nature medicine

# Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells

Tianhong Wang[1,5], Guilian Niu[2,5], Marcin Kortylewski[2,5], Lyudmila Burdelya[2,5], Kenneth Shain[3], Shumin Zhang[4], Raka Bhattacharya[2], Dmitry Gabrilovich[2], Richard Heller[3], Domenico Coppola[3], William Dalton[3], Richard Jove[4], Drew Pardoll[1] & Hua Yu[2]

**Although tumor progression involves processes such as tissue invasion that can activate inflammatory responses, the immune system largely ignores or tolerates disseminated cancers. The mechanisms that block initiation of immune responses during cancer development are poorly understood. We report here that constitutive activation of Stat-3, a common oncogenic signaling pathway, suppresses tumor expression of proinflammatory mediators. Blocking Stat-3 in tumor cells increases expression of proinflammatory cytokines and chemokines that activate innate immunity and dendritic cells, leading to tumor-specific T-cell responses. In addition, constitutive Stat-3 activity induces production of pleiotropic factors that inhibit dendritic cell functional maturation. Tumor-derived factors inhibit dendritic cell maturation through Stat-3 activation in progenitor cells. Thus, inhibition of antitumor immunity involves a cascade of Stat-3 activation propagating from tumor to dendritic cells. We propose that tumor Stat-3 activity can mediate immune evasion by blocking both the production and sensing of inflammatory signals by multiple components of the immune system.**



Recent studies in genetically deficient mice indicate that numerous components of both the innate and adaptive immune systems can act as extrinsic tumor suppressors[1,2]. However, the immune system is generally tolerant of established cancers[3,4]. It has been proposed that dendritic cells play a central role in immunologic decisions[5,6]. In the absence of inflammation, dendritic cells do not become fully activated and induce tolerance rather than immunity[5,6]. Evidence has also been emerging that tumor cells can secrete factors that inhibit dendritic cell maturation[7,8]. Here we evaluate the role of Stat-3 activity in blocking the initiation of antitumor immunity. Stat-3 is a potential negative regulator of inflammatory responses, as mice devoid of the Stat-3 gene in macrophages and neutrophils have enhanced inflammatory activity, leading to the development of chronic enterocolitis[9]. Recently, *Stat3*$^{-/-}$ macrophages have been shown to produce increased amounts of interleukin-12 (IL-12) and RANTES, which are involved in restoring the responsiveness of tolerant CD4$^{+}$ T cells as well as in reversal of systemic tolerance[10]. Stat-3 is commonly constitutively activated in diverse cancers of both hematopoietic and epithelial origin. Constitutively activated Stat-3 enhances tumor cell proliferation and prevents apoptosis[11–13]. We show here that constitutive Stat-3 activity in tumors inhibits the production of multiple proinflammatory cytokines and chemokines while inducing the release of factors that inhibit dendritic cell maturation through activation of Stat-3 in dendritic cells. These results indicate that Stat-3 activation in tumors negatively regulates induction of adaptive immunity.

## RESULTS

### Tumor Stat-3 activity and inflammatory mediators

To investigate whether constitutive activation of Stat-3 in cancer cells might negatively regulate the cells' ability to express inflammatory mediators, we disrupted Stat-3 signaling in tumor cell lines by transfecting either a dominant-negative variant of Stat-3, designated Stat-3β[12], or a Stat-3 antisense oligonucleotide[14]. Like human tumors, many murine tumor cell lines show constitutively activated Stat-3 (refs. 14–16). An electrophoretic mobility shift assay (EMSA) showed that Stat-3 is constitutively activated in B16, SCK-1 and CT26 mouse tumor cells (**Fig. 1a**, top). As blocking Stat-3 signaling in the tumor cells results in apoptosis[14,16], we carried out transient transfection of these tumor cell lines with Stat-3β or the Stat-3 antisense oligonucleotide. Because Stat-3β expression is coexpressed with fluorescent protein (GFP)[14], we used either FACS or fluorescence microscopy to quantify *in vitro* transfection efficiency. Transfection efficiencies were 20–40% for B16 cells and 5–15% for SCK-1 and CT26 cells. Western blot analyses showed that transient transfection of Stat-3β resulted in the expression of Stat-3β protein (**Fig. 1a**, middle), which has higher DNA-binding activity than Stat-3α and thus is expected to displace Stat-3α DNA binding[12]. Transient transfection of a Stat-3 antisense oligonucleotide reduces the expression of Stat-3 but not Stat-1 (**Fig. 1a**, bottom). We initially evaluated the effects of the proinflammatory cytokines on expression using RNase protection assays (RPAs) with multitemplate RNA probes. We found that interferon-β (IFN-β),

[1]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA. [2]Immunology Program, [3]Clinical Investigations Program and [4]Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, Department of Interdisciplinary Oncology, University of South Florida College of Medicine, Tampa, Florida 33612, USA. [5]These authors contributed equally to this work. Correspondence should be addressed to D.M.P. or H.Y. (dmpardol@jhmi.edu or huayu@moffitt.usf.edu).

