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Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A

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Neutrophils are the main effector cells during inflammation, but they can also control excessive inflammatory responses by secreting anti-inflammatory cytokines. However, the mechanisms that modulate their plasticity remain unclear. We now show that systemic serum amyloid A 1 (SAA-1) controls the plasticity of neutrophil differentiation. SAA-1 not only induced anti-inflammatory interleukin 10 (IL-10)-secreting neutrophils but also promoted the interaction of invariant natural killer T cells (*i*NKT cells) with those neutrophils, a process that limited their suppressive activity by diminishing the production of IL-10 and enhancing the production of IL-12. Because SAA-1-producing melanomas promoted differentiation of IL-10-secreting neutrophils, harnessing *i*NKT cells could be useful therapeutically by decreasing the frequency of immunosuppressive neutrophils and restoring tumor-specific immune responses.

Several types of myeloid cells can promote tumor progression either directly, by inhibiting tumor-specific immune responses, or indirectly, by promoting angiogenesis, tumor growth and tissue remodeling[1]. The best characterized of these are tumor-infiltrating macrophages, which have properties of alternatively activated macrophages[2], and myeloid-derived suppressor cells (MDSCs), a heterogenous population of immunosuppressive myeloid cells[3]. MDSCs are able to differentiate into immunosuppressive macrophages and neutrophils[4] and are characterized by abundant production of arginase and inducible nitric oxide synthase, whose metabolic products include diffusible and highly reactive peroxynitrites[5]. Reports have highlighted the ability of neutrophils not only to promote inflammation but also to demonstrate anti-inflammatory properties depending on cues from the tumor microenvironment, such as secretion of tumor growth factor-β[6]. Interleukin (IL)-10-secreting neutrophils proliferate during bacterial infection because of the cotriggering of pathways dependent on Toll-like receptors (TLRs) and the adaptor MyD88 and those dependent on C-type lectin receptors and the tyrosine kinase Syk[7].

The results noted above support the idea of a previously unanticipated plasticity for neutrophils, similar to that seen in macrophages[2], which raises the possibility that alternative polarization of neutrophils can be harnessed by tumors to dampen tumor immune responses and facilitate tumor growth. However, it remains unclear whether neutrophils acquire their anti-inflammatory properties at the site of inflammation or whether systemic signals released during acute and chronic inflammatory processes are able to modulate the phenotype of circulating neutrophils, thus affecting a much larger pool of cells. Furthermore, although neutrophils are able to directly promote inflammatory reactions by influencing the activity of other immune cells and tissues[8], it remains unclear whether the interaction of neutrophils with other immune cells may also modulate their pro- and anti-inflammatory properties.

Here we show that the main acute-phase reactant serum amyloid A 1 (SAA-1), one of the first and most abundant proteins secreted during the physiological response to infection and injury, controls the plasticity of neutrophil differentiation. SAA-1 induced IL-10 secretion from neutrophils and also promoted their ability to interact with invariant natural killer T cells (*i*NKT cells), a process that limited their suppressive activity by decreasing IL-10 production and enhancing IL-12 production. Our findings underscore the plasticity of neutrophils as cells able to have pro- and anti-inflammatory properties and highlight the role of *i*NKT cells as modulators of inflammatory responses.

RESULTS

Suppressive IL-10-secreting neutrophils in patients with melanoma

To address the issue of whether melanomas induce the population expansion of leukocytes with suppressive properties, we compared the frequency of tumor-specific $CD8^+$ T cells whose populations we expanded *in vitro* from whole blood samples of patients with melanoma and from Ficoll-purified peripheral blood mononuclear cells (PBMCs). The results of these experiments demonstrated that the population expansion of $CD8^+$ T cells specific for a peptide of

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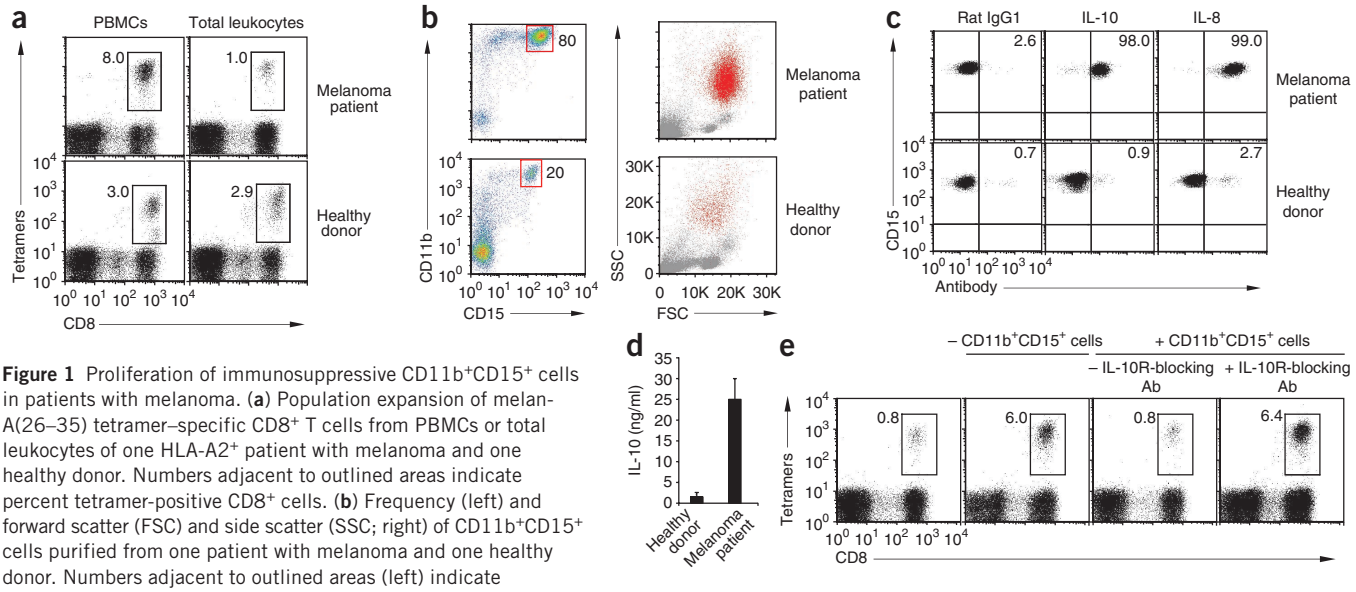

