fig. 6B, right panel). Thus, STAT3 inhibitors, which block IL-6-mediated signals, also decreased *RORC* expression in the IL-1R1$^+$ naïve $CD4^+$ T cells on day 5 of the co-cultures with anti-hDectin-1-HA1-loaded DCs (Fig. 6C). Fig. 6C also demonstrated that IL-1β-induced *RORC* expression is dependent on myeloid differentiation primary response gene 88 (MyD88), but not IL-1R-associated kinase 1 (IRAK1) or IRAK4.

Taken together, we concluded that both IL-1β and IL-6 contribute to the induction of HA1-specific Th_{17}. However, surface IL-1R1 expression on naïve $CD4^+$ T cells is essential for the enhanced induction of HA1-specific Th_{17}. Surface IL-1R1$^-$ naïve $CD4^+$ T cells did not efficiently differentiate into HA1-specific Th_{17} (Fig. 2 and Fig. 6A).

IL-6 contributes to the induction of HA1-specific Th_{17} through IL-1β induction in TCR-activated T cells

IL-6 has been known to contribute to Th_{17} induction in both mice (19) and humans (9). However, IL-6 can contribute to the induction of HA1-specific Th_{17} only when naïve $CD4^+$ T cells express IL-1R1 (Fig. 6B), although IL-6R is expressed on the surface of naïve $CD4^+$ T cells (Fig. 5A, upper left panel). To further investigate the roles of IL-6 in the induction of Th_{17}, both IL-$1R1^-$ and IL-$1R1^+$ naïve $CD4^+$ T cells were activated with anti-CD3/CD28-coated microbeads at a 1:40 ratio in the presence or absence 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 naïve $CD4^+$ T cells (data not shown). After 5 days, the expression levels of *RORC*, *TBX21*, and *GATA3* in the two groups of naïve $CD4^+$ T cells were assessed. Consistent with the data in Fig. 6A, IL-$1R1^-$ naïve $CD4^+$ 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 combination with IL-1β were variable in different experiments (data not shown). However, IL-$1R1^+$ naïve $CD4^+$ 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-$1R1^+$ naïve $CD4^+$ T cells (Fig. 6D, right panel). Indeed, IL-6 was more efficient than IL-1β at inducing *RORC* expression in IL-$1R1^+$ naïve $CD4^+$ T cells. Therefore, we hypothesized that IL-6 could induce these naïve $CD4^+$ 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-$1R1^+$ naïve $CD4^+$ T cells to upregulate *IL1B* expression. Furthermore, blocking IL-1β led to decreased IL-6-mediated *RORC* expression in IL-$1R1^+$ naïve $CD4^+$ T cells (Fig. 6F).

Taken together, IL-$1R1^+$ naïve $CD4^+$ T cells were able to differentiate into HA1-derived peptide-specific Th_{17} when they were activated via both TCRs and IL-1R1. IL-6 could argument Th_{17} responses by inducing IL-1β and thus enhanced *RORC* expression in the TCR-activated IL-$1R1^+$ naïve $CD4^+$ T cells.

Targeting antigens to DCs via Dectin-1 can elicit antigen-specific human Th_{17} responses that can be further promoted by TLR2 and TLR4 ligands

Antigen targeting to DCs via Dectin-1 using recombinant fusion protein of an agonistic anti-hDectin-1 mAb and antigen could elicit antigen-specific Th_{17} responses. IL-23 secreted from anti-hDectin-1-HA1-loaded DCs contributed to HA1-specific Th_{17} responses (Fig. 1H, 1J). 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 Th_{17} 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 Th_{17} 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 Th_{17}

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 co-cultured for 7 days 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 *P. gingivalis* lipopolysaccharide (LPS) (Fig. 7C). Consistent with the data in Fig. 4B and Fig. 7A, both Pam(3)CysSK(4) and *P. gingivalis* LPS promoted HA1-specific Th_{17} responses elicited by anti-hDectin-1-HA1-loaded DCs. *E. coli* LPS was also able to enhance HA1-specific Th_{17} responses elicited by anti-hDectin-1-HA1-loaded IFNDCs (Fig 7D, upper panels) and mDCs (Fig. 7D, lower panels). We previously reported that anti-hDectin-1 mAb and *E. coli* LPS synergize to activate DCs to secrete increased amount of both IL-1β and IL-6 (34). Taken together, healthy individuals tested in this study maintained HA1-specific memory Th_{17}, although the magnitudes of Th_{17} responses in the healthy individuals were variable. In addition, a vaccine model made of an agonistic anti-hDectin-1 and antigens along with TLR2 or TLR4 ligands could offer great potential in promoting Th_{17}-mediated host immunity against infections.

Data in Fig. 8 further support our notion that targeting antigens to DCs via Dectin-1 using recombinant fusion protein of an agonistic anti-hDectin-1 mAb and antigen is an efficient strategy to elicit antigen-specific Th_{17} responses. Anti-hDEC205-HA1 fusion protein was made (Fig. 8A). Anti-hDEC205-HA1 (Fig. 8B, lower panel) but not IgG4-HA1 (Fig. 8B, upper panel) bound to the surface of monocyte-derived IFNDCs in a dose-dependent manner. IFNDCs loaded with anti-hDEC205-HA1 resulted in HA1-specific Th_1, Th_2, and Th_{17} responses (Fig. 8C). More importantly, HA1-specific Th_{17} responses elicited with anti-DEC205-HA1 were further enhanced by anti-hDectin-1 mAb (Fig. 8C, right panel). Anti-hDectin-1 mAb did not increase HA1-specific $IFN\gamma^+CD4^+$ T cell responses (Fig. 8C, left panel), but significantly decreased $IL\text{-}13^+CD4^+$ 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 Th_1, Th_2, and Th_{17} responses. These data showed that TLR2 signaling does not synergize with DEC205 to promote HA1-specific Th_{17} responses.

Discussion

This study dissected the pathways for the de novo generation of protein antigen-derived peptide-specific human Th_{17} in the context of TCR ligation by MHC II/peptide complexes presented by DCs. It appears that the induction of protein antigen-specific Th_{17} requires more than the actions of previously known Th_{17}-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 Th_{17} activated with HA1 peptides and those activated with polyclonal activators are not the same. Finally, this study demonstrates that targeting antigens to DCs via Dectin-1 using an agonistic anti-hDectin-1 mAb, particularly with TLR2 ligands, can efficiently promote antigen-specific Th_{17} where antigen-specific memory T cells have already been established.

