CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor–β–dependent manner

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Tumor growth promotes the expansion of CD4+CD25+ regulatory T (T reg) cells that counteract T cell–mediated immune responses. An inverse correlation between natural killer (NK) cell activation and T reg cell expansion in tumor–bearing patients, shown here, prompted us to address the role of T reg cells in controlling innate antitumor immunity. Our experiments indicate that human T reg cells expressed membrane–bound transforming growth factor (TGF)–β, which directly inhibited NK cell effector functions and down–regulated NKG2D receptors on the NK cell surface. Adoptive transfer of wild–type T reg cells but not TGF–β–/– T reg cells into nude mice suppressed NK cell–mediated cytotoxicity, reduced NKG2D receptor expression, and accelerated the growth of tumors that are normally controlled by NK cells. Conversely, the depletion of mouse T reg cells exacerbated NK cell proliferation and cytotoxicity in vivo. Human NK cell–mediated tumor recognition could also be restored by depletion of T reg cells from tumor–infiltrating lymphocytes. These findings support a role for T reg cells in blunting the NK cell arm of the innate immune system.

CD4+CD25+ regulatory T (T reg) cells contribute to the maintenance of immune tolerance (1, 2). T reg cells, which constitute ~5–10% of CD4+ T cells in rodents (3), prevent the spontaneous emergence of organ–specific autoimmune diseases and contribute to the establishment of dominant tolerance on infection (4, 5) and allogeneic transplantation (6–7). T reg cells can also curtail antitumor immune responses in tumor–bearing animals (8–12). Experimental depletion of T reg cells in tumor–bearing rodents using anti–CD25 antibodies (9) or low–dose cyclophosphamide (12–13) improves T cell–based tumor clearance and augments the response to DC–based therapy (11). Cancer patients often bear increased numbers of circulating and tumor–infiltrating T reg cells that exert functional inhibition of tumor–specific T cells and predict poor survival (14–17).

NK cells may participate in tumor–immune surveillance, in particular in leukemia (18), neuroblastoma (19), and gastrointestinal stromal tumors (20). Tumor cell recognition by NK cells is dictated by a balance between inhibitory signals mediated by MHC class I molecules and activating signals triggered by specific ligands (21–23). One such activating signal is provided by the MHC class I chain–related molecule (MIC)–NKG2D system (24), which participates in the control of epithelial tumors. In cancer
T cells were cell sorted using a cell-sorter cytometer. Likewise, CD4+ cell induction in GIST patients treated with imatinib mesylate.

The Wilcoxon two sample rank sum test was used to compare the two-paired groups (*, P < 0.05; Fig. 1 A). In parallel, the percentages and absolute numbers of circulating CD4+CD25high T reg cells were monitored by flow cytometry before therapy (Fig. 1 B) (27). In contrast to CD4+CD25low T cells, these CD4+CD25high T reg cells, purified by cell sorting or immunocapture with magnetic beads, exhibited inhibition of allogeneic T lymphocyte proliferation (Fig. 1 C). The mean percentages of T reg cells among CD3+CD4+ T cells in GIST patients displaying NK cell induction were not elevated as compared with normal volunteers (1.1 ± 0.3% in GIST vs. 1.2% ± 0.4% in normal volunteers [NVs]; P = 0.5), whereas these yields were significantly increased in the group of patients with no NK cell induction (3.2 ± 0.8%, P = 0.02; absolute numbers shown in Fig. 1 D). It is noteworthy that the tumor volume or tumor growth could not account for these differences because there was no correlation between tumor volumes and the T reg cell numbers or NK cell induction (unpublished data). In addition, the frequency of circulating T reg cells was not influenced by imatinib mesylate therapy, as assessed by follow-up examinations performed at 2-mo intervals (unpublished data) in both groups of patients. We con-

RESULTS

NK cell functions inversely correlate with T reg cell frequencies in cancer patients