Figure 1 Proliferation of immunosuppressive CD11b$^+$CD15$^+$ cells in patients with melanoma. (**a**) Population expansion of melan-A(26–35) tetramer–specific CD8$^+$ T cells from PBMCs or total leukocytes of one HLA-A2$^+$ patient with melanoma and one healthy donor. Numbers adjacent to outlined areas indicate percent tetramer-positive CD8$^+$ cells. (**b**) Frequency (left) and forward scatter (FSC) and side scatter (SSC; right) of CD11b$^+$CD15$^+$ cells purified from one patient with melanoma and one healthy donor. Numbers adjacent to outlined areas (left) indicate percent CD11b$^+$CD15$^+$ cells; red dots (right) indicate back gating of CD11b$^+$CD15$^+$cells. (**c**) Intracellular staining, with anti-IL-10 and anti-IL-8, of CD11b$^+$CD15$^+$cells purified from a patient with melanoma and one healthy donor. Numbers in top right quadrants indicate percent CD15$^+$ cells stained with antibody. (**d**) Cumulative IL-10 secretion from CD11b$^+$CD15$^+$ cells from seven healthy donors and ten patients with melanoma. (**e**) Population expansion of melan-A(26–35) tetramer–specific CD8$^+$ T cells from total leukocytes of one HLA-A2$^+$ patient with melanoma, depleted (–CD11b$^+$CD15$^+$ cells) or not depleted (+CD11b$^+$CD15$^+$ cells) of CD11b$^+$CD15$^+$ cells, and frequency of melan-A(26–35) tetramer–specific CD8$^+$ T cells after the addition of 10% purified CD11b$^+$CD15$^+$ cells to CD11b$^+$CD15$^+$ populations depleted of total leukocytes with (+IL-10R-blocking Ab) or without (–IL-10R-blocking Ab) blocking antibody to the IL-10 receptor. Data are representative of five independent experiments with ten patients with melanoma and five healthy donors (**a**), more than ten independent experiments with forty patients with melanoma and thirty healthy donors (**b**), five independent experiments with five patients with melanoma and five healthy donors (**c**), five independent experiments with ten patients with melanoma and seven healthy donors (**d**; error bars, s.d.) or three independent experiments with three patients with melanoma and three healthy donors (**e**).

amino acids 26–35 of the melanoma differentiation antigen melan-A (melan-A(26–35)) was much lower in total leukocyte cultures than in cultures of Ficoll-purified PBMCs (**Fig. 1a** and **Supplementary Fig. 1a**). In contrast, blood samples from healthy donors showed similar population expansion of melan-A(26–35)-specific CD8$^+$ T cells among total leukocytes and Ficoll-purified PBMCs, albeit at a lower frequency, as expected (**Fig. 1a**).

When we compared the cell profiles of total leukocytes from patients with melanoma and healthy volunteers, we observed that neutrophil populations (as defined by staining with antibody to CD11b (anti-CD11b) and anti-CD15) were expanded in the peripheral blood of a large proportion of patients with melanoma (**Fig. 1b** and **Supplementary Figs. 1b** and **2a**). Consistent with the phenotypic features of neutrophils, CD11b$^+$CD15$^+$ cells were removed after Ficoll purification and had a polysegmented nuclear morphology (data not shown). Further phenotypic profiling showed that neutrophils from patients with melanoma, unlike CD11b$^+$CD15$^+$ cells from healthy donors, constitutively synthesized IL-10 and IL-8 (**Fig. 1c**) and had enhanced expression of arginase-1 (**Supplementary Fig. 2b**). We further confirmed by enzyme-linked immunosorbent assay (ELISA) that there was spontaneous production of IL-10 by neutrophils purified from patients with melanoma (**Fig. 1d**).

The frequency of neutrophils in the blood of patients with melanoma correlated with the staging of disease (**Supplementary Fig. 2a**), and neutrophils isolated from patients with late-stage melanoma also had a greater capacity to suppress lymphocyte proliferation in mixed lymphocyte reactions (**Supplementary Fig. 2a**). We then asked whether the presence of large numbers of IL-10-secreting neutrophils in the blood of patients with melanoma could account for the observed suppression of the proliferation of melan-A(26–35)-specific CD8$^+$ T cells. Consistent with that hypothesis, we observed that depletion of CD11b$^+$CD15$^+$ cells from the leukocyte populations of patients with melanoma restored the population expansion of melan-A(26–35) specific CD8$^+$ T cells (**Fig. 1e**). Conversely, the proliferation of melan-A(26–35)-specific CD8$^+$ T cells was again abolished after we added autologous purified CD11b$^+$CD15$^+$ cells back to the same cultures (**Fig. 1e**). The immunosuppressive property of CD11b$^+$CD15$^+$ cells purified from patients with melanoma was mediated by IL-10 secretion, as the addition of an antibody that blocked the IL-10 receptor restored the proliferation of melan-A(26–35)-specific CD8$^+$ T cells (**Fig. 1e**). Control CD11b$^+$CD15$^+$ cells purified from healthy donors (which did not produce IL-10) failed to inhibit the population expansion of melan-A(26–35)-specific CD8$^+$ T cells (**Supplementary Fig. 2d**). We combined the cumulative results of all the melan-A(26–35)-specific CD8$^+$ T cell population-expansion experiments with PBMCs in the presence or absence of purified CD11b$^+$CD15$^+$ cells and with or without the antibody blocking the IL-10 receptor (**Supplementary Fig. 1a**,**c**).

SAA-1 differentiates suppressive IL-10-secreting neutrophils

The observation that circulating IL-10-secreting neutrophils in a large proportion of patients with melanoma underwent a unimodal shift in phenotype suggested that their population expansion and phenotypic modification were the result of systemic signals, which may be harnessed by the tumor as an evasion mechanism to hamper melanoma-specific immune responses. To identify which factors were responsible for the *in vivo* population expansion of IL-10-secreting neutrophils in patients with melanoma, we compared the cytokine profiles of the plasma of 40 patients with melanoma with those of 30 normal control subjects. Although the concentrations of several cytokines, such as granulocyte-macrophage colony stimulating factor, IL-13, IL-10 and IL-8, were higher in most patients (**Supplementary Fig. 3**),

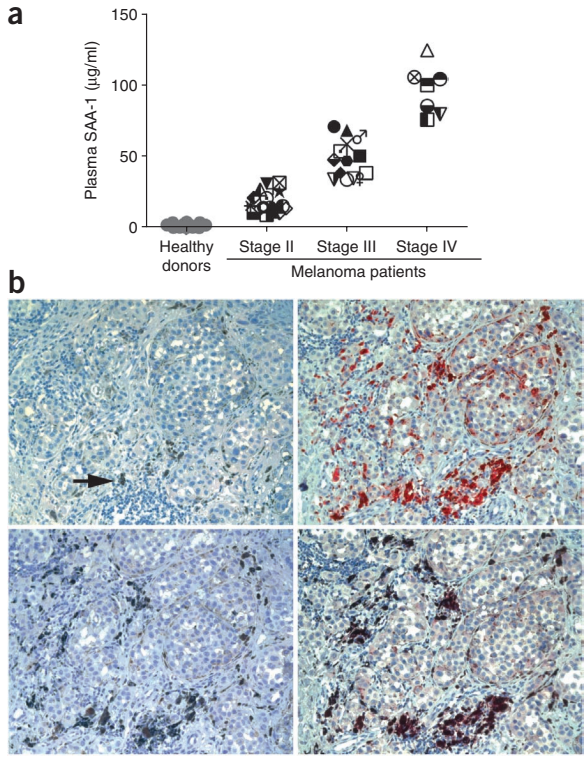

Figure 2 SAA-1 is present in plasma and primary tumors of patients with melanoma. (**a**) ELISA of SAA-1 in plasma from 40 patients with melanoma and 30 healthy donors (each symbol identifies a different patient). (**b**) Immunohistochemistry of serial sections of primary melanoma. Top left, melanin location (arrow); top right, staining with anti-SAA-1; bottom left, staining with antibody to human CD68; bottom right, double staining with anti-SAA-1 and anti-CD68. Original magnification, ×20. Data are representative of two experiments (**a**) or six experiments with five patients with melanoma (**b**).

we found that in all patients studied, the concentration of the acute-phase response protein SAA-1 was several orders of magnitude higher than that of the other cytokines measured (**Fig. 2a**). We further confirmed those results by measuring the plasma concentration of SAA-1 in the additional ten patients with melanoma in whom we characterized the melan-A(26–35)-specific $CD8^+$ T cell response (**Supplementary Fig. 1a**) and observed a correlation between SAA-1 concentration and the frequency of $CD11b^+CD15^+$ cells (**Supplementary Fig. 1b**). In addition, the concentration of SAA-1 was higher than that of C-reactive protein, another acute-phase response protein. Notably, we observed a positive correlation between the concentration of SAA-1 and disease staging (**Fig. 2a**). Although the main source of SAA-1 during inflammation is the liver, peripheral production of this protein has been reported[9]. Indeed, staining of primary melanoma sections from five patients with SAA-1-specific antibodies showed the presence of $SAA\text{-}1^+$ tumor cells and tumor-infiltrating macrophages (**Fig. 2b**).

