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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-1 β and IL-6 are directly involved in the SAA-mediated proliferation of Treg. By engaging its several cognate receptors, SAA induces IL-1 β and IL-6 secretion by monocytes and drives them towards a 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-1 β and IL-6 abrogate the ability of SAA to induce Treg proliferation. Furthermore, both IL-1 β 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.

Keywords

Serum amyloid A; Regulatory T cells; Monocytes; IL-1 β ; IL-6

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 μ M. 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

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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in plasma remain moderately elevated in patients, compared to normal, age-matched controls (Nguyen et al., 2011, and unpublished data from the Mellins laboratory).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-1 β and IL-6, by monocytes, neutrophils and other cells, and it has chemo-attractant 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-1 β 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

C57BL/6/J (male, 8 to 10 weeks old), purchased from Jackson Laboratory, were injected intra-peritoneally with recombinant human SAA1 (Peprtech, #300-53, 30 μ g in 100 μ l PBS) or E.coli-derived endotoxin (Sigma Aldrich, #L3024, 0.25ng in 100 μ l PBS). Animals were sacrificed 16–24 hours 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 μ l of clodronate or empty liposomes were injected intra-peritoneally 24h 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-1 β (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-1 β 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 (10µg/ml for blocking antibodies and 10µM for LPG). Neutralizing reagents were pre-incubated with monocytes for 30 minutes 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 hours after blood draw. Whole blood samples were centrifuged at 514g at 25°C for 5 minutes to remove cells, and plasma then underwent two additional rounds of centrifugation at 1730g at 4°C for 5 and 15 minutes 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 hours 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 ³H-thymidine-based suppression assays, autologous Treg and Teff were cultured (together, or alone as controls) at 3,750 cells each per 50µl 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 50µl per well. Anti-CD3 antibodies (BD Biosciences, clone UCHT1) at 5µg/ml were pre-coated on U-bottom, 96-well plates for 4 hours at 37°C before suppression assays. Media was added, so the final volume in each well was 200µl. On day 6, cells were pulsed with 1µCi ³H-thymidine (25µl) per well and harvested on day 7 with a Tomtec cell harvester. ³H-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 10µM, according

to manufacturer's instructions. Assays with labeled cells were performed as described for ^3H -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 $40\mu\text{m}$ 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 ^3H -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-1 β and IL-6

We previously observed that Treg proliferate without losing suppressive capacity in co-cultures of Treg, Teff, irradiated antigen presenting cells (CD3-depleted peripheral blood mononuclear cells; APC) and anti-CD3 antibody to which SAA-rich plasma from systemic juvenile idiopathic arthritis (sJIA) subjects was added (Nguyen et al., 2011). We call these assays "suppression assays" hereafter, because of their similarity to standard *in vitro* Treg suppression assays (see *Materials and Methods* for details). Clinical information on the subjects providing plasma for the studies in this report is shown in the Supplementary Table 1. To examine the possibility that SAA stimulates Treg proliferation by induction of soluble effector molecules, we measured levels of several cytokines in supernatants collected from the suppression assays with sJIA plasma. IL-1 β and IL-6, but not TNF- α and other inflammatory cytokines and growth factors tested, were elevated in suppression assays with sJIA plasma in comparison to similar assays with healthy control (HC) plasma (Fig. 1A and Supplementary Fig. 1A). The increased expression of IL-1 β and IL-6 positively correlated

with SAA concentration in sJIA plasma samples used in the suppression assays (Supplementary Fig. 1B) and did not result from high levels of IL-1 β and IL-6 in the plasma samples themselves (Fig. 1A). Furthermore, the increases in IL-1 β and IL-6 production by APC treated with sJIA plasma were abrogated by immuno-depletion of endogenous SAA from plasma (Supplementary Fig. 1C) and, conversely, could be induced in a dose-dependent manner with recombinant human SAA (Supplementary Fig. 1C).

