Serum amyloid A overrides T_{reg} anergy via monocyte-dependent and T_{reg}-intrinsic, SOCS3-associated pathways

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The acute phase protein serum amyloid A (SAA) has been well characterized as an indicator of inflammation. Nevertheless, its functions in pro versus anti-inflammatory processes remain obscure. Here we provide unexpected evidences that SAA induces the proliferation of the tolerogenic subset of regulatory T cells (T_{reg}). Intriguingly, SAA reverses T_{reg} anergy via its interaction with monocytes to activate distinct mitogenic pathways in T_{reg} but not effector T cells. This selective responsiveness of T_{reg} correlates with their diminished expression of SOCS3 and is antagonized by T_{reg}-specific induction of this regulator of cytokine signaling. Collectively, these evidences suggest a novel anti-inflammatory role of SAA in the induction of a micro-environment that supports T_{reg} expansion at sites of infection or tissue injury, likely to curb (auto)-inflammatory responses. (Blood. 2011;117(14):3793-3798)

Introduction

Inflammation is a highly regulated physiologic response that has evolved as a mechanism to respond to infection as well as to promote healing in settings such as tissue injury. CD4⁺ regulatory T cells (T_{reg}) are observed at sites of acute and chronic inflammation,¹,² raising questions as to the molecular basis of their accumulation at these sites. Initial evidence suggested that immunosuppressive activities of T_{reg} are diminished in the presence of inflammatory signals.³,⁴ However, in several studies, the apparently opposite picture emerged, wherein T_{reg} exposed to inflammatory signals retain potent suppressive activity. For instance, murine T_{reg} at sites of viral infection or isolated from inflamed tissues still mediate regulatory function,⁵,⁶ as do human T_{reg} isolated from rheumatoid joints or inflamed colonic mucosa.⁶,⁷ Collectively, these results point to the possibility that certain signals associated with inflammation might promote T_{reg} activity. Here we unexpectedly identified serum amyloid A (SAA), an acute indicator of inflammation, as a novel factor that induces cellular and cytokine conditions to support the expansion of T_{reg} while maintaining their suppressive capacity.

Methods

In vivo effects of SAA on T_{reg} proliferation

C57BL/6J mice (male, 8-10 weeks old) were purchased from Jackson Laboratory. Mice were injected intraperitoneally with recombinant human SAA (Peprotech, 30 μg in 100 μl PBS), purified human serum albumin (Sigma-Aldrich, 30 μg in 100 μl PBS), or endotoxin (Sigma-Aldrich, 0.25 ng in 100 μl PBS). Animals were killed 16 hours later; peritoneal cells were harvested and stained for surface and intracellular markers to detect T_{reg} frequency and proliferation. In vivo depletion of monocytes was performed with clodronate liposomes (Encapsera). In these experiments, 400 μL of clodronate or empty liposomes were injected intraperitoneally 24 hours before SAA injection.

Flow cytometry and ELISA

Detection of surface markers and intracellular molecules was performed. Antibodies to mouse and human proteins used in these experiments are purchased from Biolegend except for anti–human formyl peptide receptor like-1 (FPRL-1; R&D Systems), anti–human RAGE (receptor of advanced glycation end products), suppressor of cytokine signaling 3 (SOCS3; Abcam), and anti–Ki-67, anti–human-pAKT (phosphorylated protein kinase B), pERK1/2 (phosphorylated extracellular signal regulated kinases 1 and 2; BD Biosciences). For in vitro experiments, the relevant subset was labeled with CFSE before suppression assays. Cells were pelleted at various time points and underwent standard staining protocols of the manufacturers. For in vivo experiments, cells were harvested from peritoneal cavity and underwent flow cytometric analysis.

To detect cytokines in the plasma, cytometric bead arrays (BD Biosciences) and ELISA (R&D Systems) were used according to manufacturers’ protocols.

