Immune Dysregulation by the Rheumatoid Arthritis Shared Epitope

Denise E. De Almeida, Song Ling, Xiujun Pi, Anne M. Hartmann-Scruggs, Paul Pumpens and Joseph Holoshitz

J Immunol published online 30 June 2010
http://www.jimmunol.org/content/early/2010/06/30/jimmunol.0904002

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/30/jimmunol.0904002.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Immune Dysregulation by the Rheumatoid Arthritis Shared Epitope

Denise E. De Almeida,* Song Ling,*† Xiujun Pi,* Anne M. Hartmann-Scruggs,* Paul Pumpens,† and Joseph Holoshitz*

Rheumatoid arthritis (RA) is closely associated with HLA-DRB1 alleles that code a five-amino acid sequence motif in positions 70–74 of the HLA-DRβ-chain, called the shared epitope (SE). The mechanistic basis of SE–RA association is unknown. We recently found that the SE functions as an allele-specific signal-transducing ligand that activates an NO-mediated pathway in other cells. To better understand the role of the SE in the immune system, we examined its effect on T cell polarization in mice. In CD11c+CD4+ dendritic cells (DCs), the SE inhibited the enzymatic activity of indoleamine 2,3 dioxygenase, a key enzyme in immune tolerance and T cell regulation, whereas in CD11c+CD8+ DCs, the ligand activated robust production of IL-6. When SE-activated DCs were cocultured with CD4+ T cells, the differentiation of Foxp3+ regulatory cells was suppressed, whereas Th17 cells were expanded. The polarizing effects could be seen with SE* synthetic peptides, but even more so when the SE was in its natural tridimensional conformation as part of HLA-DR tetrameric proteins. In vivo administration of the SE ligand resulted in a greater abundance of Th17 cells in the draining lymph nodes and increased IL-17 production by splenocytes. Thus, we conclude that the SE acts as a potent immunostimulatory ligand that can polarize T cell differentiation toward Th17 cells, a T cell subset that was recently implicated in the pathogenesis of autoimmune diseases, including RA. The Journal of Immunology, 2010, 185: 000–000.

*Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI 48109; †Latvian Biomedical Research and Study Center, Riga, Latvia

Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to joint destruction and early death (1, 2). The pathogenesis of the disease is not fully understood, but previous studies showed that RA is closely associated with HLA-DRB1 alleles that code a five-amino acid sequence motif in residues 70–74 of the DRβ-chain (3, 4), commonly referred to as the shared epitope (SE). The disease in SE+ patients begins earlier and is more erosive than that in SE– individuals (5). The mechanism underlying the effect of SE in RA is unclear. Based on the known role of MHC molecules in Ag presentation, the prevailing hypotheses postulate that presentation of antigenic self-peptides (6), molecular mimicry with foreign Ags (7), and/or T cell repertoire selection (8) are involved.

Although these hypotheses are plausible, evidence to support them is inconclusive. We recently discovered a novel functional role of the SE: acting as a signal-transduction ligand that activates innate immune signaling in other cells. Our data showed that whether expressed in its native conformation on the cell surface, as a cell-free HLA-DR tetrameric molecule, engineered into large recombinant proteins, or as a short synthetic peptide, the SE activated NO-mediated signaling in trans in a strictly allele-specific manner (9–11).

SE-triggered signaling is transduced via cell surface calreticulin (CRT) (12), a known innate immunity receptor (13) that is expressed on the surface of many cells (14, 15). CRT serves as the signal-transducing receptor for members of the collectin family and other innate immune system ligands (16). Importantly, CRT plays a pivotal role in the junction between tolerance and autoimmunity as a result of its critical role in the elimination of apoptotic cells (17). Ablation activation of the CRT-mediated pathway can lead to autoimmunity, as exemplified by conditions that involve defective CRT-mediated clearance of apoptotic cells (18).

CRT is expressed on dendritic cells (DCs), which are believed to play a role in the pathogenesis of RA (19). DCs are strategically positioned in the interface between the innate and adaptive immune systems. In addition to their Ag-presentation role, DCs induce tolerance through cross-talk with regulatory T (Treg) cells) (20).