3.2 SAA induces IL-1 β and IL-6 production from monocytes

Among the cells in suppression assays, APC are the predominant producers of pro-inflammatory cytokines. Therefore, we hypothesized that SAA stimulated APC under suppression assay conditions to produce IL-1 β and IL-6. To examine this possibility, we first measured IL-1 β and IL-6 levels in supernatants from cultures with only irradiated APC. Compared to irradiated APC cultures exposed to HC plasma, supernatants from irradiated APC cultures exposed to SAA-rich sJIA plasma for 24 hours had significant increases in IL-1 β and IL-6 levels (Supplementary Fig. 2A). This finding suggested that a cellular subset from the irradiated APC mixture, which consisted mostly of B cells and monocytes, was the chief producer of IL-1 β and IL-6. To rule out the possibility that irradiation affected the function of APC, we analyzed cytokine secretion profile of irradiated and non-irradiated APC after exposure to sJIA plasma. Cytokine analysis showed no significant differences in IL-1 β and IL-6 between irradiated and non-irradiated APC, suggesting that our irradiation dosage did not significantly alter APC responsiveness to sJIA plasma (Supplementary Figs. 2A and B).

We next investigated which immune cell subsets are the major source of IL-1 β and IL-6. Intracellular cytokine staining showed that IL-1 β and IL-6 levels in monocytes were significantly higher than those of B and T cells in non-irradiated PBMC cultures with sJIA plasma for 24 hours (Fig. 1B and Supplementary Fig. 2C). Consistent with this result, isolated non-irradiated monocytes produced significantly more IL-1 β and IL-6 after 24 hour exposure to sJIA plasma (Fig. 1C). In rodents, intra-peritoneal injection of SAA also led to increased levels of IL-1 β and IL-6 in peritoneal fluid (Fig. 1D). Importantly, monocytes were required for the induction of IL-1 β and IL-6 by recombinant SAA *in vivo* as intra-peritoneal injection of SAA failed to augment IL-1 β and IL-6 levels in fluid of mice that were pharmacologically depleted of monocytes with clodronate liposomes (Fig. 1E). Collectively, these results demonstrate that SAA induces IL-1 β and IL-6 production under *in vitro* and *in vivo* conditions.

3.3 SAA signals via several cognate receptors to elicit IL-1 β and IL-6 secretion by monocytes

SAA is known to interact with various cell surface receptors including formyl peptide receptor 2 (FPR2, formerly called FPRL1) (Shim et al., 2009), thrombospondin receptor (CD36) (Baranova et al., 2005), receptor of advanced glycation end-product (RAGE) (Okamoto et al., 2008), toll-like receptor 2 (TLR2) (Cheng et al., 2008), toll-like receptor 4 (TLR4) (Sandri et al., 2008). As these receptors are expressed by monocytes (Nguyen et al., 2011), we utilized pharmacologic inhibitors and neutralizing antibodies to determine which SAA receptors participate in the induction of IL-1 β and IL-6 in these cells. We showed that

induction of IL-1 β and IL-6 in monocytes by recombinant SAA could be significantly reduced by blocking TLR2, TLR4, RAGE, and FPR2 (Figs. 1F and G). Similarly, blocking TLR2, TLR4, and RAGE abrogated IL-1 β and IL-6 production in monocytes induced by SAA-rich sJIA plasma (Supplementary Figs. 2D and E). We also observed a synergistic effect of these blocking reagents on SAA stimulated IL-1 β and IL-6 production in monocytes (Figs. 1F and G). These results implied that several SAA receptors cooperated to elicit IL-1 β and IL-6 production in monocytes.

3.4 IL-1 β and IL-6 are required for the proliferation of Treg induced by SAA

We found significant positive correlations between IL-1 β and IL-6 levels in suppression assay supernatants collected 24 hours after plasma stimulation and the magnitude of cell proliferation at day 7 in H³ thymidine-based suppression assays (Supplementary Fig. 3A). To test whether IL-1 β and IL-6 were required for SAA-mediated proliferation of Treg, we performed suppression assays with sJIA plasma in the presence of IL-1 receptor antagonist (IL-1Ra) or blocking antibody against IL-6 (Figs. 2A and B) at various concentrations (Supplementary Fig. 3B). Each of these reagents mediated significant inhibition of Treg proliferation. TNF- α neutralization did not significantly alter the mitogenic effects of SAA (Supplementary Fig. 3C). Interestingly, blocking other STAT5 activating growth factors (IL-2, IL-7, IL-15) showed varying degrees of inhibition of cellular proliferation in suppression assays (Supplementary Fig. 3C), of which the most potent neutralizing effect was seen with blocking IL-2, a cytokine produced by Teff in suppression assays (not shown).