Published online 21 December 2003; doi:10.1038/nm976

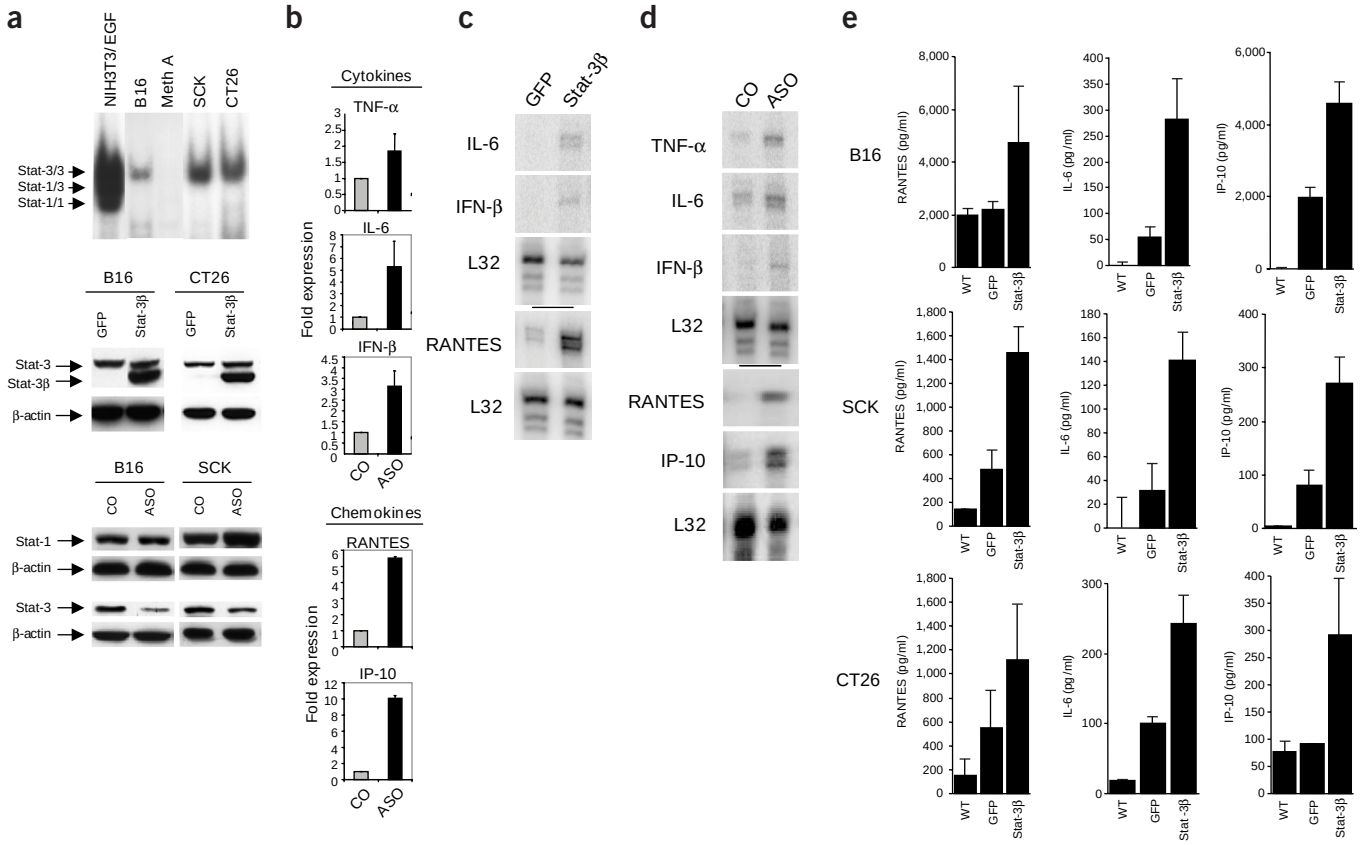


**Figure 1** Blocking Stat-3 in tumor cells increases proinflammatory cytokine and chemokine expression. (**a**) Stat-3 activity and expression in tumor cells. Top, Stat-3 activation in tumor cells, as detected by EMSA. 3/3, Stat-3 homodimer; 3/1, Stat-3/Stat-1 heterodimer; 1/1, Stat-1 homodimer. Middle, Stat-3β protein expression after transient transfection detected by western blot. Bottom, western blot showing specificity of a Stat-3 antisense oligonucleotide (ASO) as compared to its control (CO). NIH3T3/EGF, NIH3t3 cells stimulated with EGF as markers of activated Stat-1 and Stat-3; MethA, mouse sarcoma tumor cells. (**b**) Histograms representing the means of three RPA experiments with oligonucleotides normalized to RNA expression of L32 and GAPDH (internal controls). Data are presented as fold difference relative to control-transfected B16 cells. (**c**,**d**) RPA analysis of RNA from CT26 (**c**) and SCK-1 (**d**) tumor cells transfected with the indicated vectors or oligonucleotides. Autoradiograms are representative of two or three independent experiments. (**e**) Detection of cytokines and chemokines by ELISA in transiently transfected tumor cells. Results are presented as the mean ± s.e.m., $n = 3$, for each ELISA. WT, untransfected tumor cells. Both GFP- and Stat-3β-transfected cells were sorted before ELISA analysis.

tumor necrosis factor-α (TNF-α), IL-6 and the chemokines RANTES and IP-10 were elevated in B16 melanoma cells transiently transfected with either Stat-3β (data not shown) or the antisense Stat-3 oligonucleotide (**Fig. 1b**). The upregulation of these proinflammatory mediators was selective, as we detected no increases in the expression of many other genes, including IL-4 and IL-10 (see Methods for cytokine and chemokine RNAs tested). Because interrupting Stat-3 signaling induces growth arrest and apoptosis of B16 cells[17], we prepared RNA before the initiation of any apoptotic changes. In addition, we examined the effects of apoptosis-inducing doses of UV irradiation on cytokine and chemokine RNA expression. UV irradiation had no detectable effects on expression of the indicated cytokines and chemokines (data not shown), indicating that their induction after Stat-3 blockade was not a general response to an apoptotic stimulus.

The expression of proinflammatory mediators is not specific to B16 melanoma cells. Interruption of Stat-3 signaling in the CT26 mouse colon carcinoma line and the SCK-1 breast carcinoma cell line, each of which shows constitutive Stat-3 activity (**Fig. 1a**), similarly induced expression of these cytokines and chemokines (**Fig. 1c,d**). We confirmed these results at the protein level by ELISA, which showed that Stat-3 blockade induced the proinflammatory mediators IL-6, RANTES and IP-10 in each tumor cell type (**Fig. 1e**).

To further verify the role of Stat-3 in downregulating the expression of inflammatory mediators, we asked whether constitutive Stat-3 activity could inhibit induced expression of proinflammatory cytokines and chemokines. Treatment of normal BALB/c 3T3 fibroblasts, which lack constitutively activated Stat-3, with IFN-γ (50 U/ml) and lipopolysaccharide (LPS; 2.5 μg/ml) resulted in upregulation of IL-6 and RANTES (data not shown). We induced Stat-3 activation in BALB/c 3T3 cells by two independent approaches—transformation with v-Src, which induces constitutive Stat-3 activation and enforced expression of a constitutively activated Stat-3 mutant, Stat-3C[11]. v-Src transformation resulted in Stat-3 activation and inhibition of cytokine and chemokine induction by IFN-γ and LPS in 3T3 fibroblasts (**Fig. 2a**). Similarly, Stat-3C-transfected 3T3 cells showed higher Stat-3 DNA-binding activity and lower IFN-γ- and LPS-induced IL-6 and RANTES RNA expression than did 3T3 cells transfected with a control empty vector (pcDNA3) (**Fig. 2b**). These results show that Stat-3 activation can inhibit expression of cytokines and chemokines induced by IFN-γ and LPS.