We recently reported that therapy of GIST (gastrointestinal stromal tumor—bearing) patients using the c-kit tyrosine kinase inhibitor Gleevec, STI571 (imatinib mesylate) elicited enhanced NK cell effector functions in ~50% of cases within 2 mo and that therapy-induced NK cell activation correlated with objective responses, prolonging the time to progression (20). We collected 18 patients for which the levels of IFN-γ secreted by circulating NK cells after ex vivo stimulation were significantly enhanced by therapy with imatinib mesylate and 13 cases for which NK cells were not induced (P < 0.05; Fig. 1 A). In parallel, the percentages and absolute numbers of circulating CD4+CD25high T reg cells were monitored by flow cytometry before therapy (Fig. 1 B) (27). In contrast to CD4+CD25low T cells, these CD4+CD25high T reg cells, purified by cell sorting or immunocapture with magnetic beads, exhibited inhibition of allogeneic T lymphocyte proliferation (Fig. 1 C). The mean percentages of T reg cells among CD3+CD4+ T cells in GIST patients displaying NK cell induction were not elevated as compared with normal volunteers (1.1 ± 0.3% in GIST vs. 1.2% ± 0.4% in normal volunteers [NVs]; P = 0.5), whereas these yields were significantly increased in the group of patients with no NK cell induction (3.2 ± 0.8%, P = 0.02; absolute numbers shown in Fig. 1 D). It is noteworthy that the tumor volume or tumor growth could not account for these differences because there was no correlation between tumor volumes and the T reg cell numbers or NK cell induction (unpublished data). In addition, the frequency of circulating T reg cells was not influenced by imatinib mesylate therapy, as assessed by follow-up examinations performed at 2-mo intervals (unpublished data) in both groups of patients. We con-

CD4+CD25high T cells from all GIST patients classified as stated in A as “NK induction” versus “no NK induction” and from a panel of normal volunteers using FACS analyses. The data are represented as the means ± SEM of the absolute numbers of CD4+CD25high T cells/mm3 of blood, *, P < 0.05 using Student’s t test.
firmed that T reg cell numbers could predict NK cell induction induced by immunotherapy in another trial (unpublished data). Hence, only those patients that have low T reg cell levels manifested an increase in NK cell function after in vivo stimulation.

Human T reg cells inhibit NK cell functions in vitro
To directly assess the capacity of T reg cells to interfere with NK cell activity, we co-cultured NK cells isolated from either GIST patients or NVs with T reg cells from NVs. Although CD4⁺CD25⁺ conventional T cells (T conv) did not

Figure 2. Inhibition of NK cell functions by T reg cells in vitro: rescue by IL-2Rγ chain-dependent cytokines. NK cells purified from GIST patients (A) or NVs (B) were cultured 4 h with T reg cells or with T conv isolated from NVs before incubation with ⁵¹Cr-labeled K562 or GIST 882 tumor targets. The T cell/NK cell/target ratio was 10:10:1. Results of the chromium release assay are represented as means ± SEM of triplicate wells from one representative experiment out of three. (C) Same as in A and B, but with a variable T cell/NK cell ratio (as indicated) against GIST 882. (D) Same as in A and B, but using T reg cells fixed in 0.5% paraformaldehyde for 10 min, *, P < 0.05 using Fisher’s exact method. (E) Preincubation of NV-derived NK cells with T reg cells at 1:1 ratio for 2 h before stimulation with IL-2 or IL-12 (at increasing dosages overnight) or 500 IU/ml IL-4 or 10 ng/ml IL-7. The cytolytic activity against K562 was determined in a ⁵¹Cr release assay as a percentage of residual inhibition compared with NK cell activity in the absence of T reg cells. Data from one representative experiment out of two are shown as mean percentages of lysis inhibition ± SEM of triplicate wells. (F) NK cells isolated from NVs were co-cultured with T reg cells or with T conv or with 1 ng/ml of TGF-β1. After a 6-h incubation, 200 IU/ml rhuIL-2, 2 ng/ml rhuIL-12, or 1 ng/ml IL-15 was added to the co-culture. 18 h later, supernatants were harvested, and IFN-γ levels were determined using an ELISA test. The means ± SEM of triplicate wells from one representative experiment out of three are shown. **, P < 0.05.
hamper NK cell recognition of K562 and GIST882 targets, T reg cells strongly decreased NK cell cytotoxicity, and this inhibition was similar for NK cells from tumor-bearing patients (Fig. 2 A) and NVs (Fig. 2 B) at a T reg cell/NK cell ratio of 1:1. T reg cell–mediated NK cell inhibition was significant up to a 1:5 T reg cell/NK cell ratio using NK cells from GIST patients ($P<0.05$; Fig. 2 C and Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051511/DC1) or NVs (not depicted). Importantly, T reg cells maintained their inhibitory functions even after fixation in formaldehyde, suggesting the involvement of membrane-bound molecules (Fig. 2 D and Fig. S1 B). The inhibitory effect of T reg cells on NK cell lysis could be overcome by physiological doses of cytokines signaling via the IL-2Rγ chain (IL-2, IL-4, and IL-7) and by supraphysiological doses of IL-12 (Fig. 2 E and Fig. S2). T reg cells, like soluble TGF-β, inhibited NK cells to secrete IFN-γ on stimulation with IL-12 (at any dosing, i.e., 0.5, 10, or 50 ng/ml; not depicted) but not IL-2 and IL-15 (Fig. 2 F). Hence, T reg cells inhibit NK cell cytolysis and IL-12–mediated IFN-γ secretion, but such an inhibition is a selective process depending on the NK cell–stimulating trigger.