Human plasma preparation

The study was approved by the Institutional Review Board at Stanford University. All subjects provided informed consent before participating in the study in accordance with the Declaration of Helsinki. Plasma was prepared from whole, anticoagulated blood within 2 hours after blood draw. Whole blood samples were centrifuged at 25°C at 514g for 5 minutes to remove cells, and then underwent 2 additional rounds of centrifugation at 4°C at 1730g for 5 and 15 minutes, respectively, to remove platelets. Final plasma samples were stored at −80°C until analysis. Depletion of SAA from plasma samples was performed with anti-SAA antibodies (Santa Cruz Biotechnology) via immunoprecipitation for 4 consecutive rounds. Negative control for depletion experiments was performed with L243 (anti–HLA-DR) antibodies.

Human cell isolation

CD4⁺ T cells were purified with CD4⁺ Rosette Kit (StemCell Technologies) fromuffy coats. The CD4⁺ T cell fraction was then incubated with
anti-CD25 microbeads (Miltenyi Biotec) to isolate CD4^+CD25^- cells. The flow-through fraction after magnetic purification contained CD4^+CD25^- T_eff. All procedures were performed according to manufacturers' standard protocols. CD4^+CD25^-T cells were incubated with anti-CD127-APCs, anti-CD25-PE, and anti-CD4-FITC antibodies (BD Biosciences) before undergoing flow cytometric sorting for CD4^+CD25^-CD127^-/low Treg and CD4^+CD25^+CD127^- activated T_eff. Purity of sorted cells was confirmed to be higher than 95% by Foxp3 staining (eBioscience; data not shown). Cells were rested for 2 hours in 37^°C incubator before being used in suppression assays.

**Suppression assays**

Standard ^3^H-thymidine-based suppression assays were performed. Autologous T_reg and T_eff were cultured at 3750 cells per 50 μl per well in complete media (RPMI + 10%FBS + 1%L-glutamine) with allogeneic irradiated CD3-depleted peripheral blood mononuclear cells (APCs), at 37 500 cells per 50μl per well. Anti-CD3 antibodies (clone UCHT1; BD Biosciences) were precoated on U-bottom 96 well plates at 5 μg/mL for 4 hours at 37^°C before suppression assays. Additional 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 suppression assays without APCs, anti-CD3 antibodies at 5 μg/mL final concentration (clone UCHT1; BD Biosciences) were plate-bond in U-bottom 96-well plates for 4 hours at 37^°C. Soluble anti-CD28 (5 μg/mL; BD Biosciences) was added, followed by T_eff (40 0000 cells per 50 μl per well) and T_reg (40 000 cells per 50 μl per well). For CFSE dilution assay, T_reg were labeled with CFSE using Cell Tracer CFSE Cell Proliferation kit (Molecular Probes) at a final concentration of 10μM, according to manufacturer’s instructions. Assays with labeled cells were performed as described for ^3^H-thymidine-based suppression assays.

To evaluate effects of plasma on suppression assays and immune cell cultures, frozen plasma samples were thawed at 25^°C and debris were removed with sterile 40μm filters (BD Biosciences). All plasma samples were tested in duplicates or triplicates. To control for variations in suppressive and proliferative potentials of T_reg and T_eff, 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 cpm in assays with plasma compared with 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 (shown by dye dilution) was used to analyze the effects of plasma on cell proliferation.

**Statistical analysis**

All statistical procedures were performed with Prism Version 5.0 software (GraphPad). Data were tested for normality (Kormonov-Smirnov test) and variance equality (Bartlett test) before being subjected to appropriate statistical tests. Differences with P < .05 were considered statistically significant. Correction for multiple comparisons was performed via Bonferroni method.

**Results**

**SAA induces T_reg proliferation**

To dissect the effects of molecules associated with inflammation on T_reg, we first sought to simulate this environment in vitro using plasma from children with systemic juvenile idiopathic arthritis (SJIA), and auto-inflammatory disease, in which elevated levels of both markers of inflammation and inflammatory cytokines are...
present. Addition of a titrated volume of SJIA plasma (15%) from subjects with various degrees of disease activity flare (F, active disease), quiescence (Q, inactive disease on medication), or remission (R, inactive disease off medication), but not healthy control (HC) plasma, to 3H-thymidine-based suppression assays, stimulated cell proliferation (supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Surprisingly, CFSE-based suppression assays revealed that the presumably anergic population of Treg was proliferating and continued to suppress the proliferation of effector T cells (Teff; Figure 1A).