Evidence argues that some Treg develop the phenotype of Th17 cells after exposure to inflammatory cytokines, in particular, IL-1 β and IL-6 (Baecher-Allan et al., 2006; Beriou et al., 2009; Deknuydt et al., 2009). Treg in our suppression assays contained a minor subset of IL-17 producing cells, but there were no significant differences between cultures with SAA-rich sJIA plasma and HC plasma (Supplementary Fig. 4A). Consistent with this finding, HLA-DR, a molecule that is highly expressed by Treg undergoing terminal differentiation (Beriou et al., 2009) showed a significant increase in SAA-induced Treg the 7-day suppression assay culture, compared to HC-plasma-exposed cultures (Supplementary Fig. 4B).

3.5 IL-1 β and IL-6 activate mitogenic signaling pathways of Treg

To determine whether IL-1 β and IL-6 could affect Treg directly, we first measured expression of their surface receptors. We found that IL-1R1 (the high affinity IL-1 receptor) and gp130 (the signaling component of the IL-6 receptor complex) were expressed at low levels in freshly isolated Treg (Supplementary Figs. 4C and D). However, expression of these molecules on Treg was up-regulated during the suppression assays (Supplementary Figs. 4C and D). Higher expression of IL-1 β and IL-6 receptors on Treg was induced by day 4 in the presence of SAA-rich sJIA plasma compared to HC plasma (Supplementary Figs. 4C and D). These results suggested that Treg are able to directly respond to IL-1 β and IL-6.

The proliferation of Treg mediated by SAA was associated with the activation of AKT and ERK1/2 mitogenic signaling pathways in these cells (Nguyen et al. 2011). Therefore, we

next examined whether IL-1 β and IL-6 had any impact on these signaling cascades. As expected, blocking IL-6, but not IL-1 β , abrogated STAT3 activation in Treg by SAA-rich sJIA plasma (Fig. 3A). Surprisingly, we found that blocking either IL-1 β or IL-6 could reduce the increased expression of ERK1/2 activation in Treg by SAA-rich sJIA plasma (Fig. 3B). Furthermore, neutralization of IL-1 β and IL-6 appeared to inhibit phosphorylation of AKT but only IL-1Ra treatment led to significant reduction in phosphorylation of AKT in Treg induced by SJIA-rich SJIA plasma (Fig. 3C). Altogether, these results demonstrated that IL-1 β and IL-6 act as effector molecules downstream of SAA to induce mitogenic pathways in Treg.

3.6 SAA-stimulated monocytes exhibit an immature-dendritic-cell-like phenotype

Immature dendritic cells have been implicated in the process of immune tolerance via their ability to induce Treg proliferation and enhance their suppressive function (Marguti et al., 2009). We asked whether SAA, besides its ability to elicit cytokine production in monocytes, can induce their maturation towards dendritic cells. Non irradiated CD14⁺ monocytes were isolated from PBMC and stimulated with sJIA plasma or HC plasma. We measured expression of several cardinal markers of dendritic cell differentiation in these monocyte cultures (Aradeshna et al., 2000; Brinster and Shevach, 2005). We found that, at day 4, sJIA-induced, monocyte derived DC express significantly higher levels of DR and lower levels of HVEM, a TNF-receptor superfamily member expressed on immature DC, in comparison to those in assays with HC plasma (Figs. 4A and B). Interestingly, the expression levels of these markers in sJIA plasma-stimulated monocyte derived dendritic cells in suppression assays resemble those of HLA-DR^{hi} HVEM^{lo} immature dendritic-like cells generated in culture by short term stimulation of human monocytes with LPS (24 hours) and are lower than those of (LPS-induced, 96 hours) mature dendritic-like cells (Figs. 4A and B). Other DC maturation markers CD40, CD80, and CCR7, were not induced in these cultures (Supplementary Fig. 5A). Altogether, this surface phenotype of SAA activated monocytes most resembles that of monocyte-derived immature DC.