The role of membrane-bound TGF-β in T reg cell–mediated inhibition

In view of the controversial role of membrane-bound TGF-β in T reg cell–induced T cell suppression (28–30), we performed four-color flow cytometry stainings, measuring the expression of membrane-associated TGF-β on human resting T reg cells. CD3+/CD4+/CD25high T reg cells (but not T conv or CD3+/CD4+/CD25low cells) expressed TGF-β1 on the cell surface, as detectable with the AF-101–NA polyclonal chicken IgY (Fig. 3 A). As a specificity control, this staining was abolished by competition with saturating concentrations of soluble TGF-β (Fig. 3 B), and, additionally, the latent-associated protein (LAP)—which is noncovalently linked to TGF-β—was selectively expressed on T reg cells (Fig. 3 C). However, when cultured alone or together with NK cells, T reg cells did not secrete ELISA-detectable (<1 fmol) levels of TGF-β1. Importantly, the neutralizing anti–TGF-β antibody could counteract the inhibitory effect of T reg cells on NK cell lytic activity against both tumor lines (Fig. 4 A and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20051511/DC1), whereas recombinant soluble TGF-β mimicked the inhibitory effect of T reg cells.

Figure 3. Selective expression of membrane-bound TGF-β on T reg cells. (A) TGF-β expression was investigated on both CD4+CD25high versus CD4+CD25low cells in flow cytometry using four-color staining with anti-CD3–APC, anti-CD4–PerCP, anti-CD25–PE, anti–TGF-β–FITC (AF-101–NA polyclonal chicken IgY), or a chicken IgY–FITC (AB-101-C) as a control antibody. (B) The specificity of the staining with the AF-101–NA polyclonal chicken IgY antibody was tested using saturating concentrations of recombinant TGF-β, which blocked the detection of membrane-bound TGF-β. (C) Expression of LAP and TGF-β–RII was also analyzed in flow cytometry on these CD3+CD4+ T cell subsets in four-color staining.
similarly, the neutralizing anti–TGF-β1 antibody could restore the capacity of NK cells to respond to IL-12 for IFN-γ secretion in the presence of T reg cells (Fig. 4 B). Soluble TGF-β reportedly (31) down-regulates the expression of natural cytotoxicity receptor NKp30 and NKG2D receptors, thereby hampering recognition of target

Figure 4. Role of membrane-bound TGF-β in T reg cell-mediated NK cell inhibition. (A) NK cells were co-cultured for 4 h with T reg cells or soluble TGF-β1 and/or a TGF-β1 blocking antibody or IgY control antibody before incubation with 51Cr-labeled GIST 882 tumor targets. The T cell/NK cell/target ratio was 10:10:1. y axis = % specific lysis K562. The results of one representative experiment out of three are shown. Values represent means ± SEM of triplicate wells. *, P < 0.05 using Fisher’s exact method. (B) Incubation of NK cells with T reg cells for 6 h before stimulation with 2 ng/ml of IL-12 for 18 h. Supernatants were collected for ELISA assaying IFN-γ levels. Values represent means ± SEM of triplicate wells. The results of one representative experiment out of three are shown. *, P < 0.05. (C) NK cells were cultured alone or with T reg cells at a 1:1 ratio or with 1 ng/ml of TGF-β1. After an overnight incubation, cells were analyzed by three-color staining flow cytometry (CD3–FITC, NKG2D–PE, and CD56–PC5). (right) Same as in the left panel, but comparing T reg cells with T conv or adding anti–TGF-β blocking antibody or IgY control antibody along with T reg cells.