We reasoned that the large amounts of systemic plasma SAA-1, together with its production in primary melanomas, could account for the global shift in the phenotype of circulating neutrophils. To address this possibility, we sorted $CD11b^+CD15^+$ cells from healthy donors and observed that exposure to increasing concentrations of SAA-1, but not to C-reactive protein (**Fig. 3a**) or the potent neutrophil activator formyl peptide (**Fig. 3b**), induced IL-10 secretion in a dose-dependent manner. This effect was not caused by the presence of endotoxin in the SAA-1 preparation, as the endotoxin-removing compound polymyxin failed to decrease IL-10 production[10] (**Supplementary Fig. 4**). Consistent with the ability of SAA-1 to induce production of IL-10, we observed that SAA-1-treated $CD11b^+CD15^+$ cells isolated from healthy donors acquired the ability to suppress T cell proliferation (**Fig. 3c**).

The results presented above demonstrated the presence of SAA-1 in the plasma and primary tumors of patients with melanoma. They also defined SAA-1 as an acute-phase response protein able to induce IL-10 production in neutrophils. These findings are consistent with the observation that both SAA-1 concentrations in blood and the frequency of IL-10-producing neutrophils positively correlate with staging of the disease.

Regulation of IL-10 secretion from SAA-1-treated neutrophils

The best characterized receptors for SAA-1 are the G protein–coupled protein FPRL1 (formyl peptide like 1)[11,12], which has been renamed FPR2 (ref. 13), and TLR2 (refs. 11,14), each of which is expressed by neutrophils (**Supplementary Fig. 5a**). To identify the receptor involved in the SAA-1-dependent production of IL-10, we treated neutrophils purified from healthy donors with SAA-1 in the presence or absence of FPR2- and TLR2-blocking antibodies. The addition of FPR2-blocking antibody prevented the SAA-1-dependent release of IL-10 (**Fig. 3b**). In contrast, the TLR2-blocking antibody blocked the effect of the TLR2 agonist Pam_3Cys but had no effect on SAA-1-dependent activation of neutrophils (**Fig. 3b**).

As it has been shown before that FPR2 triggers activation of the mitogen-activated protein kinases Erk1/2, p38 and PI(3)K[15,16], which are also important for IL-10 production by neutrophils[7], we pretreated purified neutrophils for 1 h with inhibitors of p38, Erk and PI(3)K and then stimulated them for 24 h with SAA-1. The addition of each of the three kinase inhibitors alone at doses that did not affect neutrophil viability (data not shown) inhibited IL-10 production by SAA-1-treated neutrophils from patients with melanoma and healthy donors (**Fig. 3d**). Notably, the inhibitors described above also prevented spontaneous secretion of IL-10 by neutrophils purified from patients with melanoma (**Fig. 3d**), which indicated that the p38, Erk and PI(3)K pathways are constitutively activated in these cells. Indeed, *ex vivo* intracellular staining with antibody to Akt (a PI(3)K target), anti-Erk or anti-p38 (**Supplementary Fig. 5b**) or with antibodies specific for the phosphorylated forms of those three kinases confirmed that they were constitutively phosphorylated in neutrophils purified from patients with melanoma (**Fig. 3e**). Thus, the modulation of IL-10 production by SAA-1 in neutrophils is mediated by the G protein–coupled receptor FPR2 via activation of multiple nonredundant kinase pathways.

SAA-1 promotes crosstalk between neutrophils and *i*NKT cells

The finding that SAA-1 induces the differentiation of immunosuppressive neutrophils has general implications, as SAA-1 is among the first acute-response proteins to be secreted during microbial infection and the differentiation of IL-10-producing immunosuppressive neutrophils is probably a common feature of inflammatory responses. Indeed, hepatic acute-phase response proteins induce the differentiation of MDSCs in a model of sepsis[17]. In addition, we found that the population expansion of MDSCs in mice ($CD11b^+Gr\text{-}1^+$ cells) and humans ($CD11b^+CD15^+$ cells) infected with influenza virus[18] correlated with higher concentrations of SAA-1 in the serum (data not shown).

It is known that *i*NKT cells are activated by TLR-dependent dendritic cell (DC)-maturation events through the combined secretion of soluble factors[19] and upregulation of endogenous lipid agonists presented by the antigen-presenting molecule CD1d[20,21]. Activated *i*NKT cells exert a variety of immunomodulatory functions mediated largely by their

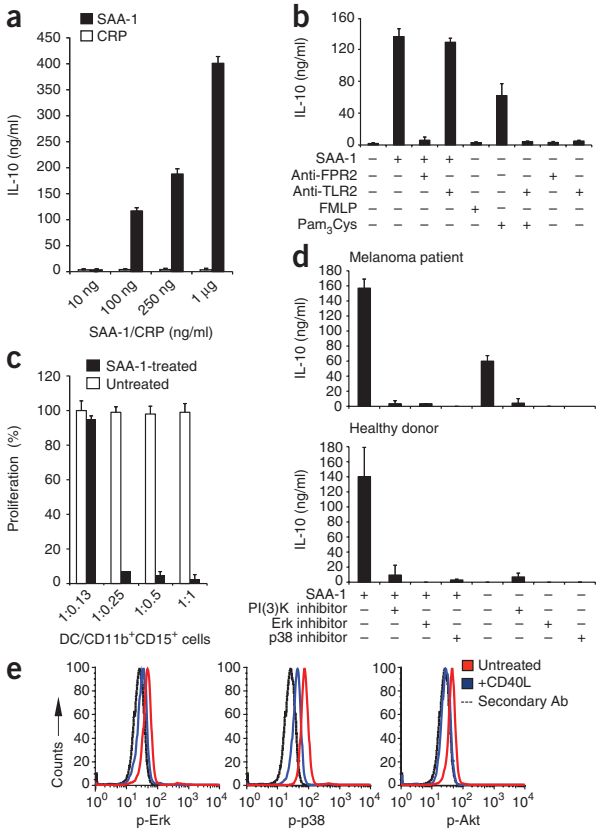

Figure 3 SAA-1 induces IL-10 production from human neutrophils. (**a**) IL-10 secretion by $CD11b^+CD15^+$ cells purified from healthy donors, in response to increasing concentrations of SAA-1. (**b**) IL-10 secretion by $CD11b^+CD15^+$ cells from healthy donors, treated with various combinations of SAA-1, Pam_3Cys or formyl peptide (FMLP) in the presence or absence of blocking anti-FPR2 and anti-TLR2. (**c**) Proliferation of alloreactive T cells stimulated by allogeneic DCs plus graded numbers of $CD11b^+CD15^+$ cells from third-party healthy donors left untreated or pretreated with SAA-1 (100% proliferation corresponds to 7×10^4 c.p.m.). (**d**) Release of IL-10 from $CD11b^+CD15^+$ cells purified from a patient with melanoma and a healthy donor, preincubated or not with the PI(3) kinase inhibitor LY294002, the Erk inhibitor U1026 and the p38 inhibitor SB203580 and then left untreated or treated with SAA-1. (**e**) Flow cytometry of $CD11b^+CD15^+$ cells obtained from patients with melanoma and left untreated or incubated with soluble CD40L (+CD40L), assessed by intracellular staining with antibodies specific for phosphorylated (p-) kinases. Ab, antibody. Data are representative of five independent experiments (**a**–**d**; error bars, s.d.) or three independent experiments (**e**).

