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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.

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Abbreviations used: BrdU, 5-bromo-2′deoxyuridine; CTX, cyclophosphamide; GIST, gastrointestinal stromal tumor–bearing; iDC, immature DC; imatinib mesylate, Gleevec, STI571; LAP, latent-associated protein; MIC, MHC class I chain–related molecule; MICA, MHC class I–related chain A; NV, normal volunteer; T conv, conventional T cells; T reg, regulatory T.

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

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F. Ghiringhelli and C. Ménard contributed equally to this work.
The online version of this article contains supplemental material.

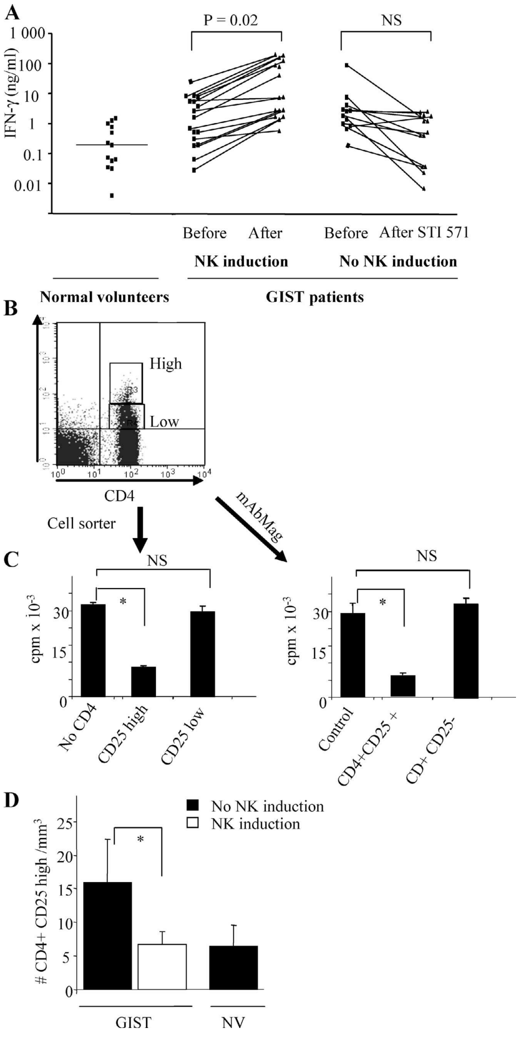

Figure 1. Inverse correlation between T reg cell numbers and NK cell induction in GIST patients treated with imatinib mesylate. (A) Prospective analysis of NK cell IFN-γ secretion after stimulation with allogeneic iDCs in the presence of LPS for 40 h at a ratio of 10:1 as previously described (reference 20) was conducted on 31 GIST patients before and 2 mo after the onset of therapy with imatinib mesylate. The levels of IFN-γ accumulating in the co-culture were assessed using an ELISA kit. Dots correspond to individual patients or controls. Intraindividual variations for the standardized assay of co-culture was <10%. NK cell induction was defined as a 5–10-fold increase at 2 mo over baseline levels (<1,000 pg/ml) of IFN-γ produced by NK cells stimulated by the same allogeneic iDCs (*n* = 18, NK cell induction; *n* = 13, no NK cell induction; and *n* = 13, NVs). The Wilcoxon two sample rank sum test was used to compare the two-paired groups (*, $P < 0.05$). The horizontal line indicates the mean. (B) FACS analyses were performed on $CD3^+CD4^+CD25^+$ T cells using three-color staining with anti-CD3-FITC, anti-CD4-PerCP, and anti-CD25-PE to identify T reg cells ($CD4^+CD25^{high}$). The y axis indicates CD25. High, $CD4^+CD25^{high}$ T cells; Low, $CD4^+CD25^{low}$ T cells. (C) $CD4^+CD25^{high}$ (T reg cells) or $CD4^+CD25^{low}$ T cells were cell sorted using a cell-sorter cytometer. Likewise, $CD4^+CD25^-$ or T reg cells ($CD4^+CD25^+$) were selected by magnetic selection. Each of these subsets were cultured with bulk T cells and allogeneic iDCs in a 10:10:1 ratio. Proliferation was determined after 5 d of culture by measurement of [^{3}H]thymidine incorporation. The values represent the means ± SEM. *, $P < 0.05$ using Student's *t* test. (D) Absolute numbers of circulating $CD4^+CD25^{high}$ 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 $CD4^+CD25^{high}$ T cells/mm^3 of blood. *, $P < 0.05$ using Student's *t* test.

patients, NK cell activation can be hampered by tumor-mediated shedding of MICs (25), but other mechanisms might blunt NK cell responses during therapy with systemic cytokines (26).