In the mouse, intra-peritoneal injection of SAA leads to a rapid accumulation of monocytes in peritoneal cavity (Supplementary Figs. 5B and C) and enhanced their expression of the maturation marker CD80 to a level comparable to that of immature dendritic cells generated *in vivo* by LPS stimulation for 24 hours (Fig. 4C), adding support to the view that SAA drives monocyte maturation towards immature dendritic cells. Collectively, our *in vitro* and *in vivo* results in both human and mouse systems, respectively, suggest a potentially novel pathway, besides the elicitation of IL-1 β and IL-6, by which SAA might be involved in enhancing proliferation of Treg.

4. Discussion

Understanding Treg behavior at sites of inflammation requires elucidation of biochemical pathways mediating Treg proliferation in response to inflammatory stimuli. Several inflammatory mediators, including IL-1 β and IL-6, typically contribute to the cytokine milieu at sites of inflammation. IL-1 β and IL-6 have many activities: they can directly exert inflammatory effector functions, provide co-stimulatory signals for cell proliferation, or

induce anti-inflammatory responses, for example through induction of IL-1Ra or SOCS3 (Dinarello 2009; Kishimoto 2009). Here we provide evidence that IL-1 β and IL-6 production from SAA-stimulated monocytes drives Treg proliferation via activation of ERK and AKT, two mitogenic signaling cascades, in these cells. This observation is consistent with our previous report of a selective gateway to Treg proliferation mediated by their reduced expression of SOCS3 (Nguyen et al., 2011), a negative regulator of IL-1 β and IL-6-driven cellular activation (Lang et al., 2003; Wong et al., 2006; Yoshimura et al., 2007).

IL-1 β and IL-6 have been previously shown to induce proliferation of Teff (Pasare and Medzhitov 2003; O'Sullivan et al., 2006). However, in our system, these cytokines are also able to induce Treg proliferation. The levels of cytokines produced by SAA-exposed monocytes as well their immature dendritic cell phenotype likely influence the effects of IL-1 β and IL-6 on Treg in our model. In studies when these cytokines are added to Treg cultures, amounts in the ng/ml or even μ g/ml range are commonly used, whereas in our system the levels of secreted cytokines induced by SAA stimulation are in the pg/ml range, likely closer to physiological levels (Kutukculer et al., 1998; Saxena et al., 2005). Our data are also consistent with a previous study (Kubo et al., 2004), which showed that LPS-primed murine DC, which produce IL-1 β and IL-6 at levels similar to those at chronic inflammatory sites (pg/ml) (Kutukculer et al., 1998; Saxena et al., 2005), are capable of inducing Treg proliferation. In contrast, high doses of exogenous IL-1 β and IL-6 induce conversion of DR-negative, undifferentiated Treg to IL-17-producers (Beriou et al., 2009; Deknuydt et al., 2009). The vast majority of Treg in our cultures do not produce IL-17, consistent with exposure to more moderate levels of IL-1 β and IL-6. Treg in our cultures also show increased expression of DR, suggesting their maturation towards a terminally differentiated phenotype, which may prevent their development of the capacity to produce IL-17 (Baecher-Allan et al., 2006).

Although it is difficult to separate Treg survival from their function, factors involved in reversal of Treg anergy and their suppressive activity can be dissociated (Kubo et al., 2004). In this study, production of IL-1 β and IL-6 by TLR-stimulated DCs reversed Treg anergy but did not seem to be involved in inhibiting their suppressive activity. Rather, it was exposure of Treg to mature DCs, without TLR stimulation, that reversed Treg suppression (Kubo et al., 2004). Interestingly, in another study, Treg anergy could be reversed without loss of their capacity of suppression of cytokine production, although Teff continue to proliferate (Brinster and Shevach, 2005). In our study, maintenance of Treg function may be associated with monocyte activation (Walter et al., 2013). Monocyte exposure to SAA induced the increased expression of DC maturation markers to levels similar to the ones observed on immature dendritic-like cells. Immature DCs have been associated with the suppressive function of Treg, either due to their profile of surface receptors or due to their secretion of cytokines (Jonuleit et al., 2000; Yamazaki et al., 2003). As such, SAA may induce the reversal of Treg anergy through induction of monocytes towards an immature DC-like phenotype.