constitutive expression of the ligand for the costimulatory molecule CD40 (CD40L) and the release of cytokines such as IL-4 and IFN-γ[22]. As the differentiation of MDSCs in mice infected with influenza virus is controlled by *i*NKT cells via a CD1d- and CD40-dependent mechanism[18] and because neutrophils express both CD40 and CD1d, which was further enhanced by their incubation with IFN-γ (**Supplementary Fig. 5c**), we sought to determine whether *i*NKT cells can modulate the activation and differentiation of IL-10-producing neutrophils. Neutrophils directly purified from patients with melanoma (**Fig. 4a**), but not those from healthy donors (**Supplementary Fig. 6a**), were able to promote the activation of *i*NKT cells in the absence of any further stimulation. In turn, incubation of neutrophils with *i*NKT cells led to less secretion of IL-10 by neutrophils from patients with melanoma (**Fig. 4b**). This effect was proportional to the number of *i*NKT cells added and was associated with the simultaneous increase in IL-12 production, as shown by ELISA (**Fig. 4c**) and by intracellular staining of $CD11b^+CD15^+$ cells (**Supplementary Fig. 6c**). The crosstalk between neutrophils and *i*NKT cells was CD1d dependent, as the addition of CD1d-blocking antibody prevented IFN-γ production by *i*NKT cells (**Fig. 4a** and **Supplementary Fig. 6a**) and restored IL-10 release by neutrophils (**Fig. 4b**).

As neutrophils from healthy donors failed to trigger activation of *i*NKT cells, we sought to determine whether SAA-1 could modulate their capacity to interact with *i*NKT cells. We found that treatment of neutrophils from healthy donors with SAA-1 triggered CD1d-dependent activation of *i*NKT cells to a degree similar to that obtained after pulsing of neutrophils with the strong *i*NKT cell agonist α-galactosylceramide (α-GalCer) (**Fig. 4a** and **Supplementary Fig. 6a**). Moreover, SAA-1 further increased the capacity of neutrophils from patients with melanoma to promote the activation of *i*NKT cells, and this activation led to less release of IL-10 by SAA-1-treated neutrophils from both patients with melanoma and healthy donors (**Fig. 4b** and data not shown). These results indicate that neutrophils purified from patients with a higher concentration of SAA-1, such as patients with melanoma, are able to spontaneously activate *i*NKT cells and that the activation of *i*NKT cells can modulate the release of IL-10 by immunosuppressive neutrophils.

Next we set out to identify the molecular mechanisms responsible for the crosstalk between *i*NKT cells and neutrophils and found that the addition of a CD40-blocking antibody abolished the ability of *i*NKT cells to diminish the release of IL-10 by immunosuppressive neutrophils (**Fig. 4b**). Three sets of experiments further confirmed that the ability of *i*NKT cells to down-modulate the SAA-1-dependent production of IL-10 by neutrophils was CD40L dependent. First, the incubation of IL-10-secreting neutrophils from patients with melanoma with increasing concentrations of soluble CD40L resulted in less production of IL-10 and more synthesis of IL-12 (**Supplementary Fig. 6b**). Second, the addition of soluble CD40L to neutrophils from patients with melanoma restored the proliferation of melan-A(26–35)-$CD8^+$ T cells (**Fig. 4d**). Finally, we observed that the CD40L-dependent decrease in IL-10 secretion in neutrophils from patients with melanoma was associated with less phosphorylation of Erk, Akt and p38 (**Fig. 3e**). Consistent with the results reported above, we observed that pulsing neutrophils isolated from patients with melanoma with the *i*NKT cell agonist α-GalCer further enhanced the ability of *i*NKT cells to abolish IL-10 production by neutrophils (**Fig. 4c**) and completely abolished their immunosuppressive activity in a CD40- and CD1d-dependent manner (**Fig. 4d**). Incubation of α-GalCer-pulsed $CD11b^+CD15^+$ cells with *i*NKT cells did not result in lysis of neutrophils by *i*NKT cells (**Supplementary Fig. 7a**), which ruled out the possibility that the loss of suppressive activity was simply due to cell death. In addition to having less secretion of IL-10 and more secretion of IL-12, *i*NKT-treated neutrophils had less production of reactive oxygen species (**Supplementary Fig. 7a**), which suggested an overall change from an immunosuppressive phenotype to a more inflammatory phenotype. These results demonstrate that in addition to driving the differentiation of IL-10-producing neutrophils, SAA-1 promotes their crosstalk with *i*NKT cells. This crosstalk is CD1d dependent, and by activating the CD40 pathway in neutrophils, it provides a negative feedback loop able to decrease IL-10 production and limit their immunosuppressive activity.

Lower frequency of suppressive neutrophils via *i*NKT cells

To further investigate the ability of *i*NKT cells to modulate the SAA-1-dependent differentiation of IL-10-producing neutrophils,

