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Immune Dysregulation by the Rheumatoid Arthritis Shared Epitope

Denise E. De Almeida,*1 Song Ling,*1 Xiujun Pi,* Anne M. Hartmann-Scruggs,* Paul Pumpsen,† 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+CD8+ 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+ T 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.

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Abbreviations used in this paper: CII, collagen type II; CRT, calreticulin; DC, dendritic cell; HBc, hepatitis B core; HVR3, third allelic hypervariable region; RA, rheumatoid arthritis; SE, shared epitope; SNAP, 5-nitrosocarbamylpenicillamine; Tg, transgenic; Treg, regulatory T.

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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...
Polarization by the Shared Epitope

C57Bl/6 mice or a DBA/1 mouse line carrying transgenic (Tg) collagen type II (CII)-specific TCR ([D1Luc.Cg-Tg[Tcra.Tcrb]24Efolo]). For brevity, the latter mouse line is designated in this article as CH-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.

mAbs against mouse CD3 (clone 2C11), IL-4 (clone 11B11), IFN-γ (clone R46A2), and IL-2 (clone S4B6) were purified from the supernatants of hybridomas obtained from the University of Michigan Hybriddoma Core Facility. Purified anti-mouse CD28 (clone 37.51) and mirlne rL-23 were purchased from eBioscience (San Diego, CA). Human rTGF-β and rIFN-γ, as well as murein rL-4, rIFN-γ, rGM-CSF, and rL-6, were purchased from PeproTech (Rocky Hill, NJ).

Cells were synthesized 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-KDLLEQKRAVDTYC-79) or 65-79*0404 (aa sequence 65-KDLLEQRRQRAVDTYC-79), corresponded to the third allelic hypervariable region (HVR3) of the DRβ-chain encoded by SE* HLA-DRB1*0401 or HLA-DRB1*0404 alleles, respectively. Control 15mer peptides 65-79*0402 (65-KDILEDERAADVTYC-79) and 65-79*0403 (65-KDLLEQRRQRAVDTYC-79) corresponded to the third allelic hypervariable region (HVR3) of the DRβ-chain encoded by SE* HLA-DRB1*0402 or HLA-DRB1*0403 alleles, respectively. The CD59–271 peptide, which corresponds to residues 259–273 of chicken CII, was kindly provided by Dr. Steven Lundy (University of Michigan, Ann Arbor, MI).

Chimeric hepatitis B core (HBc) particles engineered to express the HVR3 of the HLA-DRβ-chain were prepared as previously described (9) at the Latvian Biomedical Research and Study Center. HBc particles expressing an SE* HVR3, encoded by HLA-DRB1*0401 (designated HBc*0401), or an SE* HVR3, encoded by HLA-DRB1*0402 (designated HBc*0402), were used in this study, as previously described (9). SE* HLA-DR tetramers DRB1*0401/DRA1*0101 (designated T-DRB1*0401), SE* DRB1*1501/DRA1*0101 (T-DRB1*1501), and SE* DRB1*0301/DRA1*0101 (T-DRB1*0301), all containing identical CLIP in the peptide-binding groove, were generated by the National Institutes of Health Tetramer Core Facility (Atlanta, GA), 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-DRβ 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 (2×10^6 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, and 2 μg/ml tetracycline. After 5–7 d, cells were purified using positive-selection columns with CD11c microbeads (Miltenyi Biotec, Auburn, CA), as previously described (30).

Proliferation assays

Cells were labeled 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.

Data analysis

A two-tailed Student t test was used.

Cytokines were determined using ELISA (Quantikine; R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.

Determination of surface CRT expression on DCs

Splenocytes from DBA/1 mice were isolated following purification of DC subsets using positive-selection columns with CD11c and CD8α microbeads. Purified cells were stained for flow-cytometry using PE anti-mouse CD8 (clone 53-6.7; BD Pharmingen, San Diego, CA), FITC anti-rabbit CRT (Affinity BioReagents, Golden, CO), and isotype controls (BioLegend).

Treg cell differentiation

DBA/1 bone marrow-derived CD11c+ DCs were placed in 24-well plates (BD Biosciences) at a density of 2.5×10^5 cells per well and cultured overnight with or without 50 μg/ml peptidic (65-79*0401 or 65-79*0402) or 2 μg/ml tetracycline (T-DRB1*0401, T-DRB1*1501, or T-DRB1*1501) ligands at 37°C. On the following day, 5.0×10^4 CD4+ T cells or CD4+ CD25+CD62L−CD44− naive T cells, isolated as described above, were added to each well in addition to anti-CD3 Abs (5 μg/ml) and recombinant human TGF-β (2.5 ng/ml).

After 5 d in culture, cells were harvested and stained for flow-cytometric analysis using FITC anti-mouse CD4 (clone GK1.5), PE anti-mouse CD25 (clone 3A7), and isotype controls (BioLegend). Next day, cells were permeabilized and fixed using a Cytofix/Cytoperm kit (BD Biosciences), as recommended by the manufacturer. After permeabilization, cells were stained using an allophycocyanin-conjugated anti-mouse FcX3 Ab (clone FLK-165; eBioscience) and analyzed by a FACSCalibur flow cytometer using CELLQuest software (BD Biosciences).

Th17 differentiation

Bone marrow-derived CD11c+ DCs (2.5×10^5 cells per well) were cultured overnight in 24-well plates with or without SE ligands or controls, as above. Then, 5×10^5 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/Abs mixture containing anti-IL-4 (2 μg/ml), anti–IFN-γ (2 μg/ml), anti–IL-2 (3 μg/ml), recombinant human TGF-β (5 ng/ml), recombinant murine 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 μg/ml) and ionomycin (500 ng/ml) for the last 6 h of culture. Brefeldin A (10 μg/ml) was added to the culture for the last 5 h. Cells were then harvested and stained for surface marker using 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 Abs (clone TCL1-18H10.1; BioLegend). Mean fluorescence intensity and percentages of stained cells were determined by flow cytometry.