Figure 5. Adoptive transfer of T reg cells in nude mice. (A) Three C57BL/6 nude mice were injected with PBS or with 2 × 10⁶ T reg cells or T conv twice 3 d apart. On day 4, the cytolytic activity of splenocytes against YAC-1 cells was determined in a 51Cr release assay at a 1:200 target/effector ratio. It is noteworthy that ≤30% of NK1.1+ CD3− NK cells were recovered in all three experimental groups at day 4. Data from one representative experiment out of two are shown as means of cytolytic activity of three spleens ± SEM. *, P < 0.05 using Fisher’s exact method at 95% confidence interval. (B) Same as in A, but using T reg cells or T conv derived from TGF-β1−/− mice at a 1:100 (closed bars) or 1:50 (open bars) E/T ratio. (C) 2 × 10⁶ T reg cells or T conv activated overnight with 10 μg/ml anti-CD3, 5 μg/ml anti-CD28, and 1,000 IU/ml IL-2 were adoptively transferred into nude mice. Flow cytometry analyses of spleens were performed at 24 h using three-color staining (anti-CD3–PerCP, anti-Dx5–FITC, and anti-CD62L–APC, or NKG2D–PE). The experiments including three mice/group were performed twice with similar results. The dot plots of a representative animal are depicted, and the percentages correspond to NKG2D+ cells among Dx5+ cells. (D) 3 × 10⁶ B16-Rae or B16-WT tumor cells were inoculated in nude mice at day 0 and 12 h later, and 10⁶ activated T reg cells or controls (T conv or PBS) were infused. Mice were killed at day 25 for the enumeration of lung metastases. Student’s t test at 95% confidence interval was used to compare both treatment groups. The graph depicts the results of two independent experiments including five mice/group. Values represent the means ± SEM.
cells that express the corresponding ligands. T reg cells also down-regulated NKG2D expression (Fig. 4 C, left), yet had no effect on the expression of NKp30 (Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20051511/DC1). The T reg cell–mediated down-regulation of NKG2D expression was reversed by the neutralizing anti–TGF-β/H9252 antibody (Fig. 4 C, right). In addition, T reg cells prevented NK cell recognition of NKG2D-Fc targets (Fig. S4 B, GIST882 and K562), and this effect was again blocked by anti–TGF-β antibody. NK cell recognition of K562 and GIST882 tumor cells were found to be partially dependent on the MIC/ULBP-NKG2D molecular pathway because target lysis was impaired by anti-NKG2D antibody, NKG2D-Fc, or soluble MHC class I–related chain A (MICA) molecules (Fig. S4 C), indicating that T reg cell–mediated, TGF-β–dependent inhibition of NK cytotoxicity is associated with the down-regulation of NKG2D.

**Inhibition of NK cell function by T reg cells in the murine system**

Athymic nu/nu mice lack thymus-dependent T conv as well as T reg cells. The NK cell function of such mice can be blocked by adoptive transfer of histocompatible T reg cells (but not T conv), which abolished the natural cytotoxicity of splenocytes against the NK target cell line YAC-1 (Fig. 5 A). However, in line with the role of membrane-bound TGF-β expressed by human resting T reg cells on human NK cell inhibition (Fig. 4), we show that adoptive transfer of T reg cells derived from TGF-β−/− mice (at 6–10 d of age before the onset of lymphoproliferation...
tion) into nude mice did not result in the inhibition of YAC-1 lysis by Dx5/H11001 splenocytes (Fig. 5 B).

As in the human system (Fig. 4 C), in vivo injection of T reg cells (but not T conv) reduced the expression of NKG2D (but not CD62L; not depicted) on Dx5+ splenic NK cells within 24 h (Fig. 5 C). Moreover, the antitumor effector function of NK cells was reduced by T reg cells in vivo. A melanoma cell line (B16) transfected with the murine NKG2D ligand Rae produced more lung metastases in nu/nu mice injected with T reg cells than in control mice, but no such difference was observable for the WT B16 cell line lacking NKG2D ligands (Fig. 5 D).

If T reg cells controlled NK cells in homeostatic conditions, their absence should stimulate the NK cell system. In accord with this speculation, the mouse mutant Scurfy, which lacks the transcription factor necessary for the development of T reg cells, Foxp3 (32), exhibited a marked, >10-fold augmentation of proliferating (5-bromo-2′-deoxyuridine [BrdU]–incorporating) NK cells as compared with WT littermates (Fig. 6 A). Although CD69 molecules were not up-regulated on those dividing NK cells (Fig. 6 A), the natural cytotoxicity of purified Dx5+ splenic cells was markedly enhanced in Scurfy mice (Fig. 6 B). The absence of T reg cells was hence permissive for enhanced NK cell activity. Similarly, the depletion of T reg cells achieved by injection of anti-CD25 mAb or immunostimulatory doses of cyclophosphamide (CTX) into C57BL/6 mice promoted the baseline proliferation of splenic NK cells (Fig. 6 C). However, the T reg cell–mediated NK cell inhibition was tightly regulated because CTX enhanced the proliferation of LN NK cells after local injection of immature DCs but not LPS (Fig. 6 C). i.p. injection of transporter asso-