To gain insights into the role of the SE in immune regulation, we examined its functional effects on DCs. We show that the SE inhibits IDO activity in the CD11c+CD8+ subset of murine DCs and increases IL-6 production by CD11c+CD8− DCs. This leads to enhanced differentiation and expansion of Th17 cells with a reciprocal effect on Treg cells.

Materials and Methods

Mice and reagents

All mice were from The Jackson Laboratory (Bar Harbor, ME). Experiments were carried out in 5–10-wk-old male DBA/1, BALB/c, or
C57BL/6 mice or a DBA/1 mouse line carrying transgenic (Tg) collagen type II (CII)-specific TCR (D1Lac.Cg-Tg[TCRa,TCRb]24Efro/J). For brevity, the latter mouse line is designated in this article as CII-TCR Tg. The animals were housed in the University of Michigan Unit for Laboratory Animal Medicine facility. All experiments were performed in accordance with protocols approved by the University of Michigan Committee on Use and Care of Animals. mouse against mouse CD3 (clone 2C11), IL-2 (clone 1B11), IFN-γ (clone R46A2), and IL-2 (clone 5A6B) were purified from the supernatants of hybridomas obtained from the University of Michigan Hybridoma Core Facility. Purified anti-mouse CD28 (clone 37.51) and murine rIL-2 were purchased from eBioscience (San Diego, CA). Human rTGF-β and rIFN-γ, as well as murine rIL-2, rIFN-γ, GM-CSF, and rIL-6, were purchased from PeproTech (Rocky Hill, NJ). DCs were purified and HPLC purified to >90% by the University of Michigan Protein Structure Facility, as previously described (9, 12). SE-expressing 15mer peptides, designated as 65-79*0401 (aa sequence 65-KDLLEQKRAA VDTYC-79) or 65-79*0402 (aa sequence 65-KDLLEQKRAAVDTYC-79) and 65-79*0403 (aa sequence 65-KDLLEQKRAAVDTYC-79), corresponded to the HVR3 of the DRβ-chain encoded by SE* HLA-DRB1*0401 or HLA-DRB1*0404 alleles, respectively. Control 15mer peptides 65-79*0402 (65-KDIDELEERAAVDTYC-79) and 65-79*0403 (65-KDILLEQKRAAVDTYC-79) corresponded to the third alllic hypervariable region (HVRV3 of the DRβ-chain encoded by SE* HLA-DRB1*0402 or HLA-DRB1*0403 alleles, respectively. The CD25-27peptide, which corresponds to residues 259-273 of chicken CII, was kindly provided by Dr. Steven Lundy (University of Michigan, Ann Arbor, MI).

Chronic experimental arthritis (CEA) was performed in the BALB/c mice as previously described (26). Unless stated otherwise, all chemicals were from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of cells

Murine L cell transfectants expressing human HLA-DR/β heterodimers (29) and human fibroblast line M1 (9) were maintained as previously described.

For generation of CD11c+ DCs, mouse bone marrow cells were plated in culture flasks (2 × 106 cells/ml per T150; Costar, Cambridge, MA) in RPMI 1640 medium containing 2 mM L-glutamine, 10% FBS, 1% penicillin-streptomycin, 10 mM HEPES buffer solution, 10 mM sodium pyruvate, 50 mM 2-ME, streptomycin, 10 mM HEPES buffer solution, 10 mM sodium pyruvate, and 50 mM 2-ME. GM-CSF (10 ng/ml), GM-CSF (10 ng/ml), and IL-4 (10 ng/ml). On day 3, half of the medium was removed, and fresh medium containing GM-CSF (10 ng/ml) and IL-4 (10 ng/ml) was added. After 5–7 d, DCs were purified using positive-selection columns with CD11c microbeads (Miltenyi Biotec, Auburn, CA), as previously described (30). For preparation of CD11c+CD8+ and CD11c+CD8− DCs, freshly isolated splenic DCs were subjected to positive selection with CD11c and CD8α microbeads. Purified DC subsets were then cultured in RPMI 1640 medium containing 2 mM L-glutamine, 10% FBS, 1% penicillin-streptomycin, 10 mM HEPES buffer solution, 10 mM sodium pyruvate, and 50 mM 2-ME. CD4+ T cells were isolated from the spleen, using a negative-selection immunomagnetic isolation kit (EasySep; StemCell Technologies, Vancouver, B.C., Canada), according to the manufacturer’s instructions. To purify CD11c+CD25−CD62L−CD4+ naive T cells, CD4+ T cells were incubated with FITC anti-mouse CD4 and a mixture of PE-labeled anti-CD25, allopurinol-treated labeled anti-CD62L, and Per-1G7-labeled anti-CD44 Abs (all from BioLegend, San Diego, CA). CD4+CD25−CD62L−CD44+ naive T cells were sorted using a FACSDiva instrument (BD Biosciences, San Jose, CA) with a purity >98%.