### Stat-3 blockade activates components of innate immunity

We next evaluated how the inflammatory factors (both defined and undefined) from Stat-3-disrupted tumor cells might affect the innate

immune response. Resident peritoneal macrophages treated with supernatant from Stat-3β-transfected B16 cells showed strong induction of nitric oxide release and RANTES expression (**Fig. 3a,b**). In addition, the supernatant derived from Stat-3β-transfected B16 cells stimulated TNF-α production by neutrophils (**Fig. 3c**). These results indicate that the proinflammatory mediators, including cytokines and chemokines induced by interrupting Stat-3 signaling in tumor cells, initiate an amplifying cascade of proinflammatory signals within the innate immune system. We also examined whether disrupting Stat-3 signaling in tumor cells could activate innate immune cells *in vivo*. Gene transfer of Stat-3β into established B16 tumors resulted in infiltration of a pleiotropic nature, including macrophages and neutrophils (**Fig. 3d,e**).

### Blocking Stat-3 activates dendritic cells and adaptive immunity

Innate immunity critically affects the development of adaptive immune responses[18–20]. An important mechanism of linkage involves enhancement of the antigen-presenting capacity of dendritic cells induced in part by proinflammatory cytokines[21,22]. Exposure of dendritic cells to conditioned medium containing supernatant from Stat-3β-transfected B16 cells resulted in greater expression of IL-12 (**Fig. 4a**), MHC class II and CD40 (**Fig. 4b**). No increase in dendritic cell maturation markers was seen when supernatant from UV-irradiated B16 cells was added to dendritic cell culture, indicating that the maturation induced by supernatant from Stat-3β-transduced tumor cells was not simply the result of apoptosis (data not shown). A fivefold induction of IL-12 production by dendritic cells treated with Stat-3β-transfected CT26 tumor cells was also observed (see **Supplementary Fig. 1** on line).

Because dendritic cells are the cell type responsible for regulating adaptive immunity[5,6], we next investigated whether interrupting Stat-3 signaling in tumor cells might result in activation of T cells, particularly tumor antigen-specific T cells, both *in vitro* and *in vivo*. Dendritic cells exposed to supernatants derived from Stat-3-interrupted tumor cells were more potent than control supernatant–treated dendritic cells in activating naive antigen-specific T cells to proliferate and produce IL-2 and IFN-γ (**Fig. 4c**). Blocking Stat-3 signaling in the B16 tumors *in vivo* by Stat-3β gene therapy leads to tumor infiltration of T cells (**Fig. 4d**). The MHC class I H2-K$^b$–restricted tyrosinase-related protein-2(181–188) peptide (TRP2(181–188)) is recognized by B16-specific CD8$^+$ T cells[23]. To determine whether interrupting Stat-3 signaling in tumor cells could lead to activation of tumor-specific CD8$^+$ T cells *in vivo*, we subcutaneously injected irradiated Stat-3β-transfected or control GFP-transfected B16 cells (transfection efficiencies were 20–40%) into C57BL/6 mice. We carried out both ELISPOT assays and intracellular cytokine staining to detect TRP2-specific, IFN-γ-producing CD8$^+$ T cells using splenocytes isolated from these mice. We detected larger numbers of antigen-independent IFN-γ spots after injection of either GFP- or Stat-3β-transfected cells, probably as a result of the effects of immunostimulatory CpG sequences in the plasmid. However, substantially higher TRP2-specific IFN-γ production was seen only in splenocytes from mice injected with irradiated Stat-3β-transfected B16 cells (**Fig. 4e**). Intracellular staining of IFN-γ in splenocytes cultured in the presence and absence of TRP-2 indicated that immunization with Stat-3β-transfected B16 cells resulted in

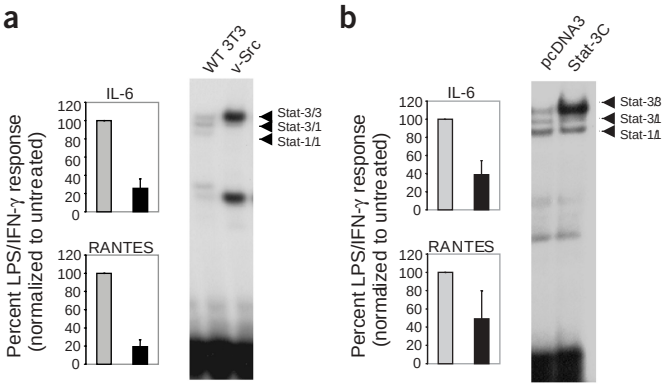


**Figure 2** Constitutive Stat-3 activity inhibits cytokine and chemokine gene expression. (**a**) v-Src transformation activated Stat-3 signaling and inhibits IFN-γ- and LPS-induced expression of IL-6 and RANTES RNA. Histograms represent the means of three RPA experiments normalized to RNA expression of L32 and GAPDH. Data are presented as percentage RNA expression from IFN-γ- and LPS-stimulated 3T3 wild-type cells normalized to untreated controls. Black bars indicate RNA levels of v-Src-transformed 3T3 cells compared with those of WT untransformed 3T3 cells (grey bars). At right, EMSA shows increased Stat-3 DNA-binding in v-Src-expressing 3T3 cells as compared to control untransfected 3T3 cells. (**b**) Expression of Stat-3C (black bars) suppressed IFN-γ- and LPS-induced RNA expression of IL-6 and RANTES as compared to control 3T3 cells transfected with the empty pcDNA3 vector (grey bars). Data presented as in **a**. Right, EMSA data.