ciated with antigen presentation–deficient RMA-S cells leads to the local recruitment/proliferation of NK cells (33).
We investigated the frequency of T reg cells in LNs from a single patient receiving systemic cytokine therapy while monitoring the cytotoxic activity of LN mononuclear cells against the autologous melanoma cell line (CURA) established before therapy. The percentages of CD3+/CD56+ NK cells infiltrating the metastatic and the nonmetastatic LNs were similar (2.9 vs. 3%; Fig. 7 A). In line with previous experiments (34), however, the percentage of T reg cells tripled in the metastatic LNs (4 vs. 1.3%; Fig. 7 A). We observed that the cytotoxicity against K562 and CURA was more important in nonmetastatic LNs than in metastatic LNs (46 ± 4% vs. 19 ± 3%, respectively; Fig. 7 B, right and left). After removal of T reg cells from both LNs, the natural cytotoxicity was greatly enhanced in the metastatic node but not in the nonmetastatic node (Fig. 7 B). Tumor cell recognition could be ascribed to NK cells because depletion of CD56+ cells abolished the tumor lysis enhanced by T reg cell depletion (Fig. 7 B). Similarly, in two other patients bearing kidney carcinoma, depletion of T reg cells from tumor-infiltrating mononuclear cells isolated from either a primary tumor or a metastatic LN restored recognition and lysis of K562 in an NK cell–dependent manner (Fig. 7, C and D) in two independent patients. Collectively, these data suggest that T reg cells, infiltrating the tumor or residing in LNs, suppress NK cell recognition of malignant cells in vivo.

**DISCUSSION**

This study demonstrates the capacity of resting T reg cells to directly inhibit NK cell lytic and secretory functions in vitro (Figs. 2 and 4) and to control NK cell proliferation and cytotoxicity in vivo (Figs. 5 and 6). Our mouse and human data show that NK cell inhibition induced by T reg cells is mediated by membrane-bound TGF-β in vitro (Figs. 3 and 4) and in vivo (Fig. 5 B). T reg cells keep NK cells in check in homeostatic conditions (Figs. 5 and 6) and during tumor growth in mice (Figs. 5 and 6) and humans (Figs. 1 and 7) but not in conditions of IL-2Rγ chain or Toll-like receptor 4 triggering (Figs. 2 and 6). Although earlier studies suggested a potential role of T reg cells in down-regulating NK cell effector functions in vitro (35–37), this is the first study to provide mechanistic insights into the inhibitory T reg cell–NK cell interaction and to formally demonstrate the in vivo relevance of the T reg cell–mediated inhibition of the NK cell system, both in steady-state and inflammatory conditions in mice and in cancer patients.

We observed that fixed human CD4+CD25+ T reg cells (from NVs or cancer-bearing patients) could restrict NK cell effector functions in vitro through TGF-β present on the surface of T reg cells. Human resting T reg cells were found to express TGF-β on the surface (Fig. 3), thus exhibiting a phenotype similar to activated mouse T reg cells, which also express TGF-β on their surface (whereas resting mouse T reg cells do not) (28, 38). In line with this finding, LAP is also selectively found on CD4+CD25high but not on CD4+CD25low cells (Fig. 3). Neutralization of TGF-β abrogated the inhibition of NK cell functions by human T reg cells in vitro (Fig. 4), and this inhibition could be mimicked by soluble TGF-β (Fig. 2). In contrast to T reg cells derived from WT animals, T reg cells derived from TGF-β−/− mice transferred into nude mice did not abrogate natural cytotoxicity of the recipient (Fig. 5 B).