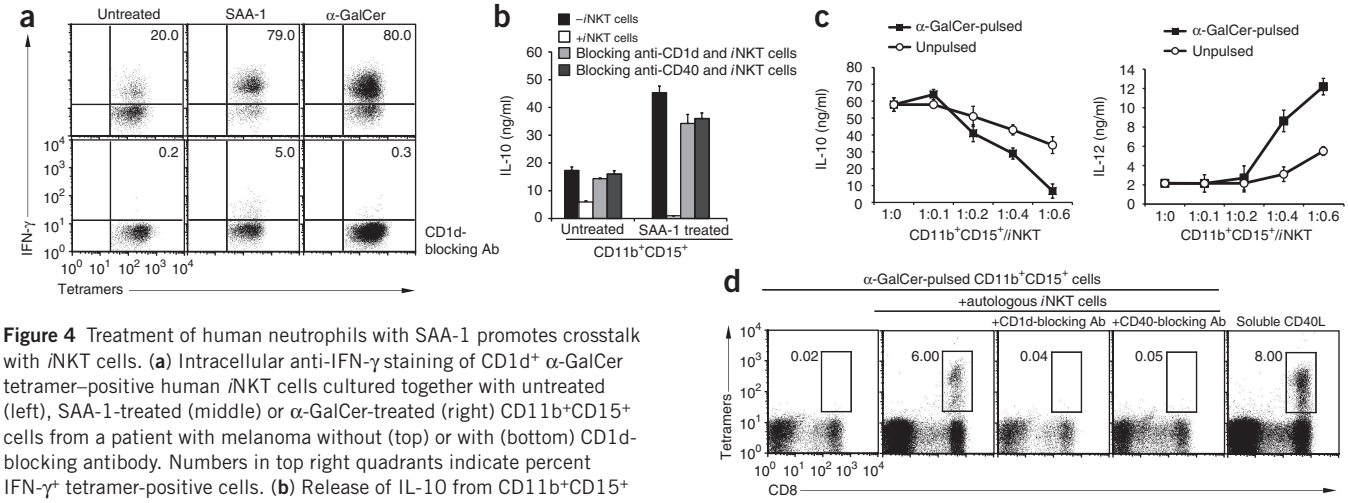

Figure 4 Treatment of human neutrophils with SAA-1 promotes crosstalk with *i*NKT cells. (**a**) Intracellular anti-IFN-γ staining of $CD1d^+$ α-GalCer tetramer–positive human *i*NKT cells cultured together with untreated (left), SAA-1-treated (middle) or α-GalCer-treated (right) $CD11b^+CD15^+$ cells from a patient with melanoma without (top) or with (bottom) CD1d-blocking antibody. Numbers in top right quadrants indicate percent IFN-γ^+ tetramer-positive cells. (**b**) Release of IL-10 from $CD11b^+CD15^+$ cells from a patient with melanoma, left untreated or treated with SAA-1 and incubated with *i*NKT cells with or without CD1d- or CD40-blocking antibodies. (**c**) Release of IL-10 and IL-12 from α-GalCer-pulsed or unpulsed $CD11b^+CD15^+$ cells from a patient with melanoma, incubated at various ratios (horizontal axes) with *i*NKT cells. (**d**) Frequency of melan-A(26–35) tetramer–specific $CD8^+$ T cells among total leukocytes obtained from a patient with melanoma and incubated with autologous melan-A(26–35) peptide–pulsed DCs and α-GalCer-pulsed autologous $CD11b^+CD15^+$ cells with or without autologous *i*NKT cells (left four plots), without (left two plots) or with blocking anti-CD1d (+CD1d-blocking Ab) or anti-CD40 (+CD40-blocking Ab). Far right, population expansion of melan-A(26–35)-specific $CD8^+$ T cells after the addition of soluble CD40L to $CD11b^+CD15^+$ cells in the absence of α-GalCer and autologous *i*NKT cells. Numbers adjacent to outlined areas indicate percent tetramer-postive $CD8^+$ cells. Data are representative of three (**a**,**b**,**d**) or two (**c**) experiments (error bars (**b**,**c**), s.d.).

we injected recombinant SAA-1 subcutaneously once a day for 5 d into wild-type and *i*NKT-deficient Tcra-J^{1Tgi} mice (which lacked the gene segment encoding T cell antigen receptor α-chain joining region 18 ($J_\alpha 18$); called '*Jα18*$^{-/-}$ mice' here) and assessed the frequency of neutrophils ($CD11b^+Ly6G^+$ cells) in blood samples at various time points during treatment. Consistent with published findings demonstrating the ability of SAA-1 to induce neutrophilia[11], we observed population expansion of $CD11b^+Ly6G^+$ cells in wild-type mice injected with SAA-1, which decreased 3 d after the final injection of SAA-1 (**Fig. 5a**,**b**). However, injection of SAA-1 induced a higher percentage of $CD11b^+Ly6G^+$ cells into *Jα18*$^{-/-}$ mice (28.2%) than in wild-type mice (13.5%), which decreased more slowly than it did in SAA-1-injected wild-type mice (**Fig. 5a**,**b**). Notably, in contrast to results obtained with wild-type neutrophils, a large proportion of $CD11b^+Ly6G^+$ cells from SAA-1-injected *Jα18*$^{-/-}$ mice produced IL-10 (**Fig. 5c**,**d**), which indicated that the lack of *i*NKT cells allowed the population expansion of IL-10-secreting neutrophils. Finally, $CD11b^+Ly6G^+$ cells isolated from *Jα18*$^{-/-}$ mice were able to suppress the proliferation of OT-I cytotoxic T lymphocytes (with a transgene for an ovalbumin-specific T cell antigen receptor) much more efficiently than were $CD11b^+Ly6G^+$ cells purified from SAA-1-injected wild-type mice (**Fig. 5e**). As a control, we demonstrated that injection of SAA-1 into TLR2-deficient mice induced the population expansion of IL-10-secreting $CD11b^+Ly6G^+$ cells similar to that in SAA-1-injected wild-type mice (data not shown).

The results reported above could have depended either on direct crosstalk between neutrophils and *i*NKT cells or on the transmodulation of neutrophils by soluble factors released from *i*NKT cells. To distinguish between those two possibilities, we reconstituted lethally irradiated B6.SJL mice ($CD45.1^+$) with a 1:1 mixture of $CD1d^+CD45.1^+$ and $CD1d^-CD45.2^+$ bone marrow

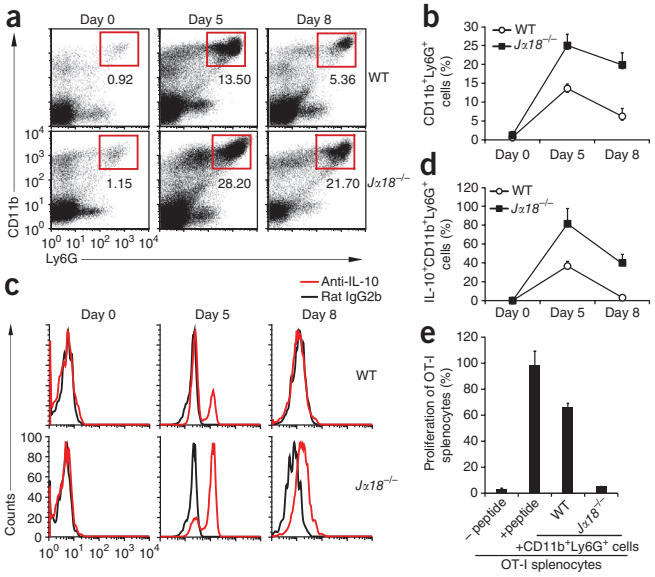

Figure 5 Population expansion of immunosuppressive IL-10-secreting neutrophils in *Jα18*$^{-/-}$ mice injected with SAA-1. (**a**) Frequency of $CD11b^+Ly6G^+$ cells in the blood of wild-type (WT) and *Jα18*$^{-/-}$ mice ($n = 6$ per genotype) injected subcutaneously daily for 5 d with SAA-1; blood samples were collected before injection (day 0) and at day 5 and day 8 (3 d after the final SAA-1 injection). Numbers under outlined areas indicate percent $CD11b^+Ly6G^+$ cells. (**b**) Cumulative frequency of $CD11b^+Ly6G^+$ cells in wild-type and *Jα18*$^{-/-}$ mice ($n = 6$) injected with SAA-1 as described in **a**. (**c**) Flow cytometry of circulating $CD11b^+Ly6G^+$ cells from wild-type or *Jα18*$^{-/-}$ mice injected subcutaneously daily for 5 d with SAA-1, stained *ex vivo* with anti-IL-10 or isotype-matched control antibody (rat immunoglobulin G2b (IgG2b)). (**d**) Cumulative frequency of $IL\text{-}10^+CD11b^+Ly6G^+$ cells in wild-type and *Jα18*$^{-/-}$ mice ($n = 6$ per genotype) injected with SAA-1 as described in **c**. (**e**) Proliferation of OT-I splenocytes labeled with the cytosolic dye CFSE and pulsed with the ovalbumin peptide SIINFEKL in the presence of 10% $CD11b^+Ly6G^+$ cells sorted from the blood of SAA-1-injected wild-type or *Jα18*$^{-/-}$ mice at day 5, analyzed at day 4. Data are representative of three (**a**–**d**) or two (**e**) experiments (error bars (**b**,**d**,**e**), s.d.).