Measurement of NO production, IDO activity, and cytokine secretion

To determine the rate of NO production, cells were loaded with 20 μM the fluorescent NO probe 4,5-diaminofluorescein diacetate, and the fluorescence level was recorded every 5 min for 500 min using a Fusion αHT system (PerkinElmer, Wellesley, MA) at an excitation wavelength of 488 nm and an emission wavelength of 515 nm. To determine IDO enzymatic activity, the generation of its product, kynurenine, was measured, as previously reported (31). Cytokine concentrations were measured in cell culture supernatants using a Lumineux platform (Millipore, Bedford, MA). In some experiments, cytokines were determined using ELISA (Quantikine; R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.

Determination of surface CRT expression on DCs

Splenocytes were isolated from the spleen of naïve C3H/HeJ mice or naïve C3H/HeJ mice. Lymphocytes were stained with the FITC-conjugated anti-CD11c mAb and PE-conjugated anti-CD11b mAb, and analyzed by a FACSCalibur flow cytometer using CELLQuest software (BD Biosciences).

Th17 differentiation

Bone marrow-derived DC11c+ DCs (2.5 × 106 cells/ml per well) were cultured overnight in 24-well plates with or without SE ligands or controls, as above. Then, 5 × 105 naive CD4+ T cells or CD4+CD25−CD62L−CD44+ naive T cells were added at the ratio of 2:1 in the presence of a Th17-polarizing cytokine mixture containing anti–IL-12 (2 μg/ml), anti–IFN-γ (2 μg/ml), anti–IL-17 (5 μg/ml), recombinant human IL-6 (20 ng/ml), recombinant murine IL-23 (10 ng/ml), anti–CD3 (5 μg/ml), and anti–CD28 (1 μg/ml), as previously described (32). After 6 d, cells were stimulated with PMA (5 ng/ml) and ionomycin (1 μg/ml) for 6 d, followed by fixation and permeabilization using a Cytofix/Cytoperm kit. Intracellular staining was performed using PE-conjugated anti-human IL-17A mAb (clone TC11-18H 10.1; BioLegend). Mean fluorescence intensity and percentages of stained cells were determined by flow cytometry.

Proliferation assays

Cells were stimulated with 1 μM CFSE (Molecular Probes, Eugene, OR), stained with CD4–PerCP, CD25–PE, Foxp3–allophycocyanin, or IL-17A–allophycocyanin Abs (BioLegend), and proliferation was determined by measuring the percentages of CFSE-labeled cycling CD4+ T cells, CD4+CD25 Foxp3+ Treg cells, or CD4+IL17A+ Th17 cells, using FACS analysis.

Determination of the SE polarizing effect in vivo

Mice were injected s.c. in the footpad with 100 μg mouse CII (Chondrex, Redmond, WA) emulsified in CFA (4 mg/ml). The inoculums contained 100 μg CII*65-79*0401 or SE*65-79*0402 ligands in PBS or an equal volume of PBS alone. Animals were sacrificed 7 d after immunization. For Th1/Th17-quantification studies, inguinal and popliteal lymph nodes were collected, and single-cell suspensions were prepared. Unfractionated lymph node cells were cultured with PMA, ionomycin, and brefeldin A for 6 h. Cells were stained with FITC anti-mouse CD4 or isotype control (BioLegend), followed by fixation and permeabilization using a Cytofix/Cytoperm kit. Intracellular staining was performed using PE-conjugated anti-mouse IL-17A mAb (clone Tc11-18H 10.1; BioLegend). Mean fluorescence intensity and percentages of stained cells were determined by flow cytometry.