T reg cell–bound TGF-β and soluble TGF-β have distinct biological effects on NK cells and are regulated independently. Although both T reg cells and soluble TGF-β down-regulated NKG2D on NK cells (Fig. 4 C) (31), only soluble TGF-β down-regulated the natural cytotoxicity receptor p30, a protein required for the NK cell–mediated lysis of DCs (Fig. S4 A) (30). Moreover, there was no correlation between TGF-β serum levels and the frequency of T reg cells in patients. Although T reg cell levels were supranormal in two different cohorts of patients failing to activate NK cells in response to imatinib mesylate (Figure. 1B), no elevation of TGF-β could be detected (not depicted). Although some of the effects of T reg cells on NK cells could be abrogated by TGF-β neutralization (e.g., NK cytotoxicity and IFN-γ production in Fig. 4), it is not clear whether the T reg cell–dependent inhibition of NK cell proliferation (detectable in the murine system) is also mediated by TGF-β or by alternative effects. However, TGF-β accounts for the T reg cell–mediated down-modulation of NKG2D on NK cells, which is detectable in vitro in the human system (Fig. 4) and in vivo in the murine system (Fig. 5). Importantly, NKG2D expression on NK cells is reduced in a fraction of patients with colon (39) and prostate carcinoma (25), constituting a negative prognostic factor. However, it remains to be elucidated whether this is effectively caused by an increase in the frequency or in the per-cell activity of T reg cells beyond the explanations that have been advanced thus far for NKG2D reduction, namely an increase in serum TGF-β levels (40) or tumor cell shedding of NKG2D ligands (25).

T reg cells have been previously shown to down-regulate the priming and the effector phase of cognate T cell responses, acting in an antigen-non-specific fashion (40–42). Thus, conditions leading to a defect in T reg cells cause autoimmune disease. This applies to the Scurfy mutant mice or Foxp3−− mice (which lack T reg cells because of the deficiency of the T reg cell–specific master transcription factor Foxp3) (32, 43), which develop a general state of autoimmunity. The contribution of NK cells to this immune pathology, however, has not been addressed thus far.

Our data reveal a novel role for T reg cells in the regulation of the innate immune system at the level of NK cells. In homeostatic conditions, the depletion of T reg cells (e-i-
ther by injection of a cytotoxic CD25-specific antibody or by administration of immunostimulatory CTX doses) enhances the proliferation of NK cells, as well as their cytotoxic potential (Fig. 6). Based on these data, it is tempting to speculate that NK cell functions are normally controlled by T reg cells and that any imbalance in the frequency of T reg cells may affect NK cell homeostasis. However, T reg cells do not influence the production of IFN-γ by NK cells stimulated by IL-2γ chain-dependent cytokines (Fig. 2), and it appears thus plausible that conditions leading to significant cytokine production (such as LPS injection in Fig. 5 C, and, speculatively, acute infection) would not involve any significant inhibition of NK cells by T reg cells (P < 0.05). However, in circumstances where IL-12 is involved to promote perforin-dependent NKG2D-dependent cell lysis, T reg cells could be potent inhibitors of tumor regression (44). Similarly, in chronic inflammatory processes, including cancer, it is possible that T reg cells influence the NK cell system. In favor of this contention, T reg cell frequencies correlated with NK cell function in patients (Fig. 1), and depletion of T reg cells can stimulate NK cell-mediated lysis of tumor cells ex vivo (Fig. 7). Thus, immunopharmacological manipulations depleting T reg cells might constitute a welcome addition to the oncologist’s armament against tumor (44). Moreover and independently from this study, LNs were resected from a stage-IV metastatic melanoma patient 2 mo after a 6-wk systemic administration of a combination of IFN-α and IL-2 that induced partial regression. Two additional patients bearing primary kidney cancer and benefitting from surgical resection of the primary lesion and LNs gave their consent to the monitoring of NK cell activity in tumor-infiltrating mononuclear cells. PB-MCs were isolated from blood by Ficoll density centrifugation (Ficoll-Paque; GE Healthcare).

Mice
C57BL/6 WT nude mice and TGF-β−/− mice were obtained from the Centre d’élevage Janvier, the Mollegard Breeding and Research Centre A/S, and from T. Doetschman (University of Cincinnati College of Medicine, Cincinnati, OH), respectively, and were maintained in the animal facility of Institut Gustave Roussy according to the animal Experimental Ethics Committee guidelines. Scurfy mice were bred at the Institut Pasteur (Paris, France) by A. Freitas. Treatment by injection of a cytotoxic CD25-specific antibody or by administration of immunostimulatory CTX doses) enhances the proliferation of NK cells, as well as their cytotoxic potential (Fig. 6). Based on these data, it is tempting to speculate that NK cell functions are normally controlled by T reg cells and that any imbalance in the frequency of T reg cells may affect NK cell homeostasis. However, T reg cells do not influence the production of IFN-γ by NK cells stimulated by IL-2γ chain-dependent cytokines (Fig. 2), and it appears thus plausible that conditions leading to significant cytokine production (such as LPS injection in Fig. 5, and, speculatively, acute infection) would not involve any significant inhibition of NK cells by T reg cells (P < 0.05). However, in circumstances where IL-12 is involved to promote perforin-dependent NKG2D-dependent cell lysis, T reg cells could be potent inhibitors of tumor regression (44). Similarly, in chronic inflammatory processes, including cancer, it is possible that T reg cells influence the NK cell system. In favor of this contention, T reg cell frequencies correlated with NK cell function in patients (Fig. 1), and depletion of T reg cells can stimulate NK cell-mediated lysis of tumor cells ex vivo (Fig. 7). Thus, immunopharmacological manipulations depleting T reg cells might constitute a welcome addition to the oncologist’s armament against tumor (44). Moreover and independently from this study, LNs were resected from a stage-IV metastatic melanoma patient 2 mo after a 6-wk systemic administration of a combination of IFN-α and IL-2 that induced partial regression.