It has been known that the immune system can favor Th_{17} 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 Th_{17}-promoting cytokines from APCs (3, 45). However, Th_{17} 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 Th_{17} are mainly effector T cells with a short life span, as they are often found in peripheral tissues and organs (46), had raised a question as to the value of such vaccine-induced Th_{17}-mediated immunity. However, recent studies have shown that human Th_{17} consist 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 Th_{17}, although the levels of Th_{17} among donors varied. Such memory Th_{17} could contribute to the protective immunity against influenza infections, presumably by enhancing $CD8^+$ T cell and antibody responses (6, 13, 16, 49). More importantly, those Th_{17} responses can be greatly enhanced by the vaccine model, recombinant fusion protein of anti-hDectin-1 and antigens along with TLR2 and TLR4 ligands.

The mechanisms of human Th_{17} differentiation remained obscure, but previous studies have revealed several key cytokines that promote Th_{17} 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 Th_{17} differentiation (19, 20). The major role of IL-23, expanding memory Th_{17} (1, 50), is recapitulated by our data. We could detect active forms of TGFβ in the co-cultures of DCs and T cells (data not shown). This suggests that TGFβ may not be a key cytokine that determines the generation of antigen-specific human Th_{17}, while 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 Th_{17} 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 antibodies specific for targeted cytokines.

We have shown that the induction of antigen-specific Th_{17} requires more than the actions of currently known Th_{17}-promoting cytokines. Foremost, the induction of surface IL-1R1 expression was the key step for naïve $CD4^+$ T cell differentiation into pathogen-specific Th_{17}. 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 Th_{17} development (51), our data indicated that these could also contribute to the generation of antigen-specific Th_{17} 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 (52) 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 Th_{17} 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 Th_{17} 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 Th_{17}. This was further supported by the facts that mutations in STAT1 or STAT3 resulted in the deficiency of Th_{17}-mediated immunity in patients with fungal infections (12, 54). Interestingly, however, IL-6 promotes the induction Th_{17} only when naïve $CD4^+$ 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-$1R1^+$ naïve $CD4^+$ T cells. The key 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 Th_{17}-mediated immunity against chlamydial infection (55, 56). Moreover, defective IL-1R/MyD88 signaling is associated with impaired Th_{17} responses (57).

In conclusion, this study revealed the complex but complementary mechanisms for the induction of protein antigen-specific human Th_{17} in the context of TCR ligation by MHC/peptide complexes. Although there could be alternative pathways for the generation of antigen-specific human Th_{17}, 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 Th_{17}-mediated host immunity against infections and certain types of cancers in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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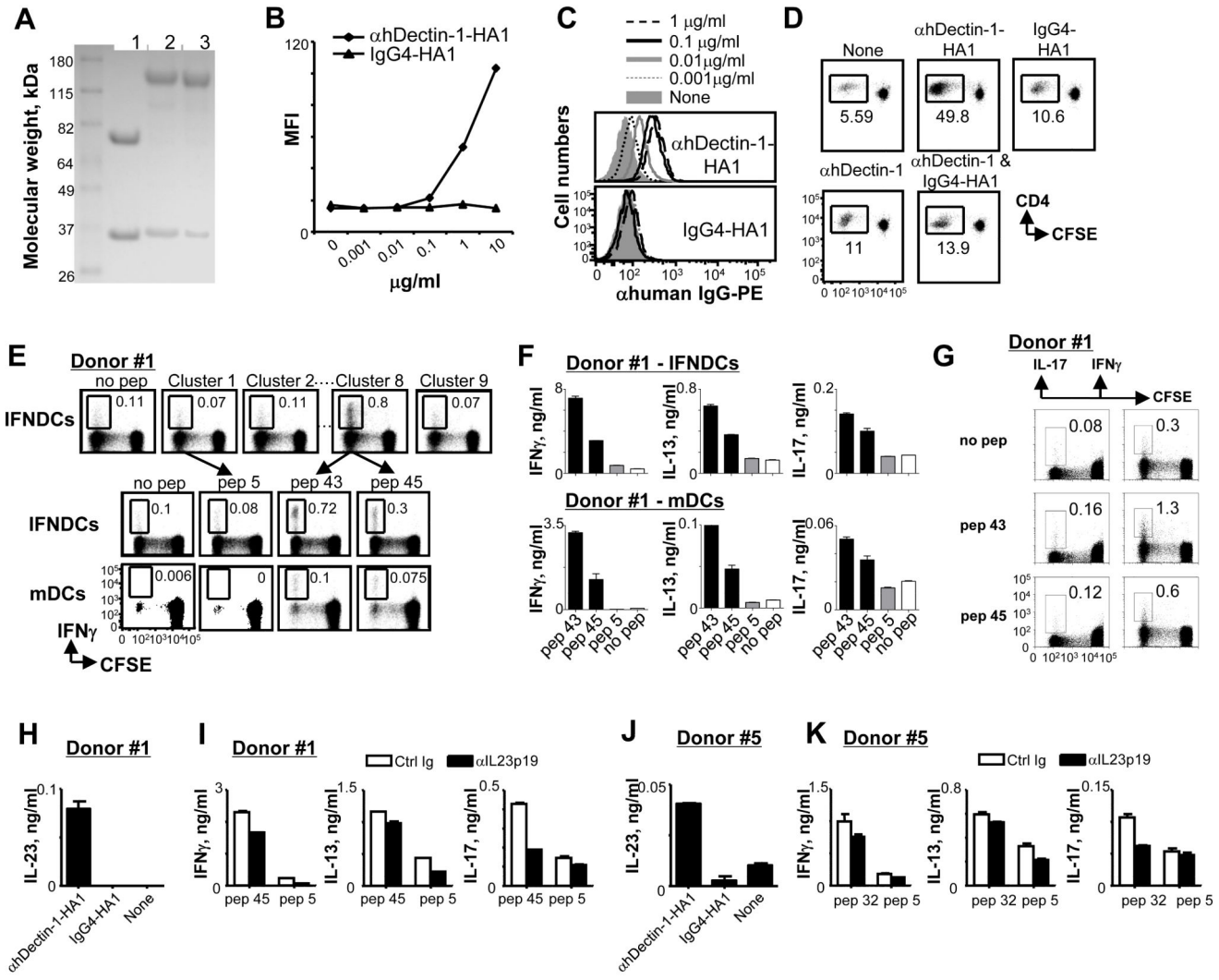

FIGURE 1.