To characterize the factor(s) in SJIA plasma that induced Treg proliferation, we measured the serum levels of various inflammatory markers and found significant increases in some cytokines in plasma samples collected at SJIA flare compared with HC plasma (supplemental Figure 2). Noticeably, SJIA plasma samples from all disease stages had significantly higher levels of SAA than samples from HC (Figure 1B). More importantly, depletion of SAA in SJIA plasma abrogated Treg proliferation (Figure 1C, supplemental Figure 3), suggesting that this acute phase protein is required for the mitogenic effects of SAA.

To determine whether SAA is sufficient to reverse Treg anergy, recombinant SAA was added to suppression assays, in the presence of polymyxin to inhibit any contaminating LPS. Recombinant SAA was able to selectively enhance proliferation of Treg without reducing their suppressive activity (Figure 2A). Furthermore, there was a significant increase in Treg abundance in the peritoneal cavity of SAA-injected mice compared with those injected with a control protein, (purified human serum albumin) or endotoxin at the level found in recombinant SAA (0.25 ng/mL). Concurrently, a significantly higher percentage of peritoneal Treg from SAA-injected mice expressed the nuclear antigen Ki-67, indicating that they were undergoing cell division (Figure 2C). In contrast, SAA injection did not enhance Teff proliferation (Figure 2C). Collectively, these results demonstrate that SAA is a novel mitogenic stimulator of Treg.

Mitogenic effects of SAA on Treg requires its interaction with monocytes

It is known that SAA interacts with at least 6 distinct receptors: FPR-1, CD36, RAGE, TLR2, TLR4, and Tanis10-14 which are found to be expressed at strikingly high levels in monocytes compared with B cells and T cells (Figure 3A). To explore the possibility that SAA might act on monocytes to indirectly induce Treg proliferation, we first performed suppression assays with anti-CD3 and anti-CD28 antibodies as T cell stimuli in the absence of APCs. Under this condition, both SJIA plasma-derived and exogenous SAA failed to induce Treg proliferation (supplemental Figure 4), indicating that cells in the APC mixture mediated the mitogenic effect of SAA on Treg. More importantly, depleting monocytes led to a significant decrease in cell proliferation in
suppression assays with SAA derived from SJIA plasma (Figure 3B). Conversely, monocytes were sufficient to induce cell proliferation at similar levels to those observed using unfractionated APCs (Figure 3B). In contrast, B cells were not required for the mitogenic effects of SAA derived from SJIA plasma on Treg proliferation in suppression assays (supplemental Figure 4B). To further investigate the role of monocytes on SAA-induced Treg proliferation, we depleted monocytes in vivo with clodronate liposomes (supplemental Figure 4C) and found a significant decrease in Treg abundance and their expression of Ki-67 in the peritoneal cavities of SAA-treated animals (Figure 3C-D). Altogether, these results showed that monocytes were essential for the mitogenic effects of SAA on Treg.

Induction of SOCS3 in Treg antagonizes their SAA-driven proliferative response

We next examined the activation state of signaling molecules that have been implicated in mitogenic processes, such as AKT and ERK1/2 in Treg in suppression assays. We found that Treg selectively exhibited increased activation of AKT (pAKT) and ERK1/2 (pERK1/2) in response to SAA derived from SJIA plasma (Figure 4A). Furthermore, Treg also expressed significantly higher levels of pAKT and pERK1/2 than Teff (supplemental Figure 5). Intriguingly, we found that SOCS3, a member of suppressor of cytokine signaling protein family,15 was expressed at significantly lower levels in Treg than in Teff (supplemental Figure 5). Remarkably, forskolin-treated Treg still suppressed Teff proliferation, but they no longer proliferated in response to SAA derived from SJIA plasma (Figure 5A). The increased activation of mitogenic signaling in Treg compared with Teff cocultured in the same assays was also abrogated (Figure 5B). Collectively, these results suggested that the level of expression of SOCS3 by Treg regulates the dynamic range of their proliferative response to SAA.