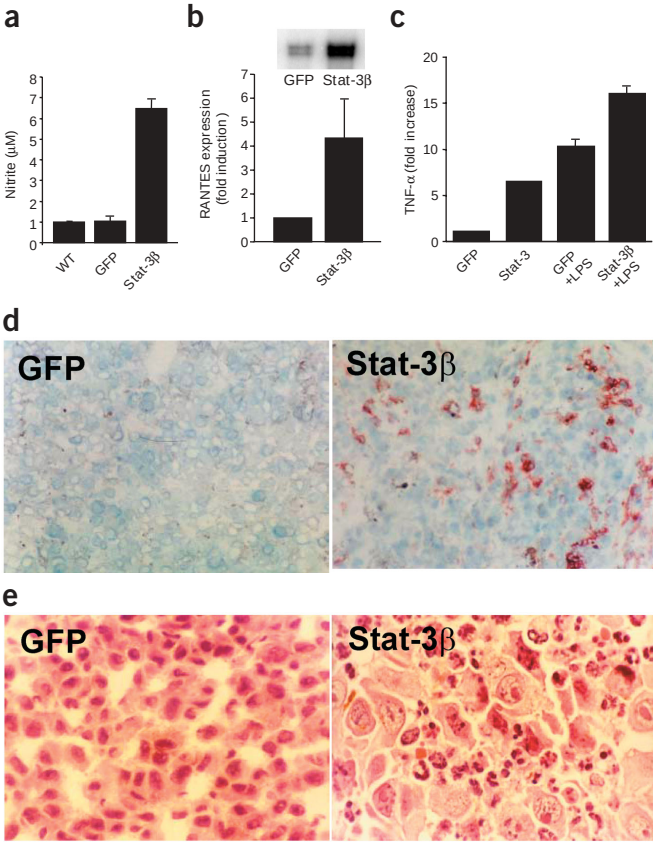


**Figure 3** Blocking Stat-3 in tumor cells stimulates macrophages and neutrophils. (**a**,**b**) Production of nitric oxide (**a**) and expression of RANTES (**b**) by peritoneal macrophages incubated with supernatants derived from B16 cells transfected with the indicated vectors or from untransfected wild-type controls (WT). RNA quantification is shown in the histogram; $n = 3$. Inset, RPA. (**c**) ELISA analysis indicated that neutrophils incubated in conditioned medium from Stat-3β-expressing B16 cells secreted elevated levels of TNF-α, which were further enhanced by LPS; $n = 3$. (**d**,**e**) Tissue sections of B16 tumors transduced *in vivo* with the indicated vectors were stained with antibody to Mac-3 for detection of macrophages (**d**) or with Giemsa for detection of neutrophils (**e**).

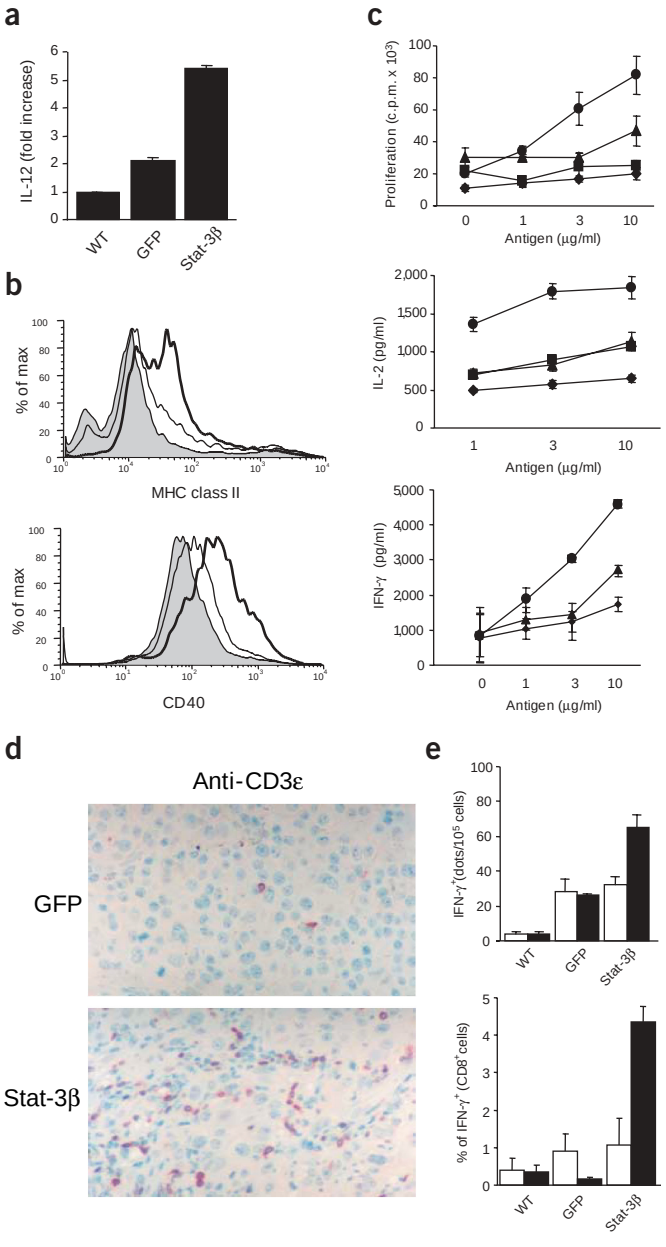


**Figure 4** Stat-3-interrupted tumor cells activated dendritic cells and naive antigen-specific T cells. (**a**) ELISA of IL-12 production by mature dendritic cells incubated with the indicated B16 tumor supernatants; $n = 3$. Fold increase as compared to untransfected wild-type (WT) is shown. (**b**) Detection of MHC class II and CD40 expression on CD11c$^+$ cells by FACS analysis of dendritic cells cultured with supernatants of WT untransfected (shaded area), GFP- (thin line) or Stat-3β-transfected (thick line) B16 cells. Data shown represent one of three experiments. (**c**) Purified CD4$^+$ T cells were incubated with dendritic cells cultured in media containing untreated (■), GFP-transfected (▲) and Stat-3β-transfected (●) B16 cells, with increasing concentrations of HA(110–120) peptide. ◆, T cells incubated with dendritic cells without tumor supernatant. Data shown are mean ± s.e.m. of three experiments. (**d**) T-cell infiltration at the B16 tumor site after Stat-3β gene transfer. (**e**) Activation of TRP2-specific CD8$^+$ T cells *in vivo*. IFN-γ ELISPOT assay (top, $n = 8$) and intracellular staining (bottom) of splenocytes from mice vaccinated with the indicated tumor cells and cultured in the presence (solid bar) or absence (open bars) of TRP2; $n = 3$.

greater numbers of activated TRP-2-specific CD8$^+$ T cells. In addition, injection of Stat-3β-transduced CT26 cells resulted in an increase in IFN-γ-producing CD8$^+$ T cells specific for the CT26-specific tumor peptide antigen AH1 (ref. 24) (**Supplementary Fig. 1** on line). These results indicate that disrupting constitutive Stat-3 signaling in tumor cells leads to activation of tumor antigen–specific T cells *in vivo*.

### Tumor Stat-3 activity and dendritic cell maturation

Our results so far show that blocking Stat-3 signaling in tumor cells leads to expression of inflammatory mediators necessary for activating innate immune cells as well as dendritic cells. Another mechanism whereby transformed cells negatively modulate immune responses is by secreting factors that inhibit dendritic cell maturation[7,8]. We therefore assessed the potential role of constitutive Stat-3 activity in increasing the production of factors that inhibit dendritic cell maturation. For these experiments, supernatants from v-Src-transformed BALB/c 3T3 fibroblasts, with or without Stat-3 signaling, or control untransformed, untreated cells were added to cultures of bone marrow progenitor cells (BMPCs) for a standard 6-d culture period in medium containing GM-CSF, then activated with LPS for an additional 2 d. v-Src-transformed BALB/c 3T3 cells indeed produced soluble factors that inhibited dendritic cell maturation, as indicated by a reduced proportion of CD11c$^+$MHC class II$^{hi}$ and CD11c$^+$CD86$^{hi}$ cells (**Fig. 5a**) and diminished IL-12 secretion (data not shown). Interrupting Stat-3 signaling in v-Src-transformed 3T3 cells by expressing a Stat-3 dominant-negative protein (Stat-3D)[25] inhibited Stat-3 DNA-binding activity (data not shown) and blocked the release of factors that inhibited dendritic cell maturation (**Fig. 5a**). Inhibition of dendritic cell maturation, as assessed by surface markers and IL-12 production, correlated with T-cell stimulatory capacity (**Fig. 5b**). LPS-treated dendritic cells matured in the presence of supernatant from v-Src-transformed 3T3 cells had reduced ability to activate antigen-specific T cells; however, blocking Stat-3 signaling in v-Src transformants completely reversed this effect (**Fig. 5b**).