DCs loaded with anti-hDectin-1-HA1 can elicit HA1-specific Th_1, Th_2, and Th_{17} responses. **(A)** Reduced SDS-PAGE analysis of anti-hDectin-1 mAb (lane 1), anti-hDectin-1-HA1 (lane 2) and IgG4-HA1 (lane 3). **(B** and **C)** Binding of anti-hDectin-1-HA1 and IgG4-HA1 to 293F cells transfected with hDectin-1 **(B)** and IFNDCs **(C)**. **(D)** Total $CD4^+$ T cell proliferation induced by IFNDCs alone or IFNDCs loaded with 1 μg/ml anti-hDectin-1-HA1, IgG4-HA1, anti-hDectin-1, or combination of IgG4-HA1 (1 μg/ml) and anti-hDectin-1 (1 μg/ml). **(E)** CFSE-labeled total $CD4^+$ T cells were co-cultured for 7 days with 1 μg/ml anti-hDectin-1-HA1-loaded IFNDCs or blood mDCs. T cells were restimulated with 1 μM of the clusters (upper panels, IFNDCs) or individual peptides from cluster 8 (middle panels, IFNDCs and lower panels, mDCs). **(F)** $CD4^+$ T cells were restimulated for 48h in the presence or absence of 1 μM peptides indicated. Cytokines in the supernatants were assessed. In **(B-F)**, three independent experiments showed similar results. **(G)** CFSE-labeled total $CD4^+$ T cells were co-cultured for 7 days with 1 μg/ml anti-hDectin-1-HA1-loaded IFNDCs. T cells were restimulated with 1 μM of the indicated peptides and IL-17 and IFNγ

expression were analyzed by flow cytometry. Two independent experiments using cells from donors #1 and #2 showed similar results. One representative data generated with donor #1 are presented. **(H)** 1×10^5 IFNDCs were incubated overnight with 1 μg/ml HA1 fusion proteins. The amount of IL-23 in the supernatants was assessed. **(I)** Total $CD4^+$ T cells were co-cultured with 1 μg/ml anti-hDectin-1-HA1-loaded IFNDCs for 7 days in the presence of 5 μg/ml anti-IL-23p19 or control antibodies. $CD4^+$ T cells were then restimulated with 1 μM peptides for 48h. Cytokines in culture supernatants were assessed. **(J** and **K)** Experiments in **(H** and **I)** were performed with blood mDCs. In **(H-K)**, Error bars indicate mean±SD of triplicate assay.

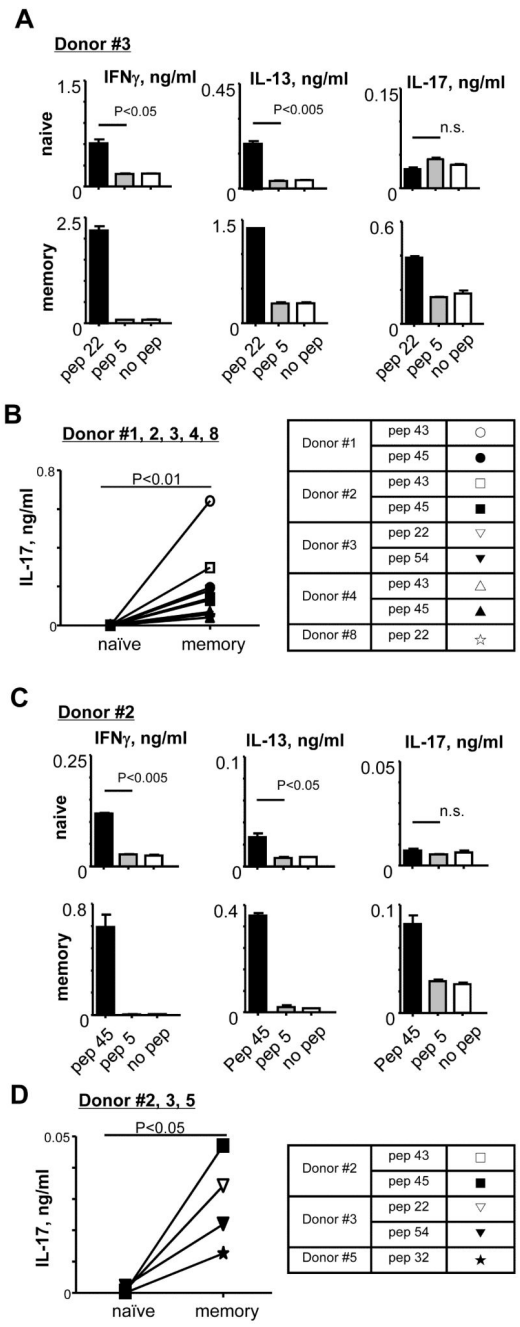

FIGURE 2.

DCs loaded with anti-hDectin-1-HA1 activate memory Th_{17}, but cannot efficiently induce HA1-specific Th_{17}. **(A** and **C)** FACS-sorted naïve and memory $CD4^+$ T cells were co-cultured for 7 days with anti-hDectin-1-HA1-loaded IFNDCs **(A)** or blood mDCs **(C)**. T cells were restimulated for 48h in the presence or absence of indicated peptides (1 μM). Pep 5 was used as a control peptide. Cytokines in the supernatants were assessed. Error bars indicate mean±SD of triplicate assay. **(B** and **D)** Summarized data generated with IFNDCs **(B)** and blood mDCs **(D**) from healthy donors. Background values acquired with pep 5 were

subtracted. P values were acquired by the Student's t-test. Indications of donors and peptides tested in **(B** and **D)** are summarized in the table.