In this study, we determined whether tumor-driven expansion of T reg cells might impair NK cell activation in cancer patients. We provide evidence that both in human and in murine systems, in vitro and in vivo, T reg cells potently suppress NK cell responses, including the NK cell–mediated control of tumor expansion. Our data are compatible with the hypothesis that eliminating T reg cells might constitute a novel strategy to stimulate the innate immune response against tumors.

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 $CD4^+CD25^{high}$ T reg cells were monitored by flow cytometry before therapy (Fig. 1 B) (27). In contrast to $CD4^+CD25^{low}$ T cells, these $CD4^+CD25^{high}$ 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-

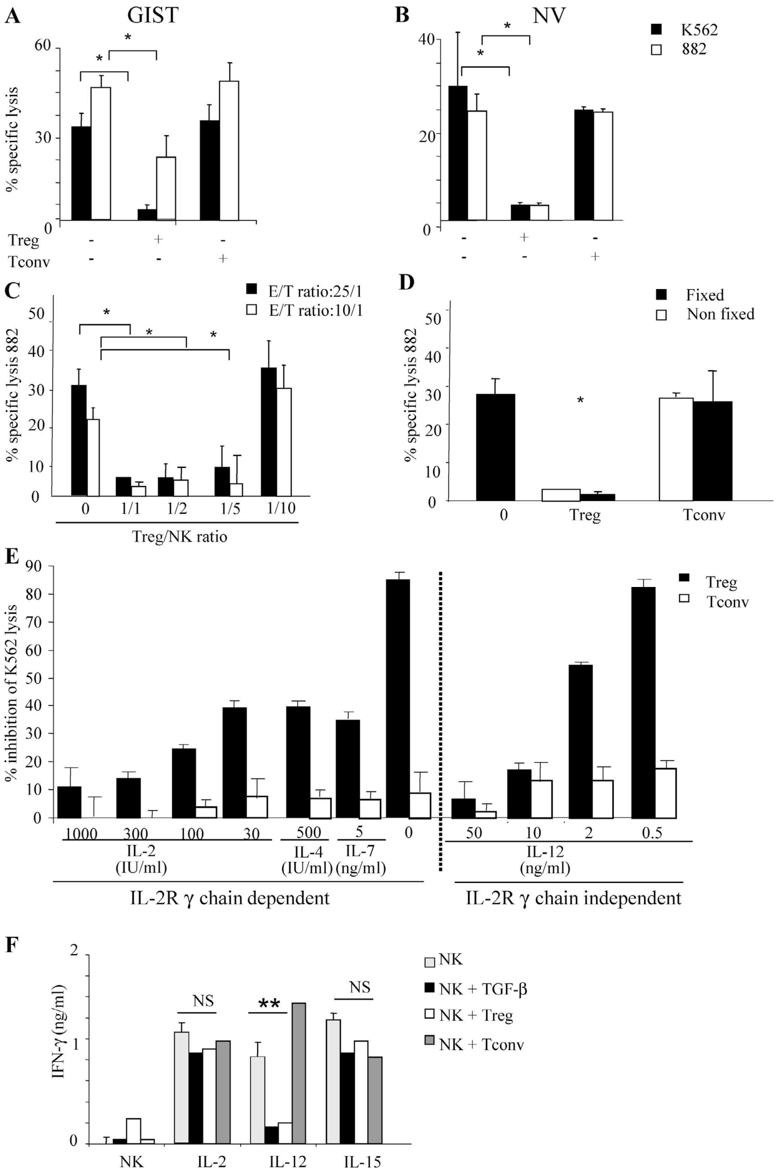

Figure 2. Inhibition of NK cell functions by T reg cells in vitro: rescue by IL-2Rγ chain–dependent cytokines. NK cells purified from GIST patients (A) or NVs (B) were cultured 4 h with T reg cells or with T conv isolated from NVs before incubation with ^{51}Cr-labeled K562 or GIST 882 tumor targets. 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 in A–D. (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 ^{51}Cr 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. **, $P < 0.05$.