The ability of constitutively activated Stat-3 to promote the production of factors that inhibit dendritic cell functional maturation was further demonstrated by enforcing expression of the constitutively activated Stat-3 mutant, Stat-3C. Expression of Stat-3C in 3T3 fibroblasts induced factors that inhibited dendritic cell maturation, as demonstrated by a reduction in CD11c$^+$CD86$^{hi}$ and CD11c$^+$MHC class II$^{hi}$ dendritic cells as well as lower IL-12 production (**Fig. 5c**). The inverse correlation between Stat-3 activity and dendritic cell functional maturation was also demonstrated with the B16 tumor model, which has moderate endogenous Stat-3 activity (**Fig. 1a**). Culture of BMPCs with supernatants from B16 tumor cells reduced dendritic cell production of IL-12 (**Fig. 5d**). Supernatant from B16 cells transfected with a Stat-3C expression vector, which increased Stat-3 activity[14], caused stronger inhibition of dendritic cell production of IL-12 (**Fig. 5d**).

### Inhibition of dendritic cell maturation by Stat-3 signaling

An attempt to ascertain the identity of the Stat-3-induced factors that inhibited dendritic cell maturation showed that they were diverse and distinct in different circumstances. For example, vascular endothelial growth factor (VEGF), which has been reported to inhibit dendritic cell maturation and whose gene is a direct target of Stat-3 (ref. 14), seems to have a role in dendritic cell inhibition by B16 supernatant, as antibodies to VEGF neutralize the supernatants inhibitory effects (data not shown) of B16 supernatant (data not shown). However, neutralizing VEGF in v-Src-transformed 3T3 cells, which secrete more VEGF than their nontransformed counterparts, did not reverse the inhibitory effects on dendritic cell maturation (data not shown).

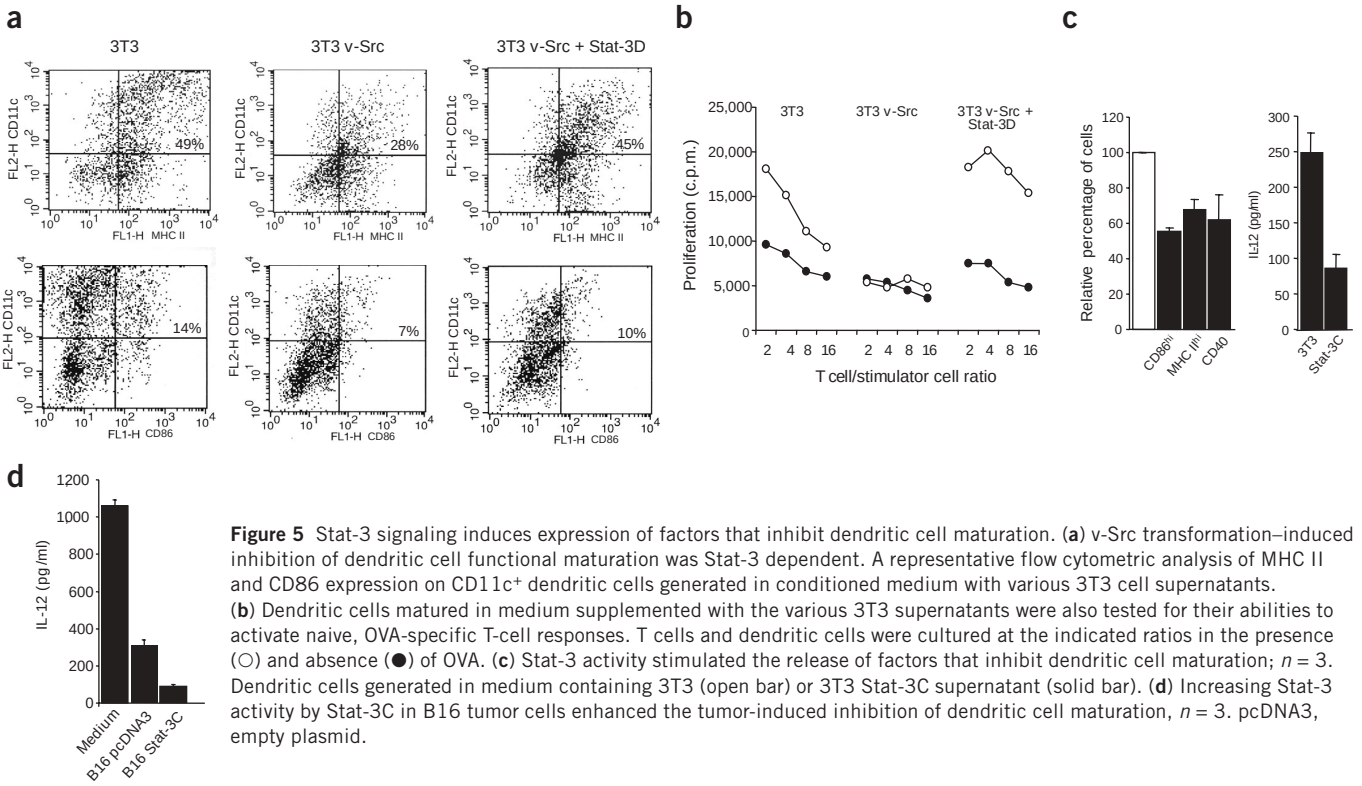


**Figure 5** Stat-3 signaling induces expression of factors that inhibit dendritic cell maturation. (**a**) v-Src transformation–induced inhibition of dendritic cell functional maturation was Stat-3 dependent. A representative flow cytometric analysis of MHC II and CD86 expression on CD11c$^+$ dendritic cells generated in conditioned medium with various 3T3 cell supernatants. (**b**) Dendritic cells matured in medium supplemented with the various 3T3 supernatants were also tested for their abilities to activate naive, OVA-specific T-cell responses. T cells and dendritic cells were cultured at the indicated ratios in the presence (○) and absence (●) of OVA. (**c**) Stat-3 activity stimulated the release of factors that inhibit dendritic cell maturation; $n = 3$. Dendritic cells generated in medium containing 3T3 (open bar) or 3T3 Stat-3C supernatant (solid bar). (**d**) Increasing Stat-3 activity by Stat-3C in B16 tumor cells enhanced the tumor-induced inhibition of dendritic cell maturation, $n = 3$. pcDNA3, empty plasmid.