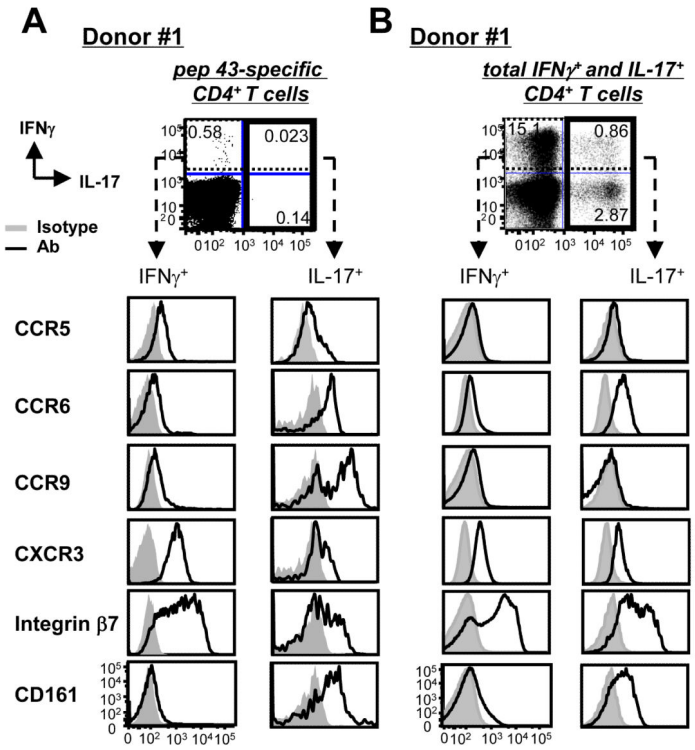

FIGURE 3.

Phenotypes of HA1-specific and PMA/ionomycin-activated Th_{17}. Total $CD4^+$ T cells were co-cultured for 7 days 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 6h. Cells were stained for intracellular IFNγ and IL-17 as well as surface receptors indicated. Four independent experiments using cells from two different healthy donors showed similar results.

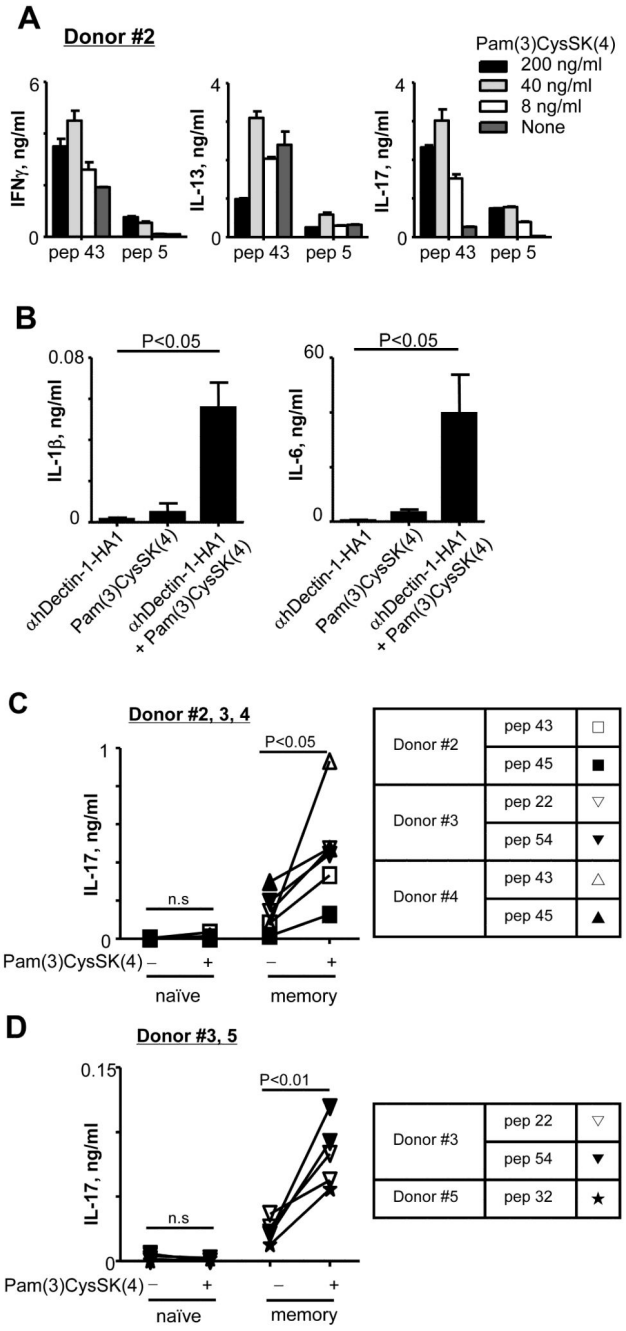

FIGURE 4.

IL-1β and IL-6 can promote HA1-specific memory Th_{17} responses but fail to induce naïve $CD4^+$ T cell differentiation into HA1-specific Th_{17}. **(A)** Total $CD4^+$ T cells were cultured for 7 days with 1 μg/ml anti-hDectin-1-HA1-loaded IFNDCs in the presence of different concentrations of Pam(3)CysSK(4). T cells were then restimulated for 48h with 1 μM pep 43 or pep 5 (control peptide). Cytokines in the supernatants were assessed. Error bars indicate mean±SD of triplicate assay. Two independent experiments showed similar results. **(B)** IL-1β and IL-6 levels in the culture supernatants of 1×10^5 IFNDCs incubated with 1 μg/ml

anti-hDectin-1-HA1, 40 ng/ml Pam3CysSK4, or 1 μg/ml anti-hDectin-1-HA1 plus 40 ng/ml Pam(3)CysSK(4). Data are pooled from three independent experiments. **(C** and **D)** FACS-sorted naïve and memory $CD4^+$ T cells were co-cultured for 7 days with IFNDCs **(C)** and blood mDCs **(D)** loaded with 1 μg/ml anti-hDectin-1-HA1 in the presence or absence of 40 ng/ml Pam(3)CysSK(4). T cells were restimulated for 48h with 1 μM peptides. The amount of IL-17 in the supernatants was assessed. In **(C)** and **(D)**, background values generated with pep 5 were subtracted. P values were acquired by the Student's t-test. Indications of donors and peptides tested in **(C** and **D)** are summarized in the table.