Data analysis

A two-tailed Student t test was used.
Results

The SE inhibits IDO activity

In previous studies, we demonstrated that the SE ligand indiscriminately activates NO signaling in different cell lineages from several species (9–12). As a prelude to investigating the immune-regulatory effect of the SE, we confirmed that it activates NO signaling in DCs. As expected, the SE activated robust NO production in CD11c+ DCs from several mouse strains in a strictly allele-specific manner (Supplemental Fig. 1). Thus, similar to its effect in many other cell lineages, the SE also activates NO signaling in mouse DCs.

Given the known inhibitory effect of NO on IDO activity (24), we next examined whether the SE could affect IDO enzymatic activity. In addition to a small subset of DCs (33), IDO is expressed in several other cell lineages, including fibroblasts. Given the much greater abundance of fibroblasts over IDO-producing DCs, we first determined the effect of the SE on IDO activity in murine fibroblast L cell transfectants expressing functionally and structurally intact HLA-DRα/β heterodimeric molecules on their surface through cDNA transfection (29). As seen in Fig. 1A, transfectants expressing SE+ HLA-DR molecules on their surface (lines L-565.5 and L-300.8, expressing SE+ DRβ 0401 or DRβ 0404 molecules, respectively) produced significantly less kynurenine in response to IFN-γ compared with transfectants expressing SE− HLA-DR molecules (lines L-514.3 and L-259.3 expressing SE− DRβ 0402 or DRβ 0403 molecules, respectively). An identical pattern was seen when M1 fibroblasts were stimulated with SE peptidic ligands 65–79*0401 or 65–79*0404. As seen in Fig. 1B, the SE ligands strongly inhibited IFN-γ–induced IDO activity. SE− controls 65–79*0402 and 65–79*0403 did not inhibit IDO activity. Consistent with previous studies (24, 34) the NO donor S-nitrosoacetylpenicillamine (SNAP) also inhibited IDO activity. Thus, these results demonstrate that the SE, whether physiologically expressed on the cell surface or added as a cell-free ligand, effectively and specifically inhibits the activity of the tolerogenic enzyme IDO in human and murine cells.

To proceed with the studies reported in this article that focused on DBA/1 mice, we confirmed (Fig. 1C) that IFN-γ–induced IDO activity in this strain is found in CD11c+CD8+ DCs but not in CD11c−CD8− DCs, similar to published data for other strains (33). To examine the effect of maturation on IDO activity in DBA/1 mice, CD11c+CD8+ DCs were incubated for 24 h with or without LPS (1 μg/ml), and IFN-γ–induced IDO activity was determined. As shown in Fig. 1D, activation of IDO in immature DCs was significantly more potent than in mature cells. We also confirmed that, similar to other mouse strains, IDO activation in DBA/1 mice is inhibited by NO by demonstrating that IFN-γ–induced IDO activity in DBA/1 immature CD11c+CD8+ DCs is potently inhibited by the NO donor SNAP (Fig. 1E). Finally, to determine the effect of the SE on IDO activity in DCs, DBA/1 immature CD11c+CD8+ DCs were preincubated with HBC particles engineered to express the HVR3 (residues 65–79) encoded by the SE+ allele DRβ1*0401 (designated HBc*0401) or the HVR3 encoded by the SE− allele DRβ1*0402 (HBc*0402). Cells were then stimulated with IFN-γ, and IDO activity was determined, as above. As seen in Fig. 1F, SE+ HBc*0401, but not SE− HBc*0402, particles significantly inhibited IDO activity in DCs. Thus, our data indicate that the SE ligand inhibits IDO activity in CD11c+CD8+ DCs.

