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Serum amyloid A induces mitogenic signals in regulatory T cells *via* monocyte activation

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ABSTRACT

Serum amyloid A (SAA) has recently been identified by our group as a mitogen for regulatory T cells (Treg). However, the molecular mechanism by which SAA induces Treg proliferation is unknown. Here we provide evidence that IL-1β and IL-6 are directly involved in the SAA-mediated proliferation of Treg. By engaging its several cognate receptors, SAA induces IL-1β and IL-6 secretion by monocytes and drives them toward an HLA-DR^{hi} HVEM^{lo} phenotype resembling immature dendritic cells, which have been implicated in tolerance generation. This monocyte-derived cytokine milieu is required for Treg expansion, as inhibition of IL-1β and IL-6 abrogate the ability of SAA to induce Treg proliferation. Furthermore, both IL-1β and IL-6 are required for ERK1/2 and AKT signaling in proliferating Treg. Collectively, these results point to a novel mechanism, by which SAA initiates a monocyte-dependent process that drives mitogenic signals in Treg.

1. Introduction

Previous evidence from our laboratory demonstrated that serum amyloid A (SAA), an acute-phase protein, induces SOCS3-regulated, mitogenic signaling pathways in Treg (Nguyen et al., 2011). SAA is normally present in the bloodstream at 0.1 μM. During acute inflammation, SAA is predominantly produced by the liver, increasing plasma SAA levels by 500–1000 fold (Steel and Whitehead, 1994). One condition in which plasma SAA levels increase dramatically during disease activity is systemic juvenile idiopathic arthritis (sJIA). sJIA is characterized by signs and symptoms of systemic inflammation, such as elevated erythrocyte sedimentation rate, fever, rash, serositis, together with arthritis. One complication of chronic, persistent sJIA is amyloidosis, in which amyloid fibrils derived from SAA are deposited in the extracellular space, causing organ dysfunction, which can be fatal (Woo, 2006). Even when sJIA is controlled by anti-inflammatory medication, SAA levels in plasma remain moderately elevated in patients, compared to normal, age-matched controls (Nguyen et al., 2011, and unpublished data from the Mellins laboratory).

The association of elevated SAA with inflammatory states has been observed for decades (Obici and Merlini, 2012), but the functions of SAA are still being elucidated. In the past few years, it has become clear that SAA, acting through its various surface receptors, is involved in immunomodulation (Eklund et al., 2012). SAA induces the synthesis of several cytokines, including IL-1β and IL-6, by monocytes, neutrophils and other cells, and it has chemoattractant properties for neutrophils, monocytes and possibly mast cells, among other functions (Eklund et al., 2012). However, the identities of the mitogens affecting Treg proliferation downstream of SAA are unknown. Here, we found that SAA induced phenotypic changes in monocytes such that they resemble HLA-DR^{hi} HVEM^{lo} immature tolerogenic dendritic cells and identified two classical inflammatory cytokines, IL-1β and IL-6, as critical factors elicited by these cells to induce the proliferation of Treg.

2. Materials and methods

2.1. Animals and in vivo studies

C57BL6/J (male, 8–10 weeks old), purchased from Jackson Laboratory, were injected intra-peritoneally with recombinant human

Abbreviations: CD36, thrombospondin receptor; FPR2, formyl peptide receptor 2; HC, healthy control; HVEM, herpesvirus entry mediator; IL1RA, IL-1 receptor antagonist; PDL1, program death ligand 1; PDL2, program death ligand 2; RAGE, receptor of advanced glycation end-product; SAA, serum amyloid A; sJIA, systemic juvenile idiopathic arthritis; Tanis, selenoprotein S; Teff, effector T cells; Treg, regulatory T cells; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; Veh, vehicle; WT, wild type.

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SAA1 (Peprotech, #300-53, 30 μg in 100 μl PBS) or *E. coli*-derived endotoxin (Sigma Aldrich, #L3024, 0.25 ng in 100 μl PBS). Animals were sacrificed 16–24 h after SAA injection and peritoneal cells were harvested in PBS. Peritoneal cells were subjected to flow cytometric analysis to examine monocyte phenotype. Peritoneal fluid was stored at −80 °C for cytokine analysis. *In vivo* depletion of monocytes was performed with clodronate liposomes (Encapsula); 400 μl of clodronate or empty liposomes were injected intra-peritoneally 24 h before SAA injection.

2.2. Flow cytometry and ELISA

Detection of surface and intracellular proteins was performed with standard protocols from BD Biosciences and Biolegend. Antibodies against mouse proteins: F4/80 (clone BM8), CCR7 (clone 4B12), CD11b (clone M170), CD80 (clone 1610A1), CD115 (clone AFS98); and human proteins: CD3 (clone HIT3), CD4 (clone RPAT4), CD14 (clone M5E2), CD19 (clone HIB19), CD25 (clone BC96), CD40 (clone 5C3), CD83 (clone HB15e), CD86 (clone IT2.2), CD127 (clone A019D5), Foxp3 (clone 206D), IL-1β (clone JK1B1), IL-6 (clone MQ213A4), HLA-DR (clone L243), PDL1 (clone 10F9G2), PDL2 (clone 24F10C12), HVEM (clone 122), CCR7 (clone G043H7) and lineage cocktail (all Biolegend). Antibodies against human-pAKT (clone M8961), pERK1/2 (clone 20A), and pSTAT3 (clone 4PSTAT3) (BD Biosciences). To detect cytokines in plasma, culture supernatant or peritoneal fluid, cytometric bead arrays (BD Biosciences) and ELISAs for IL-1β and IL-6 (R&D System) were used, according to manufacturers' protocols. For blocking SAA receptors, optimal concentrations of neutralizing antibodies against human CD36 (clone 1851G2) (Lifespan Biosciences), TLR2 (clone TL21), TLR4 (clone HTA25), IgG2 control antibody (clone MOPC173) (Biolegend), RAGE (clone 176902) (R&D Systems); and FPR2 antagonist LPG (Avanti Polar Lipids) were experimentally determined (10 μg/ml for blocking antibodies and 10 μM for LPG). Neutralizing reagents were pre-incubated with monocytes for 30 min at 37 °C before exposure to recombinant SAA or sJIA plasma.