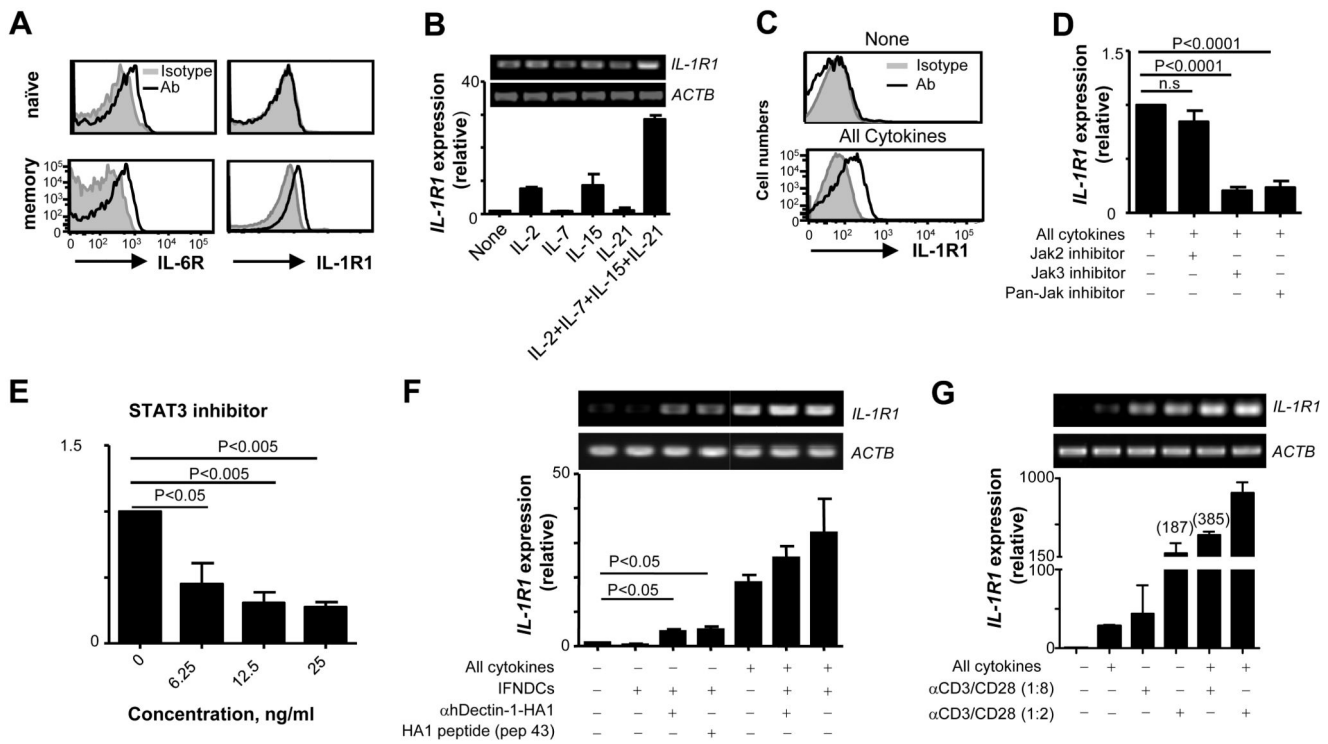

FIGURE 5.

Naïve CD4$^+$ T cells do not express IL-1R1 that is inducible by synergistic actions of signals from TCRs and common-γ chain receptors. **(A)** IL-1R1 and IL-6R expression on naïve and memory CD4$^+$ T cells in the peripheral blood. CD4$^+$ T cells from 6 healthy donors showed similar results. **(B)** Real-time (lower panel) and conventional (upper panel) RT-PCR analysis of *IL-1R1* expression in naïve CD4$^+$ T cells treated for 18h with indicated cytokines. **(C)** Surface IL-1R1 expression on naïve CD4$^+$ T cells treated for 36h with the combination of the cytokines in **(B)**. **(D** and **E)** Real-time RT-PCR analysis of *IL-1R1* expression in naïve 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. P values were acquired by the Student's t-test. **(F** and **G)** Real-time (lower panel) and conventional (upper panel) RT-PCR analysis of *IL-1R1* expression in naïve CD4$^+$ T cells co-cultured overnight with IFNDCs alone, IFNDCs loaded with HA1 peptide (Pep 43, 1 μM), or IFNDCs loaded with 1 μg/ml anti-hDectin-1-HA1 in the presence or absence of the combination of common γ-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. P values were acquired by the Student's t-test.