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 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_1 on human resting T reg cells. CD3$^+$/CD4$^+$/CD25$^{\text{high}}$ T reg cells (but not T conv or CD3$^+$/CD4$^+$/CD25$^{\text{low}}$ cells) expressed TGF-β_1 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-β_1. Importantly, the neutralizing anti–TGF-β_1 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

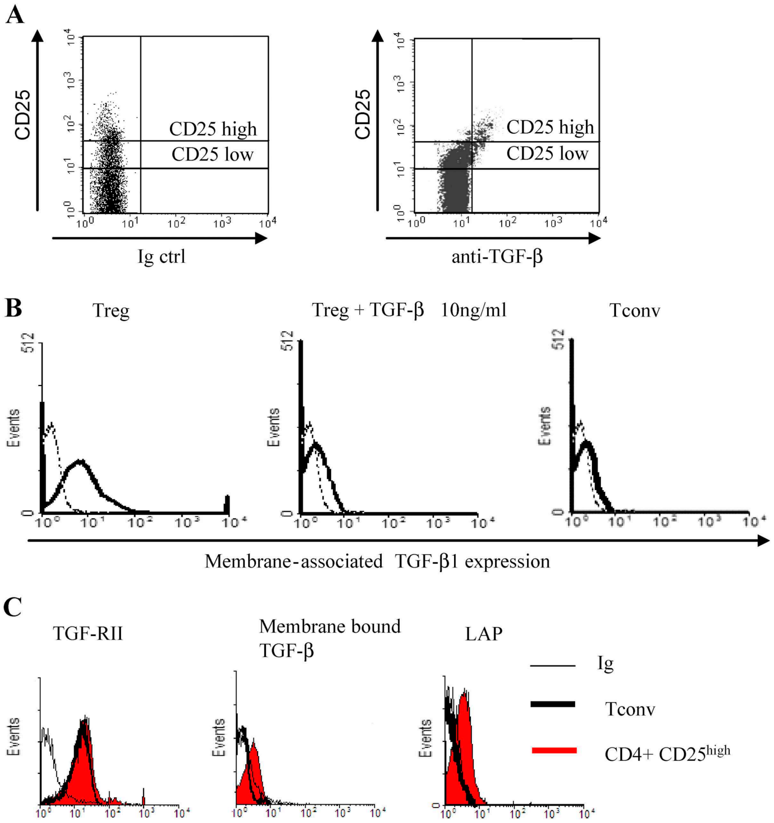

Figure 3. Selective expression of membrane-bound TGF-β on T reg cells. (A) TGF-β expression was investigated on both CD4$^+$CD25$^{\text{high}}$ versus CD4$^+$CD25$^{\text{low}}$ cells in flow cytometry using four-color staining with anti-CD3–APC, anti-CD4–PerCP, anti-CD25–PE, anti–TGF-β–FITC (AF-101-NA 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.