Cytokine production by SE-stimulated DCs

In addition to IDO-mediated T cell regulation, DCs can affect immune responses by producing cytokines capable of activating or expanding particular subsets of T cells, thereby polarizing the immune response. For example, in mice, the combination of IL-6 and TGF-β facilitates differentiation of Th17 cells, whereas IL-23 is involved in the expansion of this subset (35). To determine whether SE-activated signaling in DCs induces cytokine production, we studied supernatants of SE-stimulated DCs. As seen in Fig. 2, the SE ligand 65–79*0401 activated robust production of IL-6 in

FIGURE 1. Inhibition of IDO activity by the SE ligand. A, Murine L cells expressing SE+ (L-565.5 or L-300.8) or SE− (L-514.3 or L-259.3) functional HLA-DR molecules on their surface through cDNA transfection were incubated for 48 h with recombinant human IFN-γ (1000 U/ml), and cellular IDO activity was determined. *p < 0.001. B, M1 fibroblasts were incubated overnight with medium, NO donor SNAP, or 100 μg/ml of SE ligands (65–79*0401 or 65–79*0404), or SE− controls (65–79*0402 or 65–79*0403). Cells were cultured for 48 h with recombinant human IFN-γ, and cellular IDO activity was determined. *p < 0.005. C, CD11c+CD8+ and CD11c−CD8− DC subsets were purified from DBA/1 spleens, and their IDO activity in response to IFN-γ was determined. *p < 0.005. D, CD11c+CD8+ DCs were purified from DBA/1 spleens, matured or not with LPS, and their IDO activity in response to IFN-γ was determined. E, DBA/1 splenic CD11c+CD8+ DCs were activated, with or without IFN-γ, in the presence or absence of the NO donor SNAP. IDO activity was determined at 48 h, as above. F, DBA/1 splenic CD11c+CD8+ DCs were preincubated for 1 h with or without HBC particles engineered to express the 65–79 region of DRβ-chains, encoded by SE+ (HBc*0401) or SE− (HBc*0402) HLA-DRB1 alleles. DCs were subsequently stimulated with IFN-γ, and IDO activity was determined as above.
CD11c⁺CD8⁻ DCs but not in the CD11c⁺CD8⁺ subset. IL-6 levels peaked at a relatively early time point (24 h) and declined later. This pattern is likely a result of the short t₁/₂ of the peptidic ligand due to rapid degradation in tissue culture conditions. The SE control 65-79*0402 did not trigger any cytokine production. Other cytokines (IL-4, IL-10, IL-12, IL-1β, and TGF-β) did not show increased production, attesting to the specificity of the SE effect (Fig. 2). Thus, although the SE inhibited IDO activity in CD8⁺ DCs (Fig. 1), its IL-6–production effect was restricted to the CD8⁻ DC subset (Fig. 2). The reason for this dichotomy is unknown, but it may be attributed to differential abundance of the receptor CRT on the surface of these two DC subsets (Supplemental Fig. 2).

SE-activated IL-6 production was noted in CD11c⁺CD8⁻ DCs only when they were separated from the CD11c⁺CD8⁺ subset but not when unfractionated CD11c⁺ DCs were assayed (Fig. 2). This could be explained by the fact that although CD11c⁺CD8⁺ DCs are a small subset (∼5–15% of splenic CD11c⁺ cells), once activated by the SE, they could exert potent inhibitory effects on the activation of CD11c⁺CD8⁻ DCs, consistent with previously reported suppressive effects of DCs (36). Recent studies showed that IDO produced by a small subset of DCs can dominantly suppress the production of IL-6 in other DCs (data not shown). However, in preliminary studies using pharmacologic IDO inhibitors, we could not reverse the apparent suppressive effect of IDO-producing DCs (37, 38). To determine whether the SE interferes with Treg cell differentiation or expansion, we previously observed that peptidic SE ligands exert weaker signaling effects as a result of their flexible conformation in solution (9, 10, 12). To address this possibility, similar Treg cell-differentiation experiments were performed using SE⁺ HLA-DR tetramer (designated T-DRB1*0401) or control SE-negative HLA-DR tetramers (T-DRB1*1501 or T-DRB1*0301) instead of soluble peptides. The HLA-DR molecule in tetramers is folded in its natural tridimensional conformation; therefore, it better preserves the physiologic function of the protein. Fig. 4C shows that the SE⁺ tetramer T-DRB1*0401 had a specific and much more potent inhibitory effect on Treg cell differentiation.