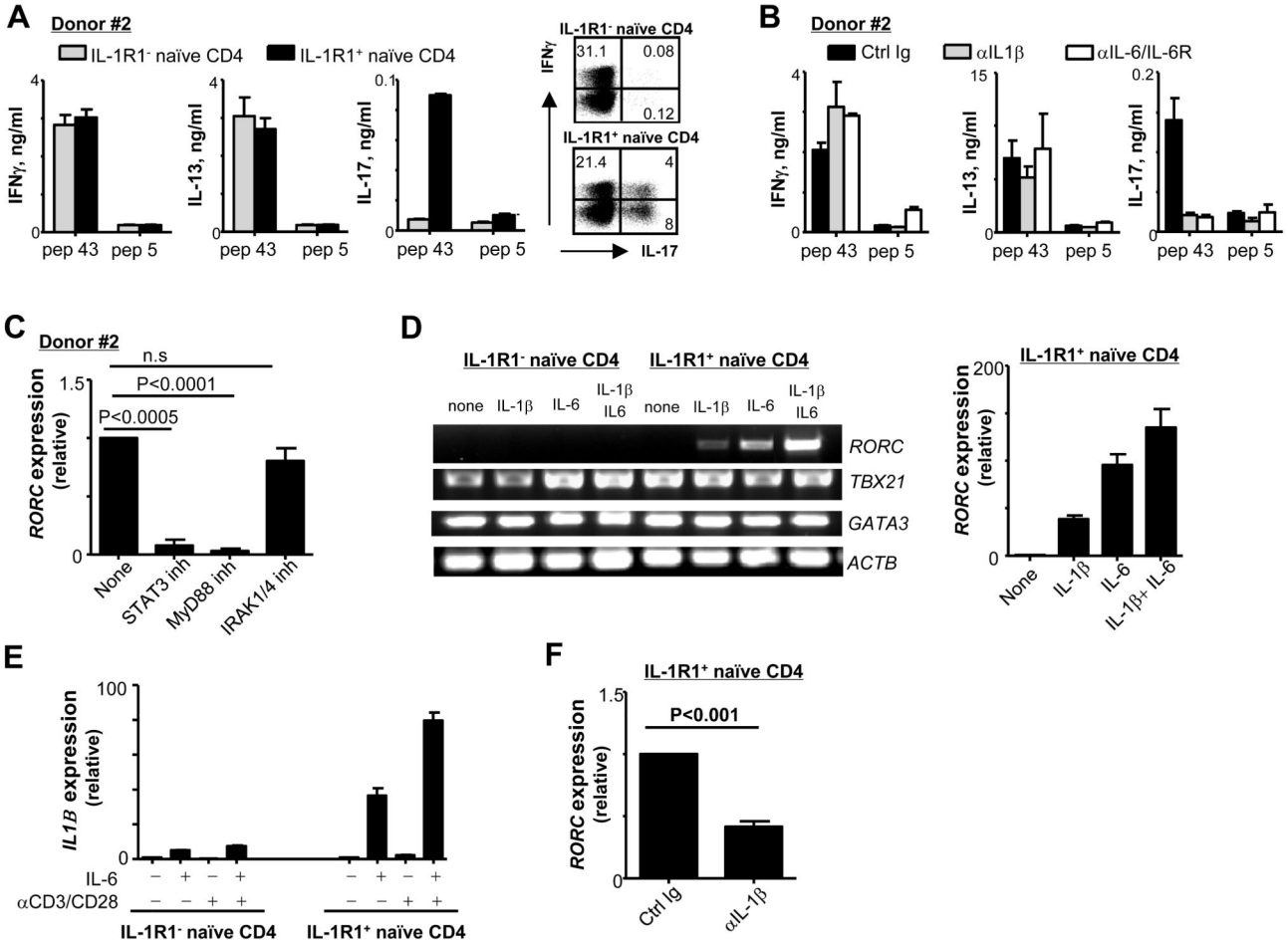

FIGURE 6.

Anti-hDectin-1-HA1-loaded DCs induce IL-1R1$^+$ naïve CD4$^+$ T cell differentiation into HA1-specific Th$_{17}$. **(A)** IL-1R1$^-$ and IL-1R1$^+$ naïve CD4$^+$ T cells were co-cultured for 7 days with anti-hDectin-1-HA1-loaded IFNDCs. **(left panels)** T cells were then restimulated for 48h with 1 μM pep 43 or pep 5. Cytokines in the supernatants were assessed. (**right panels**) T cells were then restimulated for 6h with PMA and ionomycin. Cells were stained for intracellular IFNγ and IL-17 expression. **(B)** Experiments in **(A)** were performed with IL-1R1$^+$ naïve CD4$^+$ T cells in the presence or absence of indicated antibodies (10 μg/ml). T cells were restimulated for 48h with 1 μM pep 43 or pep 5. In **(A)** and **(B)**, three independent experiments showed similar results. Error bars indicate mean±SD of triplicate assay. **(C)** Real time RT-PCR analysis of *RORC* expression in IL-1R1$^+$ naïve CD4$^+$ T cells co-cultured for 5 days with 1 μg/ml anti-hDectin-1-HA1-loaded IFNDCs in the absence or presence of indicated inhibitors. **(D)** IL-1R1$^-$ and IL-1R1$^+$ naïve CD4$^+$ T cells were incubated with anti-CD3/CD28-coated microbeads (beads: cells=1:40) in the absence or presence of indicated cytokines for 5 days. mRNA was analyzed by conventional RT-PCR (left panel) and real time RT-PCR (right panel). Two independent experiments showed similar results. Error bars indicate mean±SD of triplicate assay. **(E)** IL-1R1$^-$ and IL-1R1$^+$ naïve CD4$^+$ T cells were incubated for 4h with 50 ng/ml IL-6, anti-CD3/CD28-coated microbeads or combination of

IL-6 and anti-CD3/CD28-coated microbeads. *IL1B* expression was assessed by real time RT-PCR. Error bars indicate mean±SD of triplicate assay. One representative of three experiment is shown. **(F)** IL-1R1^{+} naïve CD4^{+} T cells were incubated with anti-CD3/CD28-coated microbeads in the presence of indicated antibodies for 5 days. mRNA was analyzed by real time RT-PCR. In **(C)** and **(F)**, Data are pooled from three independent experiments. P values were acquired by the Student's t-test.