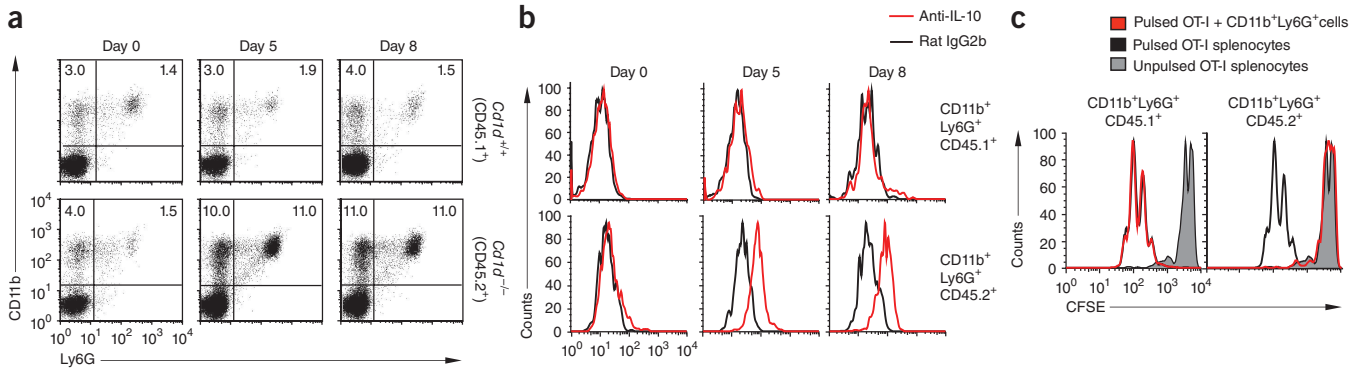

Figure 6 Population expansion of immunosuppressive IL-10-secreting *Cd1d*$^{-/-}$neutrophils in SAA-1-injected *Cd1d*$^{+/+}$*Cd1d*$^{-/-}$ mixed–bone marrow chimeras. (**a**) Expression of CD11b and Ly6G by cells from *Cd1d*$^{+/+}$*Cd1d*$^{-/-}$ chimeric mice injected subcutaneously daily for 5 d with SAA-1 (120 μg per kg body weight), in blood samples collected on days 0, 5 and 8 after the initial injection, stained with anti-CD11b and anti-Ly6G and gated on CD45.1$^+$ (*Cd1d*$^{+/+}$) cells (top) or CD45.2$^+$ (*Cd1d*$^{-/-}$) cells (bottom). Numbers in quadrants indicate percent CD11b$^+$Ly6G$^-$ cells (top left) or CD11b$^+$Ly6G$^+$ cells (top right). (**b**) Flow cytometry of CD11b$^+$Ly6G$^+$ cells derived from CD45.1$^+$ (*Cd1d*$^{+/+}$) bone marrow (top) or CD45.2$^+$ (*Cd1d*$^{-/-}$) bone marrow (bottom) stained intracellularly with anti-IL-10. (**c**) Proliferation of CFSE-labeled unpulsed or SIINFEKL-pulsed OT-I splenocytes with or without CD11b$^+$Ly6G$^+$ cells from CD45.1$^+$ (*Cd1d*$^{+/+}$) cells (left) or CD45.2$^+$ (*Cd1d*$^{-/-}$) cells (right) sorted from the blood of SAA-1 injected chimeric mice at day 5 and assessed at day 4. Data are representative of two experiments with three mice each (**a**,**b**) or two experiments (**c**).

(**Supplementary Fig. 7b**). We reasoned that if the *i*NKT cell–dependent effect on IL-10-secreting neutrophils was also CD1d dependent, injection of SAA-1 into chimeric mice should 'preferentially' induce the population expansion of IL-10-secreting CD1d$^-$CD45.2$^+$ neutrophils. Before injecting SAA-1, we confirmed equal engrafting of CD45.1$^+$ and CD45.2$^+$ hematopoietic cells (data not shown) and positive selection of *i*NKT cells (**Supplementary Fig. 7c**). After injecting SAA-1 daily for 5 consecutive days, we observed greater population expansion of circulating CD11b$^+$Ly6G$^+$ cells derived from *Cd1d*$^{-/-}$ donor (CD45.2$^+$) bone marrow than of CD11b$^+$Ly6G$^+$ cells that originated from wild-type (CD45.1$^+$) bone marrow (**Fig. 6a**). Notably, purified blood CD11b$^+$Ly6G$^+$ cells that originated from the *Cd1d*$^{-/-}$ (CD45.2$^+$) bone marrow produced IL-10 and were able to suppress the proliferation of OT-I cells (**Fig. 6b**,**c**), whereas purified blood CD11b$^+$Ly6G$^+$ cells derived from the *Cd1d*$^{+/+}$ (CD45.1$^+$) bone marrow remained IL-10$^-$ and did not have any suppressive effect on the proliferation of OT-I cytotoxic T lymphocytes (**Fig. 6b**,**c**). These findings provide conclusive evidence that the *in vivo* crosstalk between *i*NKT cells and SAA-1-treated neutrophils is CD1d dependent and results in much less IL-10 secretion.

DISCUSSION

In this study we have shown that in both humans and mice, the acute-phase response protein SAA-1 induced the population expansion and differentiation of IL-10-secreting neutrophils, which suppressed antigen-specific T cell responses. In addition, we observed that SAA-1 also promoted interaction between neutrophils and *i*NKT cells in a CD1d- and CD40-dependent manner, which reversed their suppressive phenotype by decreasing IL-10 production. The effect of SAA-1 was mediated by the binding of SAA-1 to the G protein–coupled receptor FPR2 and was dependent on the activation of mitogen-activated protein kinase and PI(3)K. Conversely, the CD40-dependent signaling pathway activated by the crosstalk with *i*NKT cells dephosporylated p38, Erk and PI(3)K.

The observation that SAA-1 also promoted interactions between neutrophils and *i*NKT cells opens an avenue to new therapeutic strategies that harness this immunomodulatory T cell subset. Many mechanisms that influence the activation of *i*NKT cells have emerged, which indicates an important link between inflammation and *i*NKT cells[23] that results in enhanced CD40-dependent activation of DCs[24,25] and B cells[26]. For example, during microbial infection, inflammatory cytokines such as IL-12 and IL-18 enhance basal *i*NKT cell autoreactivity and promote the secretion of interferon-γ[19–21,27]. In addition, during an inflammatory response, activation of *i*NKT cells can be influenced by higher expression of surface CD1d molecules by activated antigen-presenting cells[28,29], secretion of granulocyte colony-stimulating factor[30] and/or by higher expression of enzymes that lead to the biosynthesis of endogenous self lipids[20,21,31].