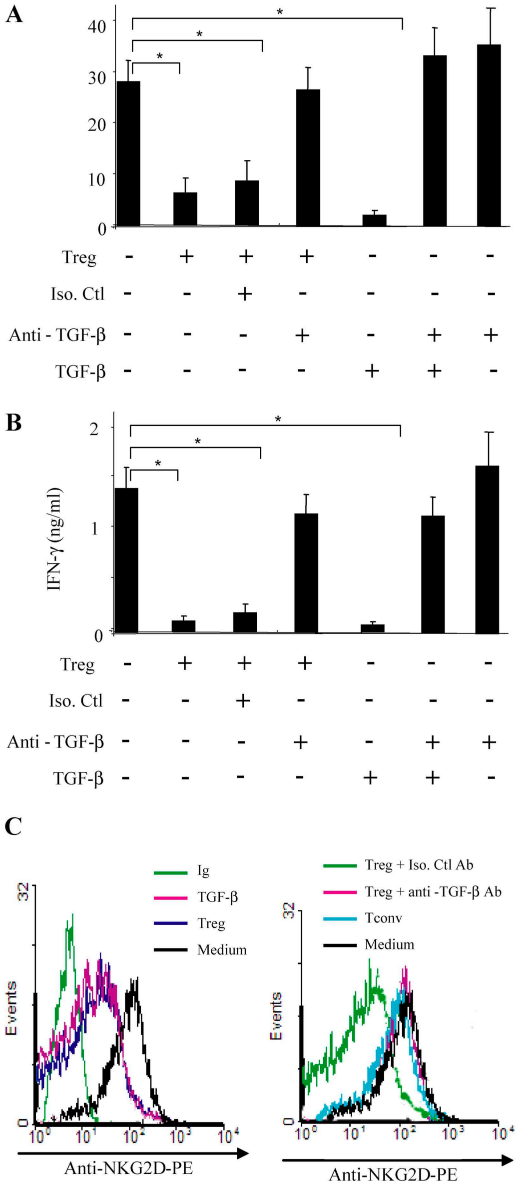

Figure 4. Role of membrane-bound TGF-β in T reg cell–mediated NK cell inhibition. (A) NK cells were co-cultured for 4 h with T reg cells or soluble TGF-β1 and/or a TGF-β1 blocking antibody or IgY control antibody before incubation with ^{51}Cr-labeled GIST 882 tumor targets. The T cell/NK cell/target 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, left) 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.

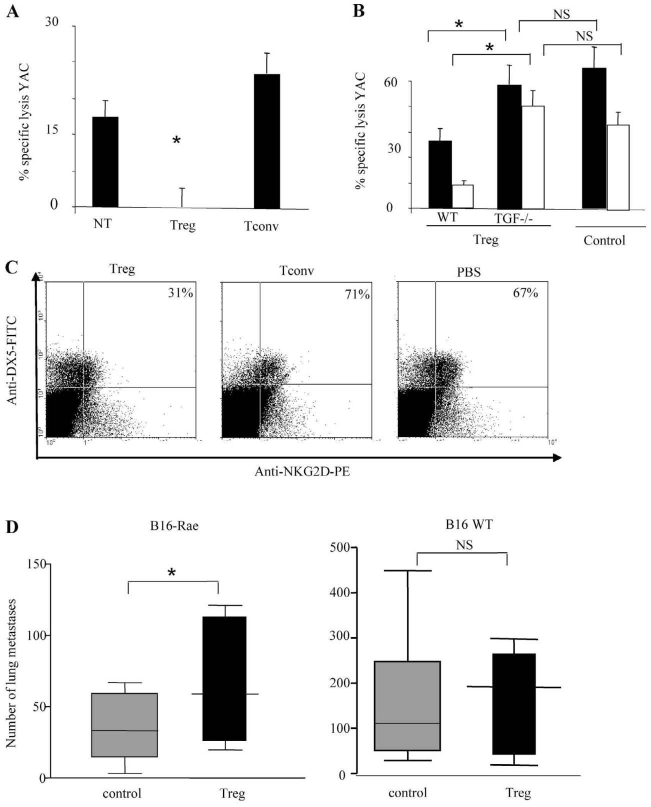

Figure 5. Adoptive transfer of T reg cells in nude mice. (A) Three C57BL/6 nude mice were injected with PBS or with 2 × 10^6 T reg cells or T conv twice 3 d apart. On day 4, the cytolytic activity of splenocytes against YAC-1 cells was determined in a ^{51}Cr release assay at a 1:200 target/effector 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.β$^{-/-}$ mice at a 1:100 (closed bars) or 1:50 (open bars) E/T ratio. (C) 2 × 10^6 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 × 10^5 B16-Rae or B16-WT tumor cells were inoculated in nude mice at day 0 and 12 h later, and 10^6 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.

on NK cell–mediated target cell lysis (Fig. 4 A). Similarly, the neutralizing anti–TGF-β_1 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