FIGURE 2. SE activates IL-6 production in CD8⁻ DCs. DBA/1 splenic unfractionated CD11c⁺ DCs (CD11c⁺) or their purified CD11c⁺CD8⁻ DCs (CD8⁻) or CD11c⁺CD8⁺ DCs (CD8⁺) subsets were cultured with the SE ligand 65-79*0401 or SE⁺ control 65-79*0402 or medium. Supernatants were collected at different time points and assayed for cytokine content using a Luminex platform. Data are from a representative experiment, one of three replications. * p < 0.05.

IL-23 levels in DCs did not increase following stimulation with the SE ligand (Fig. 3). However, in the presence of LPS (100 ng/ml), the SE had a prolonged synergistic effect in CD11c⁺ DCs. The SE had no effect when applied alone, but in the presence of LPS it had a synergistic effect that lasted for up to 72 h after stimulation, long after the LPS effect had subsided (Fig. 3). The effect was specific for IL-23, because no synergism was found in the production of another LPS-inducible cytokine, IL-6 (Fig. 3, lower panel).

Inhibition of Treg cell differentiation by the SE

It was demonstrated that IDO inhibition (39) or increased IL-6 levels (40) inhibit Treg cells. As shown above, the SE inhibited IDO activity in CD11c⁺CD8⁻ DCs and increased IL-6 production in CD11c⁺CD8⁻ DCs. Therefore, our next step was to determine whether the SE interferes with Treg cell differentiation or expansion. Accordingly, DBA/1 CD11c⁺ DCs were first incubated overnight with the SE ligand 65-79*0401, SE⁻ control 65-79*0402, or with medium. DCs were then cocultured with purified syngeneic CD4⁺ T cells (Fig. 4A) or CD4⁺CD25⁺CD62L⁻CD44⁻ naive T cells (Fig. 4B) in the presence of TGF-β (2.5 ng/ml) and anti-CD3 Abs (5.0 μg/ml). After 5 d, CD4⁺CD25⁺Foxp3⁺ Treg cell abundance was determined by flow cytometry. The SE ligand 65-79*0401 significantly inhibited Treg cell expansion (Fig. 4A) and differentiation (Fig. 4B). The inhibitory effect of 65-79*0401 on Treg cell differentiation was statistically significant, yet modest. We previously observed that peptidic SE ligands exert weaker signaling effects as a result of their flexible conformation in solution (9, 10, 12). To address this possibility, similar Tcell-differentiation experiments were performed using SE⁺ HLA-DR tetramer (designated T-DRB1*0401) or control SE-negative HLA-DR tetramers (T-DRB1*1501 or T-DRB1*0301) instead of soluble peptides. The HLA-DR molecule in tetramers is folded in its natural tridimensional conformation; therefore, it better preserves the physiologic function of the protein. Fig. 4C shows that the SE⁺ tetramer T-DRB1*0401 had a specific and much more potent inhibitory effect on Treg cell differentiation.

SE-activated DCs facilitate Th17 differentiation

As shown above, in the CD11c⁺CD8⁻ DC subset, the SE ligand 65-79*0401 triggered a robust production of IL-6, an obligatory cytokine for Th17 differentiation. IL-23 production by LPS-treated DCs was also augmented by 65-79*0401. Therefore, we next determined whether the SE ligand can facilitate Th17 differentiation or activation. To determine whether the SE affects Th17

FIGURE 3. SE augments IL-23 production in LPS-stimulated CD11c⁺ DCs. DBA/1 bone marrow-derived CD11c⁺ DCs were cultured with or without 100 ng/ml LPS in the presence or absence of SE⁺ or SE⁻ 15mer peptides (50 μg/ml). Supernatants were collected at different time points and assayed for IL-23 and IL-6 content by ELISA. Data shown are representative of two replicate experiments. * p < 0.05.
differentiation, CD11c+ DCs were stimulated overnight with peptide or tetrameric ligands, as above. Next, CD4+CD25−CD62L−CD44+ naive T cells were added and cultured in the presence of a Th17-polarizing mixture of cytokines and Abs, as discussed in the Materials and Methods. After 6 d, cells were collected and analyzed by flow cytometry. As seen in Fig. 5A, the SE ligand 65-79*0401 induced a significant increase in the differentiation of CD4+IL17A+ T cells. A more robust SE-induced Th17 polarization effect was seen when DCs were stimulated with SE + HLA-DR tetramers (Fig. 5B).