Our *in vivo* data underscore the importance of *i*NKT cell numbers in modulating the overall frequency of neutrophils and their differentiation in IL-10-secreting cells via CD1d-dependent interactions. Indeed, injection of SAA-1 resulted in fewer IL-10-secreting neutrophils in wild-type mice than in *i*NKT cell–deficient mice. In addition, injection of SAA-1 in mixed bone marrow–chimeric mice reconstituted with hematopoietic cells among which half lacked CD1d molecules resulted in the proliferation of IL-10-secreting immunosuppressive neutrophils only in the *Cd1d*$^{-/-}$ cell population. Further experiments are needed to understand in which location *i*NKT cells modulate the proliferation and differentiation of neutrophils. However, given the ability of SAA-1 to induce neutrophilia[11] and the presence of *i*NKT cells in the bone marrow[32,33], we speculate that *i*NKT cells may modulate the differentiation and population expansion of neutrophils in the bone marrow as well as in the periphery, possibly by interacting with granulocyte-monocyte precursors, which we have found to be CD1d$^+$ (data not shown).

It is known that *i*NKT cells constitute a functionally heterogeneous population of distinct subsets, differing in their CD4 expression[34] and NK1.1 expression[35,36], anatomical location[37], cytokine profiles[38] and effector functions[37]. Thus, it remains unclear whether specific *i*NKT cell subsets mediate the downregulation of IL-10 secretion from SAA-1-differentiated neutrophils *in vivo*. The functional diversity of *i*NKT cells has been extended with the discovery of a subset of IL-17-producing *i*NKT cells that lack expression of both CD4 and NK1.1 (ref. 38) and are able to promote airway neutrophilia[39] and exert effector functions in epithelia, such as the skin[40]. Given the link between IL-17 secretion and neutrophil recruitment[41], further experiments are warranted to address the role of these IL-17-producing *i*NKT cells[38,40,42,43] in the modulation of

IL-10 production by SAA-1-differentiated neutrophils. The release of IL-17 by *i*NKT cells may represent a host defense mechanism for attracting anti-inflammatory neutrophils in inflamed tissues and converting them into proinflammatory neutrophils.

The reasons for the large population expansion of IL-10-secreting neutrophils in patients with melanoma, despite the presence of circulating *i*NKT cells, remain unclear. However, it is possible that the abundance of IL-10-producing neutrophils in such patients may be accounted for by a defect in the numbers and activity of *i*NKT cells, as it has been reported that patients with cancer often have lower frequencies of circulating *i*NKT cells than do healthy volunteers[44–46], and that these *i*NKT cells have a defective activation phenotype[47]. As we have shown that the ability of *i*NKT cells from patients with melanoma to abolish the production of IL-10 by neutrophils was enhanced after their incubation with strong *i*NKT cell agonists, such as α-GalCer, pharmacologically harnessing the interaction between *i*NKT cells and neutrophils may provide a new conditioning regimen aimed at increasing the therapeutic efficacy of subsequent vaccination strategies.

The population expansion of suppressive IL-10-secreting neutrophils in patients with melanoma correlated positively with higher concentrations of SAA-1 and with later stages of disease. Our findings confirm and extend published results demonstrating a high frequency of circulating immunosuppressive neutrophils in many tumor types[48–50] and suggest that SAA-1 is a negative prognostic marker in patients with cancer[51]. As the proliferation of suppressive neutrophils during microbial infection is a mechanism for controlling the unwanted bystander effects of neutrophils[7,17], our results highlight a previously unknown mechanism used by tumors to impair tumor-specific immune responses by harnessing the anti-inflammatory properties of this acute-phase protein. By exploiting the plasticity of neutrophils, the tumor microenvironment induces the proliferation of large numbers of immunosuppressive IL-10-secreting cells, which enhances local and systemic SAA-1. Consistent with our findings, it has been shown that a proportion of renal cell carcinomas secrete SAA-1 (ref. 52) and that SAA-3, another member of the SAA family, is able to condition the premetastatic niche of tumor cell infiltration by facilitating the accumulation of myeloid cells in premetastatic lungs[53], although secretion of IL-10 in those tumors was not investigated[53]. These results indicate that SAA-1 secreted into the tumor microenvironment (by tumor cells and tumor-infiltrating macrophages) contributes to total plasma concentrations of SAA. However, as it is known that soluble factors secreted into the tumor microenvironment (such as TNF, IL-6 and IL-1) can enhance transcription of the gene encoding SAA-1 (refs. 54,55), the relative contributions of liver- and tumor-derived SAA-1 remains unknown at present.

SAA-1 is secreted rapidly during viral and bacterial infection, and we anticipate that the results we have reported here can be extended to inflammatory processes that depend on microbial infection. Indeed, infection of mice lacking *i*NKT cells with influenza virus results in the population expansion of immunosuppressive $CD11b^+Ly6G^+$ $Ly6C^+$ cells, which infiltrate inflamed lungs and greatly diminish the ability of these infected mice to mount antigen-specific immune responses[18]. As SAA-1 concentrations in plasma are much higher during influenza infection[56] and increase to much larger amounts during influenza infection than in patients with melanoma (data not shown), it is likely that the proliferation of suppressive $CD11b^+Ly6G^+$ cells observed in influenza virus–infected mice and of $CD11b^+CD15^+$ cells observed in influenza virus–infected people also correlates with the enhanced SAA-1 plasma concentrations.

In conclusion, our results have described a previously unknown mechanism for the control of inflammatory processes via local and systemic secretion of SAA-1 that not only leads to the proliferation and differentiation of anti-inflammatory neutrophils but also promotes the crosstalk of neutrophils with *i*NKT cells, which in turn modulates their suppressive properties. Our findings emphasize the plasticity of neutrophils as cells able to have pro- and anti-inflammatory properties and highlight the role of *i*NKT cells as important modulators of inflammatory responses. This powerful physiological mechanism designed to control excessive inflammation can be exploited by tumors to impair tumor-specific immune responses. Harnessing *i*NKT cells may diminish the frequency of immunosuppressive neutrophils, which may have important implications for vaccination strategies.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

C.D.S. designed and did the experiments, prepared the figures and contributed to the writing of the manuscript; R. Arscott did specific experiments; I.K. and M.M. obtained consent from patients with melanoma and collected blood samples; S.B., M.J. and R. Asher did tissue staining of melanoma sections; M.S. provided reagents and contributed to the writing of the manuscript; and V.C. designed the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. Tetramers of major histocompatibility complex class I and CD1d were prepared as described[57,58]. SAA-1 protein was from Peprotech. Soluble CD40L was from Alexis. Anti-FPR2 (FN-1D6-AI) was from GENOVAC, and antibodies to phosphorylated Erk (4377), p38 (92110 and Akt (9272) were from Cell Signaling. Other antibodies used for flow cytometry staining were from eBioscience. Anti-Ly6G (1A8) was from BD Pharmingen. Samples were acquired on a FACSCalibur with CellQuest software or on a CyAn flow cytometer and data were analyzed with FlowJo software. Antibodies for immunoblot analysis were anti-arginase-1 (N-20; sc-18351; Santa-Cruz Biotechnology) and anti-GAPDH (glyceraldehyde-3-phoshate dehydrogenase; AHP996; AbD Serotec). Kinase inhibitors were SB203580 (for p38), U1026 (for Erk) and LY294002 (for PI(3)K; Calbiochem Merck). DCFDA-H_2 (5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; Invitrogen) was used for the detection of reactive oxygen species production as described[59]. Pam_3Cys was used at a concentration of 10 μg/ml (Invivogen); α-GalCer was solubilized at a concentration of 200 μg/ml in vehicle (0.5% (vol/vol) Tween-20 in PBS). The human CD1d–blocking antibody CD1d42 was from BD Pharmingen.