MATERIALS AND METHODS
Patients
Patients enrolled in the French Phase II trial (BR1-F14; Institut Gustave Roussy/Centre Leon Berard, Novartis) testing the efficacy of imatinib mesylate in GIST patients were investigated according to the immunomonitoring protocols approved by the local research and ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale). 80 ml of peripheral blood was drawn from patients before and after 2 mo of imatinib mesylate treatment of 400 mg/day. Similarly, age- and sex-matched NVs were enrolled in parallel investigations (Établissement Français du Sang, Reims, France). Informed written consent was given by all patients. Moreover and independently from this study, LNs were resected from a stage-IV metastatic melanoma patient 2 mo after a 6-wk systemic administration of a combination of IFN-α and IL-2 that induced partial regression. Two additional patients bearing primary kidney cancer and benefitting from surgical resection of the primary lesion and LNs gave their consent to the monitoring of NK cell activity in tumor-infiltrating mononuclear cells. PB-MCs were isolated from blood by Ficoll density centrifugation (Ficoll-Paque; GE Healthcare).

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Cell lines
The GIST cell line (882) was kindly provided by J.A. Fletcher (Harvard Medical School, Boston, MA). The CURA cell line was derived in our laboratory from a primary culture of a metastatic LN in the melanoma patient described above before therapy with IFN-α and IL-2. CURA cells did not express HLA-ABC class I molecules as assessed by flow cytometry with W6/32 mAb, an anti–HLA-ABC antibody (unpublished data). YAC-1 mouse cells are NK cell–sensitive Moloney virus–induced T cell lymphoma cells of A/Sa background. B16Rae is a melanoma cell line stably transfected with a cDNA encoding Rae (45) that has been provided by D. Raulet (University of California, Berkeley, Berkeley, CA). All these cells were maintained in RPMI with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO BRL).

Cytokines, antibodies, and reagents for in vitro culture
Recombinant human IL-12, IL-15, IL-4, IL-7, and TGF-β1 were obtained from R&D Systems. rhuIL-2 was purchased from Chiron Corp. Human anti-NKG2D blocking antibody, NKGD2-Fc, and CTLA-4-Fc were obtained from R&D Systems. Soluble MICA was provided by S. Caillat-Zucman (46). To ascertain that CD4+CD25+ T reg cells do express selectively membrane-bound TGF-β, we performed flow cytometry analyses directly on fresh PBMCs (after Ficoll-Hypaque separation) using four-color staining with anti-CD3–APC, anti-CD4–PerCP, anti-CD25–PE obtained from Becton Dickinson, anti–TGF-β–FITC (using the AB-101-NA polyclonal chicken IgY from R&D Systems), or a chicken IgY–FITC (AB-101-C from R&D Systems) as a control.

Purification of NK cells and T reg cells
NK cells, T reg cells, and T conv were obtained from human PBMCs by magnetic cell separation (Miltenyi Biotech) according to the manufacturer’s instructions. In some experiments, T reg cells were cell sorted using a cell-sorter cytometer (FACSVantage; Becton Dickinson). Mouse T reg cells and T conv were obtained from spleen cells using magnetic cell separation (Miltenyi Biotech) after lysis of RBCs. CD4+CD25+ T cells isolated by the cell-sorter cytometer or magnetic sorting were enriched in population in T reg cells because they were able to inhibit an alloreactive reaction and because they express Foxp3 mRNA using RT-PCR analysis (unpublished data). For adoptive transfer experiments, 10⁶ T reg cells or T conv were injected i.v. in each nude mouse after 24 h of ex vivo activation with 10 µg/ml anti-CD3 mAb, 5 µg/ml anti-CD28 mAb, and 2,000 UI/ml of IL-2 and several cycles of washing.