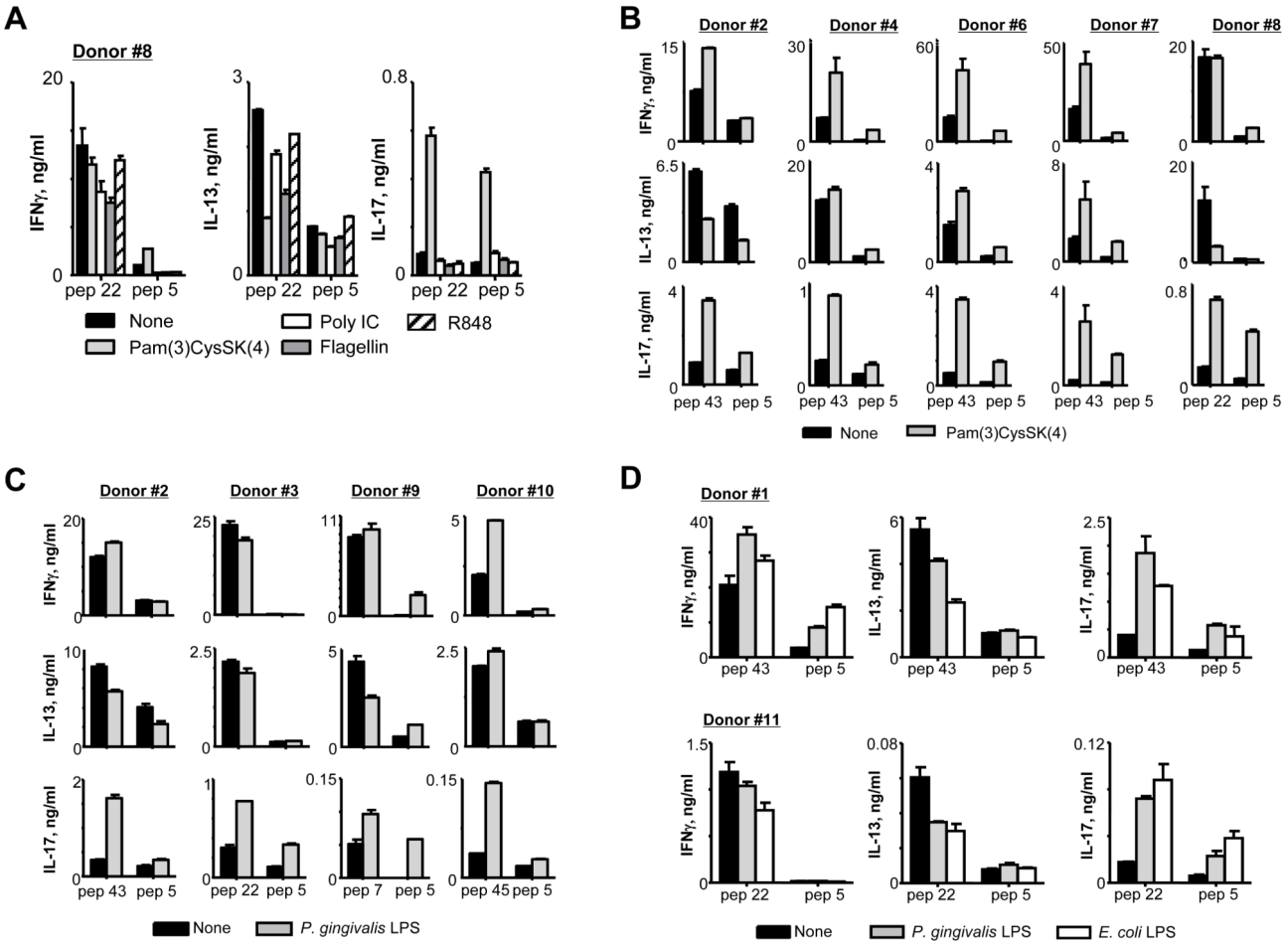

FIGURE 7.

Healthy individuals maintain influenza HA1-specific Th_{17} that can be promoted by the combination of anti-hDectin-1-HA1 and TLR2 ligands. **(A)** Total $CD4^+$ T cells were co-cultured for 7 days 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), 1μg/ml poly IC, 50 ng/ml flagellin, 500 ng/ml R848). T cells were then restimulated for 48h 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 co-cultured for 7 days 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 co-cultured for 7 days with IFNDCs (top panels) or mDCs (lower 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 48h 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.

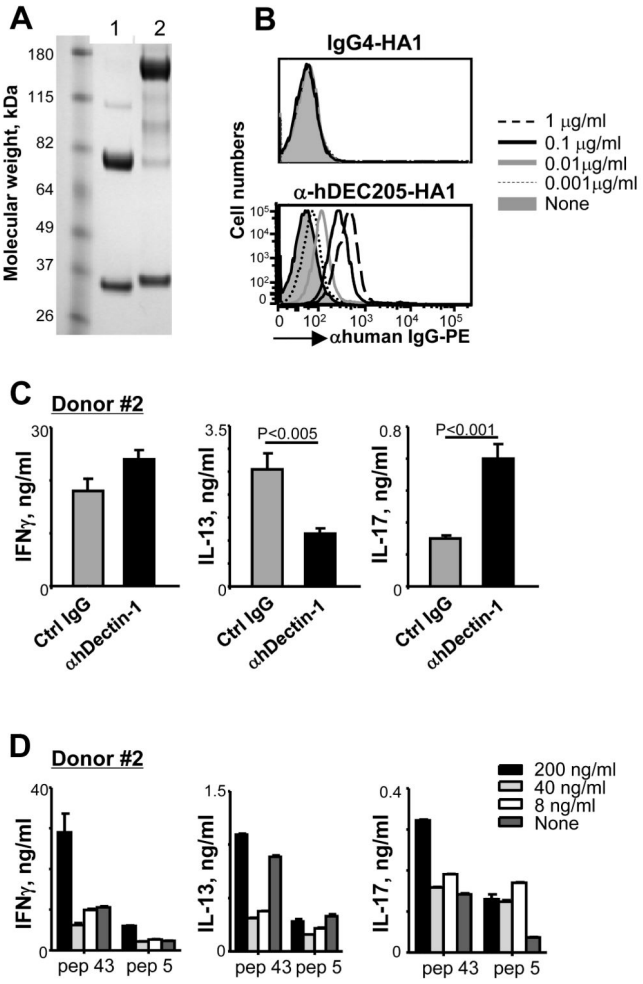

FIGURE 8.

HA1-specific Th_{17} 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-hDEC205-1-HA1 and IgG4-HA1 to IFNDCs. **(C)** Total $CD4^+$ T cells were co-cultured for 7 days with IFNDCs loaded with 1 μg/ml anti-hDEC205-HA1 in the presence 5 ug/ml control IgG or anti-hDectin-1 mAb. T cells were then restimulated for 48h with 1 μM of pep 43 or control pep 5. Cytokines in the culture supernatants were assessed. Values acquired with control pep5 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.