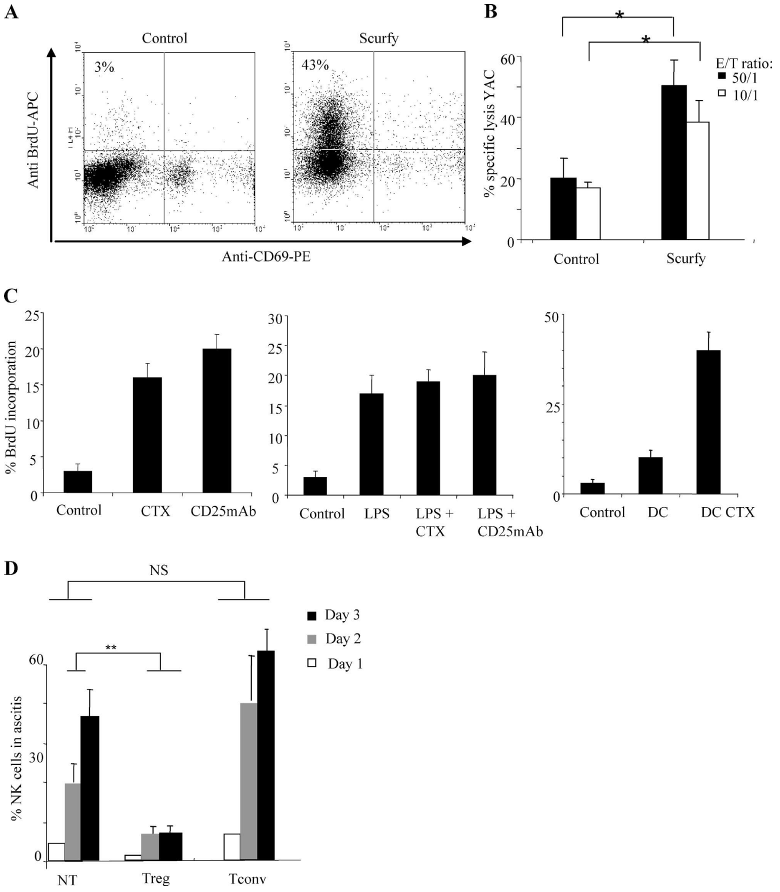

Figure 6. T reg cell depletion resulted in profound NK cell activation in vivo. (A) BrdU was injected in Scurfy mice in parallel to WT mice to assess the basal NK cell proliferation in vivo at 24 h. The percentage of proliferating CD69/BrdU$^+$ NK cells in the gate CD3$^-$/Dx5$^+$ cells was assessed by flow cytometry using four-color staining. One representative experiment out of three is shown in dot plots. (B) The natural cytotoxicity of splenic NK cells after isolation of Dx5$^+$ cells in WT and Scurfy mice was assessed against YAC-1 targets in a 4-h chromium release assay in vitro. The graph depicts means ± SEM of triplicate wells in one representative mouse out of two. *, $P < 0.05$. (C) Regulation of T reg cell–mediated NK cell inhibition in the steady state and after stimulation with LPS or DCs. WT mice received 300 μg of neutralizing anti-CD25 antibody (PC61) or rat control Ig or CTX at 100 mg/kg or PBS at day 7. At day 0, mice received a footpad injection of either 10 μg/ml of LPS or 3×10^5 BM DCs (GM-CSF + IL- 4) and, simultaneously, an i.v. injection of BrdU. The graphs depict the means ± SEM of percentages of BrdU incorporating NK cells in three animals/group. (D) 5×10^5 RMA-S cells were injected i.p. in three nude mice alone (NT) or admixed with either 2×10^6 T reg cells or T conv purified from C57BL/6 mice. Peritoneal exudates were harvested on days 1, 2, and 3 after cell injection. The percentages of NK1.1$^+$ CD3$^-$ NK cells in the exudates were determined by FACS analysis. The graph depicts the means of percentages of three mice/group ± SEM. **, $P < 0.05$.

cells that express the corresponding ligands. T reg cells also down-regulated NKG2D (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 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 lymphoprolifera-