Flow cytometry analysis
 PBMCs and cultured cells were analyzed using a cytometer (FACScalibur; Becton Dickinson). Cells were stained with directly labeled antibodies in a three-color staining analysis. The following antibodies were used: anti–human CD3–FITC (IgG1, clone UCHT1), anti–human CD25–PE (IgG1, clone M-A251), and anti–human CD4–PerCP (IgG1, clone RPA-T4). All of these antibodies were obtained from Becton Dickinson. Anti–human CD56–PC5 (IgG1, clone N901) was purchased from Beckman Coulter. Anti–human LAP and anti–TGF-βRII antibody were obtained from R&D Systems. Anti–mouse DX5 was obtained from Becton Dickinson. Magnetically selected T reg cells or CD4+CD25+ T conv were stained with anti–TGF-β–FITC (using the AB-101-NA polyclonal chicken IgY from R&D Systems) or a chicken IgY–FITC (AB-101-C from R&D Systems) as a control and/or anti–human NKGD2–PE (IgG1, clone 140/10; R&D Systems), followed by a goat anti–chicken immunoglobulin FITC-labeled antibody (R&D Systems).

In vitro assays
Cytotoxicity assay. NK cells were seeded at 2 × 10⁶ cells/well in 96-well plates to be used as effector cells. NK cells were incubated with T reg cells or T conv or reagents (1 ng/ml TGF-β1, 10 ng/ml anti–TGF-β1 antibody, 10 ng/ml isotype control antibody, 10 ng/ml soluble MICA, 15 µg/ml NKGD2-Fc, 15 µg/ml anti-NKG2D mAb, and 15 µg/ml CTLA-4–Fc) at the times indicated in the figures for each experimental setting in 200 µl of AIMV culture medium (GIBCO BRL) at the indicated effector/target ratio. NK cell cytotoxicity was measured in a standard 4-h ¹¹¹Cr release assay at 37°C. Spontaneous release was assessed from wells that con-
tained labeled target cells alone, and maximum 51Cr release was assessed by addition of 2% cetrimide (Sigma-Aldrich). Specific cytotoxicity was calculated as follows: percent 51Cr release = 100 × (cpm experimental – cpm spontaneous release) / (cpm maximum release – cpm spontaneous release).

Cytokine detection. 106 NK cells were cultured alone or with T reg cells or T conv on a 1:1 ratio and/or reagents (1 ng/ml TGF-β1 or 10 ng/ml anti–TGF-β1 antibody or 10 ng/ml isotype control antibody) for 6 h in 200 μl of AIMV medium. After a 6-h incubation, 2 ng/ml IL-12 or 200 UI/ml IL-2 or 1 ng/ml IL-15 was added for 18 h. Supernatants of these co-cultures were harvested, and IFN-γ concentrations were determined by an ELISA test kit (OptEIA; BD Biosciences) with a detection limit of 20 pg/ml.

Mixed lymphocyte alloimmune reactions. Allogeneic monocyte–derived DCs propagated in GM-CSF and IL-4 were obtained as previously described (20). 106 DCs were cultured with 106 T conv alone or with 105 CD4+CD25high or CD4+CD25low T cells (isolated by cell sorting or immunomagnetic beads with anti-CD25/CD4 mAb) for 5 d. 0.5 μCi/well of [3H]thymidine was added during the final 18 h. [3H]thymidine incorporation was measured by liquid scintillation counting after harvesting the cells on glass fiber filters using an automatic cell harvester (Tomtec).

In vivo proliferation assays, assessment of natural cytotoxicity, and B16Rae lung metastases

Proliferation assay. In brief, animals were treated or not with 100 mg/kg anti-CD25 mAb (PC61) or CTX at day −7 before inoculation of PBS, or BM DCs propagated in GM-CSF and IL-4 (47) (10 μg/injection) in the footpad and BrdU i.v. to monitor NK cell proliferation in the draining LNs at 24 h.

Tumor models. We examined the number of lung mets of B16Rae melanoma cells injected at day 0 in nude mice receiving or not, 12 h later, an infusion of 2 × 106 T reg cells or T conv activated overnight with 10 μg/ml anti-CD3, 5 μg/ml anti-CD28, and 1,000 IU/ml IL-2 by adoptive transfer. Combination therapy of STI571 and CTX to treat lung metastases revealed alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J. Exp. Med. 194:823–832.

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