To determine whether SE-activated DCs can increase IL-17 production in CD4+ T cells, CD11c+ DCs were incubated overnight with SE ligands or control reagents. Cells were then cocultured with total CD4+ T cells. As shown in Fig. 6, SE-activated DCs induced greater intracellular (Fig. 6A) and extracellular (Fig. 6B) IL-17 expression in cocultured CD4+ T cells compared with cells cultured with DCs preincubated with a control reagent or medium. The effect was seen with a soluble SE peptidic ligand, as well as with a conformationally preserved ligand in the form of an Hbc particle (Fig. 6B).

We also found that the SE affected the proliferation of Th17 and Treg cells in a reciprocal manner. Enhanced expansion through increased proliferative activity of Th17 cells and decreased proliferative activity of Treg cells was seen with TCR-independent (Supplemental Fig. 3) and TCR-mediated (Supplemental Fig. 4) T cell activation conditions.

Finally, to determine the biologic significance of the in vitro data shown above, we characterized the SE-polarizing effect in vivo (Fig. 7). In draining lymph nodes of control DBA/1 mice immunized with CII, the abundance of Th17 cells was 0.65%, consistent with published data showing frequencies for Th17, 1% in draining lymph nodes (41, 42). Coadministration of the SE ligand 65-79*0402 had no effect on Th17 abundance. However, coadministration of

**FIGURE 5.** SE facilitates Th17 differentiation. DBA/1 bone marrow-derived CD11c+ DCs were cultured overnight in the presence or absence of SE ligand 65-79*0401 or SE− control 65-79*0402 (A) or SE− T-DRB1*0401 tetramers versus SE− T-DRB1*0301 or T-DRB1*1501 tetramers (B). Syngeneic splenic CD4+CD25−CD62L−CD44− naive T cells plus a Th17-differentiation cytokine/Ab mixture were added to the culture and incubated for 6 d. Intracellular IL-17A was determined by flow cytometry. Left panels, a representative experiment of three repetitions, showing the percentages of CD4+IL17A+ cells as dot plots. Right panels, bar graphs show the data as the percentage (mean ± SD) of replicate samples. *p < 0.001.

**FIGURE 4.** SE inhibits Treg cell generation. A, DBA/1 bone marrow-derived CD11c+ DCs were cultured overnight with 50 μg/ml SE ligand 65-79*0401, SE− control 65-79*0402, or medium. Syngeneic splenic CD4+ T cells were added to the culture and incubated with anti-CD3 and TGF-β for 5 d. Left panels, flow-cytometry dot plots show the percentages of CD25Foxp3+ cells obtained from gated CD4+ T cells in each treatment. Each plot is representative of three experiments. Right panel, bar graphs show the results as the percentage (mean ± SD) of replicate samples. *p < 0.01, compared with medium and 65-79*0402. B, Cultures were performed as in A, with the exception that CD4+CD25−CD62L−CD44− naive T cells, instead of CD4+ T cells, were added to the CD11c+ DCs. *p < 0.05, compared with medium and 65-79*0402. C, DBA/1 bone marrow-derived CD11c+ DCs were incubated overnight with 2 μg/ml tetramers (SE− T-DRB1*0401 versus SE− T-DRB1*0301 or T-DRB1*1501). Syngeneic CD4+CD25−CD62L−CD44− naive T cells, anti-CD3, and TGF-β were added to the culture, incubated for 5 d, and analyzed as above. *p < 0.0005, compared with medium, T-DRB1*0301, and T-DRB1*1501.
the SE ligand 65-79*0401 dramatically increased the frequency of these cells; the SE-induced expansion was specific for Th17 cells because there was no change in the frequency of Th1 (IFN-γ+) cells (Fig. 7A). Additionally, splenocytes from DBA/1 mice immunized with CII in the presence of the SE ligand 65-79*0401 showed significantly more robust CII-stimulated IL-17 production compared with splenocytes obtained from mice coimmunized with the SE2 control 65-79*0402 (Fig. 7B). Thus, when taken collectively, our data indicate that the SE facilitates Th17 polarization in vitro and in vivo (Fig. 8).