Discussion

In human diseases that are characterized by inflammation, increased numbers of Treg have been found in inflamed tissues where local SAA production occurs.18-20 SAA is elevated within the same time-frame of accumulation of Treg during tissue injury.21 Together with our in vivo data, these observations are consistent with the possibility that early induction of SAA at inflammatory sites, coupled with its effects on innate immune cells, generates a milieu that drives Treg proliferation. Our study also suggests that modulating the level of expression of SOCS3 in Treg and, consequently, the relative expression of SOCS3 in Treg versus Teff by pharmacologic means abrogates the selective activation of mitogenic signaling pathways in Treg on exposure to the micro-environment generated by interaction between SAA and monocytes. These results imply that maintenance versus resolution of inflammatory processes might depend on the relative responsiveness of proinflammatory/effector and anti-inflammatory/regulatory cell subsets. Reduced SOCS3
expression in regulatory cell subsets, such as T_{reg}, might increase their sensitivity to inflammation-derived mitogenic signals, leading to their rapid activation and effective suppression of inflammatory cell types. It is of interest that mediators that could induce SOCS3 expression, such as IL-10, are also produced by T_{reg} and therefore might serve as negative feedback regulators of T_{reg} proliferation to exert a fine balance on tolerance versus immunity under inflammatory conditions.

Inflammation associated with infection or tissue injury affords an opportunity for molecular mimicry or exposure of previously cryptic tissue antigens to increase the risk of autoimmunity. These possibilities highlight the need for timely recruitment and/or expansion of tolerogenic cell subsets at inflammatory sites. The mitogenic effects of SAA on T_{reg} shown here may represent a mechanism for protection against the potential breach of tolerance unleashed by inflammation.

Figure 4. SAA activates distinct mitogenic pathways in T_{reg}. (A left) Real-time quantitation of ERK1/2 and AKT phosphorylation (pERK1/2 and pAKT) by phospho flow cytometry. T_{eff} (top) or T_{reg} (bottom) were labeled with CFSE. Data were collected at different time points (days 1, 4, and 7) during suppression assays with SJIA plasma (n = 6), HC plasma (n = 6) or complete media (FBS, n = 4). (Right) Representative FACS plots of pERK1/2 and pAKT in T_{reg} and T_{eff} in suppression assays. (B left) Expression of SOCS3 in T_{reg} and T_{eff} (n = 7). (Right) Representative FACS plots of expression of SOCS3 in T_{reg} and T_{eff}.

Figure 5. SOCS3 regulates mitogenic signaling cascade in T_{reg} in response to SAA. (A left) Percentages of proliferating T_{reg} and T_{eff} in suppression assays with HC plasma (n = 5) and SJIA plasma (n = 5). (Right) Percentages of proliferating forskolin-treated T_{reg} and untreated T_{eff} in suppression assays with HC plasma (n = 5) and SJIA plasma (n = 5). T_{reg} were rested in complete media or treated with forskolin for 24 hours before being used in these assays. (B left) Effects of T_{reg} specific-SOCS3 modulation on phosphorylation status of ERK1/2 and AKT by T_{reg} and T_{eff} in suppression assays. Data were collected at day 4 during suppression assays with SJIA plasma (n = 6), HC plasma (n = 6) or complete media (FBS, n = 4). (Right) Representative FACS plots of pERK1/2 and pAKT in T_{reg} and T_{eff} in suppression assays. Unpaired 2-tailed t tests (A,C,D) and paired 2-tailed t tests (B) were used for statistical analyses. Horizontal bars represented median values.
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Authorship

Contribution: K.D.N., C.M., K.C.N., and E.D.M. are involved in project planning; K.D.N., C.M., K.C.N., P.T., and T.Y. performed experiments and analyzed the results; T.L. and J.L.P. provided clinical samples for the project; and K.D.N. and E.D.M. wrote the manuscript.

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