Table 1

MHC class II types of healthy subjects and peptide binding scores of individual peptides to corresponding MHC class II

Donors	HLA types	Peptides	Amino acid residues	Peptide sequence	ARB score (IC50 values)
#1 (Fig. 1E, 1F, 1G, 1H; Fig. 2A, 2B; Fig. 3A, 3B; Fig. 7B, 7C, 7D)	HLA-DRB1*01	pep 43	250-266	LEPGDTIIFEANGNLIA	10.7
		pep 45	262-278	GNLIAPWYAFALSRGFG	9.6
	HLA-DQB1*05:01	NA			NA
#2 (Fig. 2B, 2D; Fig.4A 4C, 4D; Fig. 6A, 6B, 6C; Fig. 7A, 7B, 7C)	HLA-DRB1*13:02	pep 43	250-266	LEPGDTIIFEANGNLIA	0
		pep 45	262-278	GNLIAPWYAFALSRGFG	10004
	HLA-DRB1*15:01	pep 43	250-266	LEPGDTIIFEANGNLIA	41.6
		pep 45	262-278	GNLIAPWYAFALSRGFG	54.6
	HLA-DRB3*03:01	NA			NA
	HLA-DQB1*06:04	NA			NA
#3 (Fig. 2B, 2C, 2D; Fig, 4D, 4E; Fig. 7C)	HLA-DRB1*01	pep 22	126-142	SSFERFEIFPKESSWPN	826
		pep54	316-332	IDECPKYVRSAKLRMVT	6.7
	HLA-DRB1*13	pep 22	126-142	SSFERFEIFPKESSWPN	25784.9
		pep54	316-332	IDECPKYVRSAKLRMVT	1354
	HLA-DQB1*03:01	NA			NA
	HLA-DQB1*05	NA			NA
#4 (Fig. 2B; Fig. 4D Fig. 7B)	HLA-DRB1*04:01	pep 43	250-266	LEPGDTIIFEANGNLIA	346.8
		pep 45	262-278	GNLIAPWYAFALSRGFG	10.4
	HLA-DRB1*09	pep 43	250-266	LEPGDTIIFEANGNLIA	378.9
		pep 45	262-278	GNLIAPWYAFALSRGFG	6.1
	HLA-DQB1*03:01	NA			NA
	HLA-DQB1*03:03	NA			NA
#5 (Fig. 1I, 1J; Fig. 2D; Fig. 4E)	HLA-DRB1*04:02	NA			NA
	HLA-DRB1*13	pep32	186-202	EKEVLVLWGVHHPPNIG	351.1
	HLA-DQB1*06:04	NA			NA
	HLA-DQB1*03:02	NA			NA
#6 (Fig. 7B)	HLA-DRB1*07	pep 43	250-266	LEPGDTIIFEANGNLIA	103.4
		pep 45	262-278	GNLIAPWYAFALSRGFG	1.6
	HLA-DRB1*08	pep 43	250-266	LEPGDTIIFEANGNLIA	79.5
		pep 45	262-278	GNLIAPWYAFALSRGFG	596.9
	HLA-DQB1*02	NA			NA
	HLA-DQB1*04:02	NA			NA
#7 (Fig. 7B)	HLA-DRB1*01	pep 22	126-142	SSFERFEIFPKESSWPN	826
		pep 54	316-332	IDECPKYVRSAKLRMVT	6.7
	HLA-DRB1*11	pep 22	126-142	SSFERFEIFPKESSWPN	50.6

Donors	HLA types	Peptides	Amino acid residues	Peptide sequence	ARB score (IC50 values)
		pep 54	316-332	IDECPKYVRSAKLRMVT	6.3
	HLA-DQB1*03	NA			NA
	HLA-DQB1*05	NA			NA
#8 (Fig. 7C)	HLA-DRB1*03	pep 22	126-142	SSFERFEIFPKESSWPN	16976.1
		pep 54	316-332	IDECPKYVRSAKLRMVT	726.5
	HLA-DRB1*11	pep 22	126-142	SSFERFEIFPKESSWPN	50.6
		pep 54	316-332	IDECPKYVRSAKLRMVT	6.3
	HLA-DQB1*02	NA			NA
	HLA-DQB1*06	NA			NA
# 9 (Fig. 7C)	HLA-DRB1*03	pep 7	37-53	LEKNVTVTHSVNLLEDS	478.4
		pep 45	262-278	GNLIAPWYAFALSRGFG	53285.8
		pep 46	268-284	WYAFALSRGFGSGIITS	61616.3
		pep 52	304-320	SSLPFQNVHPVTIGECP	7485.1
	HLA-DRB1*07	pep 7	37-53	LEKNVTVTHSVNLLEDS	13.6
		pep 45	262-278	GNLIAPWYAFALSRGFG	1.6
		pep 46	268-284	WYAFALSRGFGSGIITS	1.6
		pep 52	304-320	SSLPFQNVHPVTIGECP	13.6
	HLA-DQB1*02	NA			NA
# 10 (Fig. 7C)	HLA-DRB1*07	pep 22	126-142	SSFERFEIFPKESSWPN	2641.5
		pep 45	262-278	GNLIAPWYAFALSRGFG	1.6
		pep 46	268-284	WYAFALSRGFGSGIITS	1.6
		pep 52	304-320	SSLPFQNVHPVTIGECP	13.6
	HLA-DRB1*11	pep 22	126-142	SSFERFEIFPKESSWPN	50.6
		pep 45	262-278	GNLIAPWYAFALSRGFG	104.5
		pep 46	268-284	WYAFALSRGFGSGIITS	104.5
		pep 52	304-320	SSLPFQNVHPVTIGECP	2981.4
	HLA-DQB1*02	NA			NA
	HLA-DQB1*03	NA			NA
# 11 (Fig. 7D)	HLA-DRB1*03	pep 7	37-53	LEKNVTVTHSVNLLEDS	478.4
		pep 45	262-278	GNLIAPWYAFALSRGFG	53285.8
		pep 46	268-284	WYAFALSRGFGSGIITS	61616.3
		pep 52	304-320	SSLPFQNVHPVTIGECP	7485.1
	HLA-DRB1*11	pep 22	126-142	SSFERFEIFPKESSWPN	50.6
		pep 45	262-278	GNLIAPWYAFALSRGFG	104.5
		pep 46	268-284	WYAFALSRGFGSGIITS	104.5
		pep 52	304-320	SSLPFQNVHPVTIGECP	2981.4
	HLA-DQB1*02	NA			NA
	HLA-DQB1*03	NA			NA

Peptides were predicted by the algorithm in the web: (http://tools.immuneepitope.org/analyze/cgi-bin/mhc_II_binding.py).

NA: alleles are not available in the algorithm.