Discussion

In prior studies we demonstrated that the SE activates innate immune signaling in many cell lineages (12). In this study, we showed that when murine DCs were exposed to the SE ligand, they enhanced Th17 differentiation and inhibited the generation of Treg cells. The SE effect in DCs was allele specific and particularly potent when the ligand was presented in its physiologic tridimensional conformation.

The functional dichotomy between DC subsets relative to their response to the SE ligand is intriguing. As shown in this study, in CD11c+CD8- DCs, the SE triggered robust production of the proinflammatory and immunostimulatory cytokine IL-6, whereas in CD11c+CD8+ DCs it inhibited induction of IDO. These data demonstrate that identical ligands can produce markedly different functional effects in different cellular contexts. The variability of the functional response might be attributed to differential abundance of the receptor, as suggested in Supplemental Fig. 2, or modulatory effects by other signaling pathways. Such factors could vary markedly among different lineages in different tissue sites. Specifically relevant to immune regulation, the data in this study reiterate the known functional heterogeneity of different DC subsets and add new insights into the distinct effects of the SE in...
those subsets. Several studies showed that IDO is selectively expressed by a small subset of murine plasmacytoid DCs that express the B cell marker CD19 (43, 44). The relevance of that small DC subset to the findings shown in this article is unknown. More research is needed to dissect the fine phenotypic and functional characteristics of different DC subpopulations relative to their role in SE-triggered IDO inhibition.

Although the SE ligand alone did not activate IL-23 production in DCs in our in vitro experiments, it produced a potent synergistic effect in the presence of low concentrations of LPS (Fig. 4). The effect was specific for the SE ligand, because a SE− control agent failed to produce it. The synergistic effect with LPS shown in this article is reminiscent of the LPS effect on high molecular group binding protein-mediated activation (45). Different from that synergism, however, the SE–LPS effect in our study selectively involved IL-23 production but not that of IL-6. Because IL-23 induces Th17 cell expansion, it is tempting to speculate that under certain costimulatory in vivo conditions, the SE may accelerate pathogen-triggered expansion of Th17 cells.

To simulate stringent structural conditions, the T cell-polarization studies were repeated with physiologically folded SE ligands in the form of tetrameric molecules (Figs. 4C, 5B). Tetramers are made of four identical units of the HLA-DR heterodimeric molecule, each folded in its native tridimensional conformation. To assure consistency among different tetramers, they were all loaded with identical groove peptide, CLIP (26). Likewise, SE-expressing HBc particles have been engineered to allow the SE to preserve its native α helical structure outside of the HLA-DR molecule context and without the presence of groove peptide (10). Therefore, the significance of the experiments using these reagents is 2-fold: they demonstrate high consistency among data generated with the SE in several formulations and, most importantly, given their intact tridimensional conformation, the results attest to the physiologic relevance of the findings.

The data presented in this article provide the first direct indication that the SE may be acting as an immune-stimulatory ligand. The study demonstrates that the SE, independent of groove peptides, activates pathogenically relevant immune-system events. In addition to its generic Ag-independent Th17 polarization effects, the SE particularly facilitates Ag-specific immune responses. One scenario to consider is that the SE could allow promiscuous augmentation of various Ag-specific immune responses and increase their autoimmune potential. Depending on genetic and environmental cofactors, the nature of the specific Ags, and their tissue distribution, the SE could conceivably facilitate the onset of distinct autoimmune diseases. This scenario could provide a plausible explanation for the counterintuitive, promiscuous association of the SE, a highly allele specific and particularly potent with physiologically folded ligands. Studies are underway to determine whether the polarizing effect of the SE ligand can facilitate autoimmunity in mice.

Acknowledgments
We thank the National Institutes of Health Tetramer Core Facility for providing tetrameric HLA-DR reagents. We also thank Laura Tesmer for technical advice and Dr. Steven Lundy for providing CII-TCR Tg mice and a CII synthetic peptide.

Disclosures
The authors have no financial conflicts of interest.

References