IL-10, which is secreted by many tumors, has also been reported to inhibit dendritic cell maturation[26]. Increasing Stat-3 activity in 3T3 fibroblasts by v-Src transformation led to higher IL-10 expression, which was abrogated when Stat-3 signaling was blocked by a Stat-3 dominant-negative mutant (see **Supplementary Table 1** online). These data indicate that there are diverse factors produced by each tumor capable of inhibiting dendritic cell maturation and that tumor Stat-3 signaling contributes to their production. Because the IL-10 receptor[27] and VEGF receptor[28] signal through Stat-3, we analyzed whether activation of Stat-3 in dendritic cells represented a common pathway of maturation inhibition by the pleiotropic factors produced by different tumors. Indeed, supernatants from tumors showing high constitutive Stat-3 activity (CT26 and SCK-1, **Fig. 1a**) and v-Src-transformed 3T3 cells, as well as IL-10, strongly activate Stat-3 in dendritic cells (**Fig. 6a**). Recombinant VEGF weakly but reproducibly induces Stat-3 activity in BMPCs (data not shown). The central role of Stat-3 activation in inhibiting dendritic cell maturation was demonstrated in two different ways. First, we showed that a phosphopeptide inhibitor of

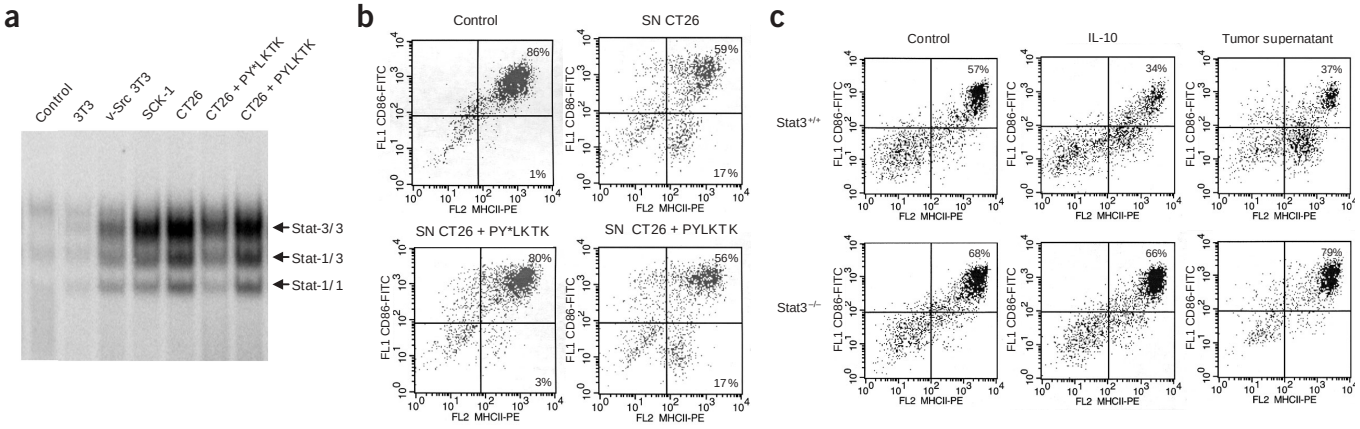


**Figure 6** Stat-3 signaling mediates inhibition of dendritic cell maturation. (**a**) Tumor-derived factors and IL-10 activated Stat-3 signaling in BMPCs as determined by EMSA. pY*LKTK, Stat-3 inhibitor; PYLKTK, control peptide. (**b**) Blocking Stat-3 signaling in BMPCs abrogated tumor supernatant–induced inhibition of dendritic cell maturation. CD11c$^+$ dendritic cells were gated in the FACS analysis. The plot represents one of three experiments. (**c**) IL-10- and tumor-supernatant-induced inhibition of dendritic cell maturation was completely abrogated in *Stat3*$^{-/-}$ BMPCs, as indicated by the percentage of CD11c$^+$CD86$^{+hi}$MHCII$^{+hi}$ cells relative to that of the control. SN, supernatant. Data for flow cytometry with IL-10 represents 1 of 11 experiments. For CT26 supernatant experiments, data represents one of three experiments.

Stat-3 (ref. 29), which reduced Stat-3 DNA-binding in bone marrow–derived progenitor cells (**Fig. 6a**), reversed the inhibition of dendritic cell maturation by CT26 supernatants (**Fig. 6b**). In a second approach, we evaluated the ability of CT26 tumor supernatant and IL-10 to inhibit dendritic cell maturation in wild-type and *Stat3*-null BMPCs. Dendritic cell maturation in *Stat3*-null BMPCs is completely unaffected by either tumor supernatants or IL-10, whereas maturation of $Stat3^{+/+}$ dendritic cells is similarly and substantially inhibited by both (**Fig. 6c**).

## DISCUSSION

Taken together, these findings support the notion that Stat-3 signaling in tumors negatively regulates inflammation, dendritic cell activity and T-cell immunity. Although constitutive activation of Stat-3 in tumor cells has been shown to upregulate cell cycle regulatory and antiapoptotic genes crucial to the transformation process[11–13,15], its role in modulating interactions between tumor cells and the immune system has not been previously recognized. It is logical that successful development of invasive, metastatic cancer would require the modulation of genes in a manner that inhibits activation of both innate and adaptive elements of the immune surveillance system. The Stat-3 signaling pathway in tumor cells seems to accomplish this by both inhibiting the production of proinflammatory danger signals and inducing expression of factors that inhibit dendritic cell functional maturation. In a previous study we showed that Stat-3β gene therapy of B16 tumors resulted in growth inhibition and regression of tumors, although *in vivo* gene transfection efficiency was only about 15% (ref. 16). Infiltration of immune cells may contribute to the 'bystander' effects associated with Stat-3β gene therapy[16]. Although we cannot formally rule out the possibility that some of the immune infiltration is a result of apoptosis after Stat-3 blockade, detection of inflammatory cytokines and chemokines in cultured tumor cells in the absence of apoptosis indicates that *in vivo* targeting of Stat-3 may contribute to leukocyte infiltration.