Our model suggests that, at sites of inflammation, SAA may be able to induce Treg proliferation but at the same time maintain their suppressive capacity. Stimulation of Treg expansion via monocyte production of cytokines is reminiscent of SAA effects on neutrophil

number. SAA stimulates TLR2-mediated induction of G-CSF production by human PBMC and murine macrophages; the latter has been shown to lead to proliferation of neutrophils in SAA-treated mice (He et al., 2009). Notably, SAA also directly drives a regulatory (immunosuppressive) program in neutrophils, stimulating them to produce IL-10 through binding to their surface FRP2 molecules (De Santo et al., 2010).

In our assays with sJIA plasma, IL-1 β and IL-6 levels in the supernatants correlate with the frequency of proliferating Treg. In dose-response assays with a uniform set of APC, IL-1 β and IL-6 production is proportional to the amount of SAA used as a stimulus. However, the potency of the APC response to SAA also differs between donors (not shown). Thus, the donor-dependent differential sensitivity to SAA-induction of cytokine production is consistent with known functional polymorphisms in some SAA receptors such as TLR2, TLR4, and RAGE (Hofmann et al., 2002; Misch and Hawn 2008). Furthermore, the relationship between levels of IL-1 β and IL-6 produced by APC and extent of Treg proliferation may represent a mechanism to link Treg homeostasis to the intensity of the inflammatory insult.

It is worth noting that besides pro-inflammatory cytokines IL-1 β and IL-6, T cell growth factors are essential for the reversal of Treg anergy in our system, as blocking IL-2, IL-7, and IL-15 abrogated activation of STAT5 and reduced proliferation of Treg in our assays. However, levels of these cytokines did not differ significantly in sJIA versus HC plasma or in supernatants from suppression-type cultures with sJIA versus HC plasma (Nguyen et al., 2011) (Supplementary Fig. 1A). Although Teff are a major source of IL-2, this cytokine is also produced by various innate immune cells such monocytes, DC, and NK cells. Indeed, IL-2 production by highly phagocytic immature DC can induce Treg proliferation (Marguti et al., 2009). Therefore, it remains possible that production of common-gamma chain and pro-inflammatory cytokines by innate immune cells might be sufficient to reverse Treg anergy during tissue repair or pathogen encounter. In fact, it has been shown that IL-1 β and IL-6 cooperate to enhance the response of Treg to IL-2 and suggest that the potentiation of the IL-2/Treg response could be the crucial role of IL-1 β and IL-6 (Kubo et al., 2004).

IL-1 β and IL-6 are thought to play a major role in the pathogenesis of sJIA as IL-1RA anakinra and IL-6 inhibitor tocilizumab are effective in the treatment this chronic auto-inflammatory disease (Sikora and Grom 2011, Martini 2012). In this context, the chronic detrimental effects of IL-1 β and IL-6 might be different from their acute functions which are shown to be associated with induction of Treg in our experimental system (Nguyen et al., 2011). Treg expansion has been observed at inflammatory sites which are enriched for SAA, IL-1 β and IL-6 in arthritic disorders (de Kleer et al. 2004, Cao et al. 2004, Walter et al. 2013). In addition, it has been reported that Treg levels in the circulation are reduced in active sJIA (de Kleer et al., 2006). Our findings would predict the presence and expansion of Treg at sites of inflammation where cell/cell contact in a particular cytokine milieu would be possible, at least upon initial expression of SAA. This might lead to some depletion of Treg in the circulation as we presume the initial Treg presence at sites of inflammation likely derives from movement of circulating Treg into the tissue. However, it should be noted that our experiments do not address how long term exposure to SAA, as can occur in persistent sJIA, might affect Treg numbers.

5. Conclusion

Our findings identify a mitogenic signaling pathway in Treg that is induced via the secretion of IL-1 β and IL-6 from immature-DC-like SAA-activated monocytes. Induction of Treg via inflammatory signals may help to limit spreading of inflammation beyond local tissues. However, excessive stimulation of Treg proliferation may also generate a state of immune silencing, leading to insufficient protective immunity against pathogens or attenuated tumoricidal activities (Khan et al., 2010). Therefore, appropriate control of inflammatory signal strength is critical to produce a beneficial Treg response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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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Highlights

1. SAA induces IL-1 β and IL-6 secretion from monocytes.
2. IL-1 β and IL-6 are required for the expansion of Treg by SAA.
3. IL-1 β and IL-6 activate ERK1/2 and AKT signaling in Treg in response to SAA.
4. SAA induces monocyte maturation towards tolerogenic HLA-DR^{hi}HVEM^{lo} dendritic cell.