2.3. Human plasma preparation

The study was approved by the Institutional Review Board at Stanford University. All subjects (clinical data on Supplementary Table 1) provided informed consent before participating in the study. Plasma was prepared from whole, anti-coagulated blood within 2 h after blood draw. Whole blood samples were centrifuged at 514 g at 25 °C for 5 min to remove cells, and plasma then underwent two additional rounds of centrifugation at 1730 × *g* at 4 °C for 5 and 15 min to remove platelets. Final plasma samples were stored at −80 °C until analysis. Depletion of SAA from plasma samples was performed with anti-human SAA antibody (Abcam, #18713) *via* immunoprecipitation for 4 consecutive rounds. Negative control for depletion experiments was an anti-HLA-DR antibody (BD Biosciences, clone L243), also used for 4 rounds.

2.4. Human cell isolation

$CD4^+$ T cells were purified with $CD4^+$ Rosette Kit (Stemcell Technologies) from buffy coats. The $CD4^+$ T cell fraction was then incubated with anti-CD25 microbeads (Miltenyi Biotech) to isolate $CD4^+CD25^+$ cells. The flow-through fraction after magnetic purification contained $CD4^+CD25^-$ Teff. All procedures were performed according to manufacturers' standard protocols. $CD4^+CD25^+$ T cells were incubated with anti-CD127, anti-CD25, and anti-CD4 antibodies (Biolegend), before undergoing flow cytometric sorting for $CD4^+CD25^{hi}CD127^{lo/-}$ Treg. Purity of sorted cells was confirmed to be higher than 95% by Foxp3 staining (not shown). Cells were rested for 2 h in a 37 °C incubator before being used in suppression assays.

2.5. Suppression assays

In our suppression assay system, heat inactivation completely abrogated the ability of sJIA plasma to induce cell proliferation (data not shown). Therefore, for ^{3}H-thymidine-based suppression assays, autologous Treg and Teff were cultured (together, or alone as controls) at 3750 cells each per 50 μl per well in complete media (RPMI + 10% heat inactivated FBS + 1%L-glutamine, which has been shown to contain no detectable level of SAA) (Cocco et al., 2010) with allogeneic, irradiated (at 5000 rads) CD3-depleted peripheral blood mononuclear cells (antigen presenting cells or APC), at 37,500 cells per 50 μl per well. Anti-CD3 antibodies (BD Biosciences, clone UCHT1) at 5 μg/ml were pre-coated on U-bottom, 96-well plates for 4 h at 37 °C before suppression assays. Media was added, so the final volume in each well was 200 μl. On day 6, cells were pulsed with 1 μCi ^{3}H-thymidine (25 μl) per well and harvested on day 7 with a Tomtec cell harvester. ^{3}H-thymidine incorporation was determined using a 1450 microbeta Wallac Trilux liquid scintillation counter.

For CFSE dilution assay, Treg were labeled with CFSE using Cell Tracer CFSE Cell Proliferation kit (Invitrogen Molecular Probes) at a final concentration of 10 μM, according to manufacturer's instructions. Assays with labeled cells were performed as described for ^{3}H-thymidine-based suppression assays, with flow cytometry analysis to determine proliferation. To assay for expression of different surface and intracellular molecules of APC in mixed cell cultures, the relevant subset was labeled with CFSE prior to suppression-type assays. Cells were pelleted out at various time points and underwent standard flow cytometry staining protocols of the manufacturers.

To evaluate effects of plasma on suppression assays and immune cell cultures, frozen platelet-poor plasma samples were thawed at 25 °C, and debris was removed using sterile 40 μm filters (BD Biosciences). All plasma samples were tested in duplicates or triplicates. To control for variations in suppressive and proliferative potentials of Treg and Teff, respectively, both HC and sJIA plasma samples were used in parallel suppression assays with the same set of purified cells for each round of experiments. In addition, fold change in ^{3}H-thymidine counts per minute in assays with plasma compared to those in complete media alone, was computed to analyze the effects of plasma in suppression assays or stimulation assays. In CFSE assays, percentage of proliferating cells (detected by dye dilution on flow cytometry) was used to analyze the effects of plasma on cell proliferation. Recombinant human IL-1Ra (#80RA010CF), and neutralizing antibodies against human IL-6 (clone 6708), TNF-α (clone 28401), IL-2 (clone 5334), IL-7 (clone 7417), IL-15 (clone 34505), and IgG1 control antibody (clone 11711) (R&D Systems) were used to evaluate the role of these cytokines in suppression assays.

2.6. Statistical analyses

All statistical procedures were performed with Prism software (GraphPad). Data were tested for normality (Koromonov–Smirnov's test) and variance equality (Bartlett's test), before being subjected to appropriate statistical tests (*t*-tests and Mann–Whitney tests for two-group analyses or Kruskal–Wallis for multiple comparisons). Differences with p values <0.05 were considered statistically significant.

3. Results

3.1. SAA-mediated proliferation of Treg is accompanied by the induction of IL-1β and IL-6

We previously observed that Treg proliferate without losing suppressive capacity in co-cultures of Treg, Teff, irradiated