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 Luminex platform (Millipore, Bedford, MA). In some experiments, cytokines were determined using ELISA (Quantikine; R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.

Cytokine secretion was determined using ELISA (Quantikine; R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.
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-DRαβ 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−CD8α− DCs but not in CD11c+CD8α− DCs, similar to published data for other strains (33). To examine the effect of maturation on IDO activity in DBA/1 mice, CD11c−CD8α− 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−CD8α− 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−CD8α− DCs were preincubated with HbC particles engineered to express the HVR3 (residues 65–79) encoded by the SE+ allele DRB1*0401 (designated HbC*0401) or the HVR3 encoded by the SE− allele DRB1*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+CD8α− 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*0402), 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+CD8α− and CD11c−CD8α− DC subsets were purified from DBA/1 spleens, and their IDO activity in response to IFN-γ was determined. D. CD11c−CD8α− 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−CD8α− 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+CD8α− DCs were preincubated for 1 h with or without HbC particles engineered to express the 65–79 region of DRαβ-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 t_{1/2} 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 (37, 38). However, in preliminary studies using pharmacologic IDO inhibitors, we could not reverse the apparent suppressive effect of IDO-producing DCs (data not shown). More experimental effort is required to conclusively address this question.

**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 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.

**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
differentiation, CD11c+ DCs were stimulated overnight with peptidic 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-hapten 65-79*0402 had no effect on Th17 abundance. However, coadministration of

**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 CD25+Foxp3+ 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.

**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.
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-\(\gamma\)) 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 SE 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 particular.

FIGURE 7. SE facilitates Th17 polarization in vivo. A, DBA/1 mice were immunized with CII in CFA in the presence of PBS, 65-79*0401, or 65-79*0402. On day 7, cells from draining lymph nodes were isolated; cultured for 6 h with PMA, ionomycin, and brefeldin A; and then stained with anti-mouse CD4, IL-17A, and IFN-\(\gamma\), as above. Left panels, representative dot plots showing percentages of IL17A+ and IFN-\(\gamma\)+ cells obtained from gated CD4+ cells. Right panel, bar graphs show results as mean ± SD of duplicate experiments. \(p < 0.001\), compared with medium and 65-79*0402. B, DBA/1 and CII-TCR Tg mice were immunized as in A. On day 7, splenic cells were isolated and cultured with the synthetic peptide CII260–267 (5 \(\mu\)g/ml). Supernatants were collected every 24 h and assayed for IL-17 levels by ELISA. Bar graphs show data as mean ± SD of duplicate samples. \(p < 0.005\).

FIGURE 8. Proposed model of the role of the SE ligand in autoimmunity. The effect of the SE ligand is the end result of its interaction with two DC subsets. The SE ligand, expressed on MHC class II+ cells, interacts with cell surface CRT (green) with resultant NO production. In CD8+ DCs, increased NO levels inhibit the enzymatic activity of IDO, which leads to decrease tryptophan metabolites and reduced generation of Treg cells. In CD8- DCs, the SE ligand activates production of IL-6 and, in the presence of LPS, could also increase production of IL-23. The end result is immune dysregulation that involves increased numbers of Th17 cells and increased production of the proarthritogenic and autoimmunogenic cytokine IL-17.
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 reagent 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 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 specific HVR3 sequence, with many antigenically unrelated human diseases and animal models (discussed in Ref. 9).

Based on the findings of this study, in Fig. 8 we propose a model describing the SE immune-polarizing effects. Given the superior potency of physiologically folded tetramers shown in this study, we propose that the tridimensional conformation of the SE plays a significant role in its immune effect. Accordingly, our model predicts that SE-expressing HLA-DR molecules are most effective in vivo when they are expressed on the cell surface and interact in trans with CRT expressed on other cells. However, it should be clarified that we cannot rule out a scenario in which SE+ HLA-DR molecules that are shed to the circulation (or SE-expressing peptideic fragments of such molecules) may exert similar effects. Additionally, SE–CRT interaction on the same cell surface (in cis) also cannot be ruled out.

Our model predicts that as a result of CRT ligation by the SE, two events take place. 1) In CD11c+CD8+ DCs, the SE ligand inhibits activation of IDO. Consequently, tryptophan catabolism diminishes, with resultant inhibition of Treg cell differentiation. 2) In CD11c+CD8+ DCs, SE-activated signaling leads to overproduction of Th17-promoting cytokines IL-6 and, in the presence of LPS, IL-23. The end result of the SE dual effect on DCs is immune imbalance and Th17 polarization.

The balance between Treg cells and Th17 was shown to play a role in the pathogenesis of inflammatory arthritis. For example, IL-17 was found to enhance angiogenesis, matrix metalloproteinase activation, osteoclastogenesis, leukocyte recruitment, and inflammation (46). Additionally, IL-17–producing cells and IL-17 are abundantly expressed in the RA joint (47, 48), and IL-17 neutralization prevents experimental arthritis development. In contrast, Treg cells were shown to modulate disease severity in the collagen-induced arthritis model (49, 50). Thus, based on the well-documented impact of Treg cell/Th17 balance on autoimmune arthritis, we propose that the mechanism described in this article may contribute to SE-associated RA severity.

In summary, we demonstrated a previously uncharacterized immune polarization by the SE ligand in vitro and in vivo. The effect is 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.

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Disclosures

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References