It is noteworthy that disruption of Stat-3 signaling in tumor cells results in the activation of proinflammatory cytokine and chemokine genes without any exogenous inductive stimuli. We hypothesize that this is because tumors represent tissue with a constitutively disrupted architecture—the pathologic hallmark of cancer. It is well established that wounding, which causes disruption of cell-cell interactions and tissue architecture, induces release of proinflammatory cytokines[30]. In fact, Stat-3 activation has been shown to be crucial to wound healing, and defective wound healing in mice with selective disruption of Stat-3 in keratinocytes is associated with increased inflammatory infiltrates at wound sites[31]. Thus, as with other aspects of cancer biology, the ability of Stat-3 activation to evade the immune system probably reflects a natural function of Stat-3 in normal physiology. As well as inhibiting expression of proinflammatory mediators, our data show that Stat-3 activation drives the secretion of factors that suppress the activity of immune cells by inhibiting dendritic cell maturation. The most likely role for this Stat-3-dependent effect relates to the fact that disruption of tissues by invading tumor would probably induce release of inflammatory cytokines from the nontransformed cells in the stroma. In addition, disruption of integumental barriers in organs such as the colon would expose the immune system to inflammatory bacterial products. Production of inhibitors of dendritic cell maturation would block immune activation by this non-cell-autonomous mechanism.

Although most previously described mechanisms whereby established tumors evade immune recognition involve various components of the antigen processing and presentation machinery critical for effector T cells[32–34], our findings underscore a crucial role of oncogenic signaling pathways within tumor cells in promoting immunologic tolerance. Our studies also provide an alternative mechanism to that proposed by Ochsenbein *et al.*[35], who suggested that tumors evade immune recognition by either failing to enter lymph nodes or walling themselves off from T cells in lymph nodes. This model ignores any role of dendritic cells in immune response decisions, whereas our model places dendritic cells as a crucial intermediary between the tumor and the resultant T-cell response. Our results here also have obvious therapeutic implications. Interrupting Stat-3 signaling with selective inhibitors would not only have direct antitumor effects through growth suppression and apoptosis induction, but could also activate innate and adaptive antitumor immunity.

## METHODS

***In vivo* experiments.** Existing B16 tumors were subjected to Stat-3β gene therapy as previously reported[16]. Tissue sections from treated tumors were stained with antibody to CD3e or Mac-3 (Transduction Laboratories). Standard Giemsa staining was used to detect neutrophils. To test whether Stat-3-interrupted tumor cells could activate tumor antigen–specific T cells *in vivo*, B16 or CT26 tumor cells were transiently transfected with vectors expressing either GFP or Stat-3β. Untransfected B16 tumor cells (denoted as WT) were also included as controls. Various groups of tumor cells were then irradiated (4,000 rad) and injected subcutaneously into C57BL/6 mice (B16 cells) or BALB/c mice (CT26 cells) ($1 \times 10^6$/mouse) twice a week for 2 weeks. Two weeks after the final injection, splenocytes were prepared from these mice for IFN-γ ELISPOT assays or intracellular staining. $Stat3^{-/-}$ BMPCs were generated using the *loxP*–Cre recombinase system as previously described[36]. Deletion of the *Stat3* gene was verified by PCR, using primer sets that distinguish *Stat3*, *loxP*-flanked *Stat3* (*Stat3*flox) and *Stat3*-deleted alleles[36] and by EMSA. Mouse care and experimental procedures were performed under approval from the Animal Care Committees of the University of South Florida and Johns Hopkins University.

**Transfection.** Tumor cells were transiently transfected with either plasmid expression vectors or oligonucleotides using Lipofectamine (Invitrogen) as previously described[14].

**Electrophoretic mobility shift assays (EMSA) and western blot analysis.** EMSA and western blot analyses to detect Stat-3 DNA-binding activity and protein abundance, respectively, were done as previously described[14,37].

**ELISAs for cytokine and chemokine detection.** $GFP^+$ B16, SCK-1 and CT26 tumor cells transfected with pIRES-EGFP or pIRES-Stat-3β were sorted by FACSVantage. $GFP^+$ cells were cultured at $5 \times 10^5$/ml for 36 h, then supernatants were analyzed by ELISA using kits from R&D Systems.

**Retroviral transduction of BALB/c 3T3 and BALB/c 3T3 v-Src cells.** Supernatants from stable cell lines producing high-titer murine stem cell virus (MSCV) encoding Stat-3C or Stat-3D[25] were used to infect nontransfected (early-passage) or v-Src-transfected BALB/c 3T3 cells. Stat-3C or Stat-3D expression was detected on the basis of fluorescence, as both Stat-3C and Stat-3D were linked to GFP, and confirmed by EMSA Stat-3 DNA-binding assays.

**RNase protection assays (RPAs).** Total RNA isolation and RPAs were carried out as previously described[17]. For B16 cells, mCK-1b, mCK-2b, mCK-3b and mCK-5 multitemplate probes (PharMingen) were used. For SCK-1, CT26, BALB/c 3T3 cells and its derivatives, only mCK-3b and mCK-5 templates were used in the RPAs. IP-10 RNA degradation under these experimental conditions is expected in the CT26 tumor cells, the gene encoding IP-10 (*Cxcl10*) as in BALB/c mice contains a nucleotide substitution resulting in incomplete RNA-RNA hybridization.

**Enzyme-linked immunospot (ELISPOT) assays.** ELISPOT assays were carried out essentially as described[38], except different antigen peptides were used. Splenocytes prepared from mice receiving irradiated, transfected tumor cells

were incubated at 37 °C for 24 h in medium with or without 10 μg/ml of either TRP2(181–188) (TYDFFVWL) or AH-1 (SPSYVYHQF).

**Intracellular staining.** IFN-γ intracellular staining was carried out according to the manufacturer's protocol using the Cytofix/Cytoperm Plus Kit (PharMingen). Briefly, fresh splenocytes were cultured at $6 \times 10^6$/ml in 24-well plates with 50 U/ml IL-2 with or without TRP-2 peptide (10 μg/ml) for 3 d.

**Bone marrow progenitor cells and functional assays.** BMPCs were prepared and bone marrow–derived mature dendritic cells were derived according to a previously described protocol[38]. Day 6 dendritic cells were then incubated in dendritic cell medium supplemented with 1:1 tumor cell supernatants.

For dendritic cell maturation inhibition experiments, BMPCs were cultured in standard dendritic cell medium supplemented with 5–20% tumor cell supernatants from day 0 or day 2. Fresh medium supplemented with supernatants was added to the culture every other day until day 6, at which point LPS was added. In some experiments, 375 μM of Stat-3 (PY*LKTK) inhibitor and control (PYLKTK) peptides[29] were added to the BMPC cultures on day 0. On day 7 or 8, cultures were processed for FACS and/or IL-12 ELISA. To examine the effects of the supernatants on dendritic cell functional maturation, the same day 7 (or day 8) dendritic cells were incubated overnight with 1 mg/ml ovalbumin (OVA; Sigma) and used to stimulate naive T cells isolated from lymph nodes of syngeneic mice. T-cell proliferation was assessed by measuring [$^3$H]thymidine incorporation over 18 h.