Figure 1. SAA induces IL-1β and IL-6 production from monocytes

A. IL-1β and IL-6 levels in sJIA and HC plasma and in supernatants of suppression assays with these plasma samples at day 1 (n=15–17 per group). Unpaired t-tests were used for statistical analyses. **B.** IL-1β and IL-6 production by non-irradiated monocytes, B and T cells stimulated with sJIA plasma for 24 hours (n=6 per group). Kruskal-Wallis tests were used for statistical analyses. **C.** IL-1β and IL-6 levels in supernatants of non-irradiated monocyte cultures with sJIA and HC plasma and at day 1 (n=6 per group). Mann-Whitney test and unpaired t-test were used for statistical analyses. **D.** IL-1β and IL-6 levels in peritoneal fluid of C57BL/6J mice 16 hours after recombinant SAA or vehicle (Veh) treatment (n=6–9 per group). Mann-Whitney tests were used for statistical analyses. **E.** IL-1β and IL-6 production in peritoneal fluid of clodronate-liposome-treated or vehicle-treated (Veh) C57BL/6J mice 16 hours after recombinant SAA injection (n=6 per group). Mann-Whitney tests were used for statistical analyses. **F–G.** IL-1β (F) and IL-6 (G) production by non-irradiated monocytes stimulated with recombinant SAA for 24 hours after antibody-mediated or pharmacologic neutralization of SAA receptors (n=6 per group). FPR2 antagonist and blocking antibodies

were used at 10 μ M and 10 μ g/ml, respectively. Kruskal-Wallis tests were used for statistical analyses. Bar graphs represent mean $\pm$ SEM.

Figure 2. L-1 β and IL-6 are necessary for SAA-mediated proliferation of Treg

A–B. (left) Treg (A) and Teff (B) proliferation in CFSE-based suppression assays with sJIA plasma in the presence of blocking reagents against IL-1 β and IL-6 (n=5 per group). IL-1Ra and anti-IL-6 antibody were used at 1 μ M and 1 μ g/ml, respectively. Kruskal-Wallis test was used for statistical analyses. (right) Representative flow cytometry plots of CFSE expression in Treg and Teff in suppression assays with sJIA plasma in the presence of blocking reagents against IL-1 β and IL-6. Bar graphs represent mean $\pm$ SEM.

Figure 3. IL-1 β and IL-6 activate mitogenic signaling pathways to induce Treg proliferation
A–C. (*upper*) Expression of phosphorylated forms of STAT3 (**A**), ERK1/2 (**B**), AKT (**C**), and in Treg (pERK1/2, pAKT, and pSTAT3, respectively) in suppression assays with sJIA plasma in the presence of IL-1 β and IL-6 blocking reagents at day 4 (n=6 per group). Kruskal-Wallis tests were used for statistical analyses. (*lower*) Representative flow cytometry plots of pSTAT3, pERK1/2, and pAKT expression in Treg in suppression assays with sJIA plasma in the presence of IL-1 β and IL-6 blocking reagents. Bar graphs represent mean $\pm$ SEM.

Figure 4. SAA induces monocyte maturation towards an immature dendritic cell phenotype A–B. (left) Expressions of HLA-DR and HVEM in non-irradiated monocyte cultures with sJIA and HC plasma at day 4 (n=6 per group). Kruskal-Wallis tests were used for statistical analyses. (right) Representative flow cytometry plots of expressions of HLA-DR and HVEM in monocyte-derived dendritic cells in suppression assays with sJIA and HC plasma in comparison to those of immature and mature dendritic-like cells, induced by LPS (50ng/ml) treatment of monocytes for 24 and 96 hours, respectively. **C.** (left) Expressions of CD80 in peritoneal monocytes from C57BL/6J mice (n=5 per group) 24 hours after

treatment with recombinant SAA or vehicle (Veh). Kruskal-Wallis test was used for statistical analysis. (*right*) Representative flow cytometry plots of CD80 expression in peritoneal monocytes from C57BL/6J mice 24 hours after recombinant SAA treatment in comparison to those of monocyte-derived immature and mature dendritic cells, induced by intra-peritoneal LPS injection (1mg/kg) for 24 and 48 hours, respectively. Bar graphs represent mean $\pm$ SEM.