Mice. Female C57BL/6 mice, OT-I mice and B6.SJL-$Ptprc^aPep3^b$/BoyJ (B6Ly5.1, CD45.1$^+$) mice were maintained in the Biological Services Unit of John Radcliffe Hospital at the University of Oxford and were used according to established institutional guidelines of the University of Oxford under the authority of a UK Home Office project license. Other mice were as follows: *Jα18*$^{-/-}$ mice, lacking $V_\alpha14$ *i*NKT cells but containing other lymphoid cell lineages[60], and *Cd1d*$^{-/-}$ (CD45.2$^+$) mice (provided by L. Van Kaer)[61].

Isolation and differentiation of human neutrophils. Neutrophils were purified from the peripheral blood of patients with melanoma and healthy donors with a Ficoll gradient: leukocytes pelleted as a layer over red blood cells were collected and then were purified with magnetic beads coated with anti-CD11b (Miltenyi Biotec). A proportion greater than 95% was stained with anti-CD11b (M1/70; M1/FO; eBioscience) and anti-CD15 (H198; eBioscience; **Supplementary Fig. 1c**). Neutrophils from healthy donors and patients with melanoma were preincubated for 1 h at 37 °C with inhibitors of Erk, p38 or PI(3) (1 μM) and with anti-CD40 (5 μg/ml; MAB617; R&D Systems) and then were cultured for 24 h with SAA-1 (250 ng/ml). SAA-1 or α-GalCer (100 ng/ml) was added to human neutrophils for 24 h in the presence of *i*NKT cells. Soluble CD40L was added to untreated neutrophils from patients with melanoma for 24 h.

Human *i*NKT cells and DCs. Both *i*NKT cells and DCs were generated from healthy blood donors or patients with melanoma as described[20].

OT-I proliferation assays. Splenocytes from OT-I mice were pulsed for 1 h at 37 °C with SIINFEKL peptide (2 μg/ml) and washed and then were labeled with 5 μM CFSE (carboxyluorescein succinimidyl ester). Sorted CD11b$^+$Ly6G$^+$ cells (2×10^4) were cultured in 96-well flat-bottomed plates with 2×10^5 CFSE-labeled OT-I splenocytes. Cells were analyzed 4 d later with a FACSCalibur with CellQuest software. Data are presented as the percentage of proliferation of SIINFEKL-pulsed, CFSE-labeled OT-I splenocytes in the presence of CD11b$^+$Ly6G$^+$ cells relative to the proliferation of SIINFEKL-pulsed, CFSE-labeled-OT-I splenocytes in the absence of CD11b$^+$Ly6G$^+$ cells (set as 100%).

Mixed-lymphocyte reactions. PBMCs (2×10^5) were mixed with allogeneic irradiated (5,000 rads) DCs (5×10^4) in 200 μl RPMI medium containing 5% (vol/vol) human AB serum in 96-well flat-bottomed plates. Cells were incubated for 5 d at 37 °C in 5% CO_2 and then [^{3}H]thymidine (1 μCi per well; Perkin Elmer life Sciences) was added for 15–18 h. The incorporation of [^{3}H]thymidine was measured with a Wallac Microbeta Jet 1450 reader (Perkin Elmer). For CD11b$^+$CD15$^+$ cell–mediated inhibition of lymphocyte proliferation, irradiated SAA-1-treated CD11b$^+$CD15$^+$ cells (5×10^4) from healthy donors were cultured together with PBMCs and irradiated DCs. Data are presented as the percentage of PBMC proliferation driven by allogeneic irradiated DCs in the presence of irradiated CD11b$^+$CD15$^+$ cells relative to the proliferation of alloreactive PBMCs in the absence of CD11b$^+$CD15$^+$ cells (set as 100%).

ELISA. Plasma was collected from patients with melanoma after 2 h of sedimentation. Blood samples were obtained according to an ethically approved protocol, after written informed consent was provided by patients. Cytokines were quantified with a FlowCytomix kit according to the manufacturer's instructions (Bender MedSystems). SAA-1 and C-reactive protein were measured with ELISA kits according to the manufacturers' instructions (Invitrogen and Elica, respectively). IL-10 and IL-12 were measured with ELISA kits (BD Biosciences).

Intracellular staining. Human or mouse neutrophils were stained for 20 min at 4 °C with antibodies to surface markers. Cells were washed twice in cold PBS, then were fixed and made permeable with a Foxp3 Fixation/Permeabilization Concentrate and Diluent kit according to the manufacturer's instructions (eBioscience). Cells were washed twice with Permabilization buffer (eBioscience) and were stained for 20 min at 4 °C with specific antibodies.

Immunohistochemistry. Tissue sections of primary tumors (4 μm in thickness) were deparaffinized and rehydrated. Antigen demasking was achieved in 50 mM Tris and 2 mM EDTA, pH 9.0, with a Philips Whirlpool Sixth Sense microwave on a steaming program. The Novolink Polymer Detection System (RE7280-K, Leica) was used for staining with anti–human CD68 (PG-M1; Dako) and substrate was developed with a silver-gray peroxidase substrate kit (SK-4700; Vector Labs). The Dako REAL Detection System with alkaline phosphatase was used for staining with anti-SAA-1 (MC1; Abcam). Samples were incubated overnight at 4 °C with primary antibody. Reactions were visualized with the Dako Fuchsin + Substrate-Chromogen system (k0625; Dako). Tissue sections were counterstained with Gill No. 3 hematoxylin (Sigma Aldrich) and were mounted in Aquatex (Merck).

Mixed–bone marrow chimera mice. Recipient mice were γ-irradiated twice with 450 rads and then were reconstituted with a mixture of bone marrow derived from *Cd1d*$^{-/-}$ and B6.SJL mice (a total of 5×10^6 cells) to achieve 1:1 chimerism. At 8 weeks after reconstitution, mice were tested for chimerism and at 10 weeks, chimeras were injected subcutaneously for 5 d with SAA-1 (120 μg per kg body weight).

Population expansion of melan-A(26–35)-specific CD8$^+$ T cells. Human DCs were pulsed for 3 h with melan-A(26–35) (100 ng/ml; sequence, ELAGIGILTV) in serum-free medium. Cells were washed thoroughly and then were incubated at a ratio of 1:10 with autologous PBMCs (1×10^6) preincubated for 1 h with IL-10 receptor–blocking antibody (10 μg/ml; 3FD1; BioLegend) in RPMI medium containing 10% (vol/vol) FCS. Autologous CD11b$^+$CD15$^+$ cells were purified as described above and were added to the culture at a ratio of 1:1 with DCs. Populations of melan-A(26–35)-specific CD8$^+$ cells were expanded in IL-2-containing medium and were analyzed at days 10–15 by tetramer staining as described[62].

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