**Enrichment of CD4$^+$ T cells for antigen-specific T-cell assays.** Enrichment of CD4$^+$ T cells was done as described[38]. TCR-transgenic mice expressing an α/β TCR specific for amino acids 110–120 of the influenza hemagglutinin (HA) protein, presented by I–E$^d$ (6.5), were used for these experiments[38]. T-cell proliferation assays and IL-2 and IFN-γ ELISAs in response to HA(110–120) peptide, SFERFEIFPKE, were carried out as previously reported[38].

*Note: Supplementary information is available on the Nature Medicine website.*

ACKNOWLEDGMENTS

We thank S. Akira and K. Takeda for *Stat3*flox mice, D. Link for the Stat-3C and Stat-3D retrovirus–producing cell lines, J. Bromberg for the Stat-3C plasmid and H. von Boehmer for CD4$^+$ TCR transgenic mice. This work is supported by US National Institutes of Health grants, by the Dr. Tsai-fan Yu Cancer Research Endowment and by gifts from Mrs. Dorothy Needle, William and Betty Topercer, Jack Goldsmith, the Janey Fund and the Seraph Foundation.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 10 October; accepted 3 December 2003
Published online at http://www.nature.com/naturemedicine/

1. Kaplan, D.H. *et al.* Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. USA* **95**, 7556–7561 (1998).
2. Shankaran, V. *et al.* IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107–1111 (2001).
3. Fuchs, E.J. & Matzinger, P. Is cancer dangerous to the immune system? *Semin. Immunol.* **8**, 271–280 (1996).
4. Pardoll, D.M. Cancer vaccines. *Nat. Med.* **4**, 525–531 (1998).
5. Steinman, R.M. & Nussenzweig, M.C. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci. USA* **99**, 351–358 (2002).
6. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1998).
7. Gabrilovich, D.I. *et al.* Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat. Med.* **2**, 1096–1103 (1996).
8. Sombroek, C.C. *et al.* Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. *J. Immunol.* **168**, 4333–4343 (2002).
9. Takeda, K. *et al.* Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* **10**, 39–49 (1999).
10. Cheng, F. *et al.* A critical role for Stat3 signaling in immune tolerance. *Immunity* **19**, 425–436 (2003).
11. Bromberg, J.F. *et al.* Stat3 as an oncogene. *Cell* **98**, 295–303 (1999).
12. Catlett-Falcone, R. *et al.* Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. *Immunity* **10**, 105–115 (1999).
13. Bowman, T. *et al.* Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc. Natl. Acad. Sci. USA* **98**, 7319–7324 (2001).
14. Niu, G. *et al.* Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* **21**, 2000–2008 (2002).
15. Bowman, T., Garcia, R., Turkson, J. & Jove, R. STATs in oncogenesis. *Oncogene* **19**, 2474–2488 (2000).
16. Niu, G. *et al.* Gene therapy with dominant-negative STAT3 suppresses growth of the murine melanoma B16 tumor *in vivo*. *Cancer Res.* **59**, 5059–5063 (1999).
17. Niu, G. *et al.* Overexpression of a dominant-negative signal transducer and activator of transcription 3 variant in tumor cells leads to production of soluble factors that induce apoptosis and cell cycle arrest. *Cancer Res.* **61**, 3276–3280 (2001).
18. Janeway, C.A., Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54**, 1–13 (1989).
19. Fearon, D.T. & Locksley, R.M. The instructive role of innate immunity in the acquired immune response. *Science* **272**, 50–54 (1996).
20. Medzhitov, R. & Janeway, J., C.A. Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* **9**, 4–9 (1997).
21. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **5**, 1249–1255 (1999).
22. Mellman, I. & Steinman, R.M. Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**, 255–258 (2001).
23. Bloom, M.B. *et al.* Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med.* **185**, 453–459 (1997).
24. Slansky, J.E. *et al.* Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity* **13**, 529–538 (2000).
25. McLemore, M.L. *et al.* STAT-3 activation is required for normal G-CSF-dependent proliferation and granulocytic differentiation. *Immunity* **14**, 193–204 (2001).
26. Brossart, P. *et al.* Tumor necrosis factor α and CD40 ligand antagonize the inhibitory effect of interleukin 10 on T-cell stimulatory capacity of dendritic cells. *Cancer Res.* **60**, 4485–4492 (2000).
27. Weber-Nordt, R.M. *et al.* Stat3 recruitment by two distinct ligand-induced, tyrosine-phosphorylated docking sites in the interleukin-10 receptor intracellular domain. *J. Biol. Chem.* **271**, 27954–27961 (1996).
28. Bartoli, M. *et al.* VEGF differentially activates STAT3 in microvascular endothelial cells. *FASEB J.* **17**, 1562–1564 (2003).
29. Turkson, J. *et al.* Phosphotyrosyl peptides block Stat3-mediated DNA-binding activity, gene regulation and cell transformation. *J. Biol. Chem.* **276**, 45443–45455 (2001).
30. Davidson, J.M. Wound repair. in *Inflammation* (ed. J.L. Gallin) 809–819 (Garland Press, New York, 1992).
31. Sano, S. *et al.* Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J.* **18**, 4657–4668 (1999).
32. Urban, J.L., Burton, R.C., Holand, J.M., Kripke, M.L. & Schreiber, H. Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunological effector cells. *J. Exp. Med.* **155**, 557–573 (1982).
33. Uyttenhove, C., Maryanski, J. & Boon, T. Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. *J. Exp. Med.* **157**, 1040–1052 (1983).
34. Wortzel, R.D., Philipps, C. & Schreiber, H. Multiple tumour-specific antigens expressed on a single tumour cell. *Nature* **304**, 165–167 (1983).
35. Ochsenbein, A.F. *et al.* Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature* **411**, 1058–1064 (2001).
36. Lee, C.K. *et al.* Stat3 is a negative regulator of granulopoiesis but is not required for G-CSF-dependent differentiation. *Immunity* **17**, 63–72 (2002).
37. Turkson, J. *et al.* Stat3 activation by Src induces specific gene regulation and is required for cell transformation. *Mol. Cell Biol.* **18**, 2545–2552 (1998).
38. Lu, Z. *et al.* CD-40-independent pathways of T cell help for priming of CD8$^+$ cytotoxic T lymphocytes. *J. Exp. Med.* **191**, 541–550 (2000).