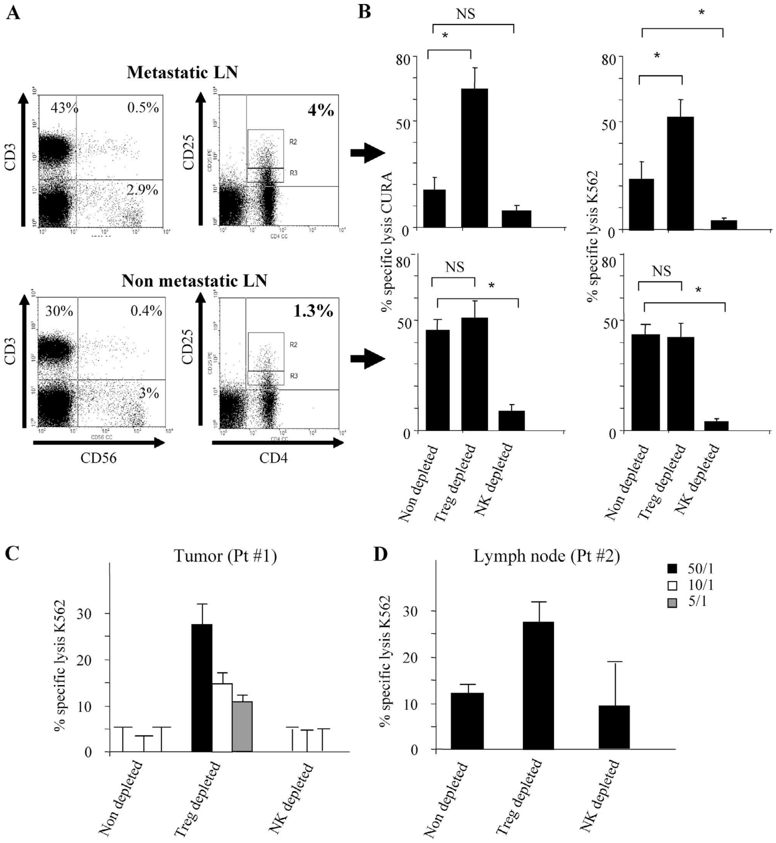

Figure 7. Depletion of T reg cells in tumors restored tumor recognition by NK cells. (A) Percentages of T and NK cells in metastatic and nonmetastatic draining LNs resected from a melanoma patient were determined by FACS analysis using a three-color staining (CD3-FITC, CD56-PC5, and CD45-APC). Percentages of T reg cells were determined using anti-CD3–FITC, anti-CD25-PE, and anti-CD4-PerCP mAbs. Data representative of three different experiments are shown. (B) LN cells from metastatic or nonmetastatic LNs depleted or not of $CD25^+$ or $CD56^+$ cells were subjected to lysis of autologous CURA cells or K562 at a 1:25 target/effector ratio. *, $P < 0.05$. (C and D) One primary tumor and one invaded LN derived from two kidney cancer patients were dissociated and centrifuged on a Ficoll-Hypaque gradient enabling isolation of mononuclear cells containing 5.6 and 9% of T reg cells, respectively, and 1.8 and 1.2% of NK cells, respectively. Mononuclear cells were treated with either mAb anti-CD56 coated onto magnetic beads to deplete NK cells or anti-CD4 mAb magnetic beads followed by anti-CD25mAb magnetic beads to deplete T reg cells or both to deplete NK cells and T reg cells before a 4-h coincubation with ^{51}Cr K562 for a chromium release assay. Cytolytic activity was determined in a 4-h ^{51}Cr release assay. Results represent means ± SEM of triplicate wells of a representative experiment.

tion) into nude mice did not result in the inhibition of YAC-1 lysis by $Dx5^+$ 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 associated with antigen presentation–deficient RMA-S cells leads to the local recruitment/proliferation of NK cells (33).

T reg cells (but not T conv) coinjected with such tumor cells strongly reduced the number of i.p. NK cells (Fig. 6 D), thus providing another line of evidence for the T reg cell–mediated inhibition of the NK cell system in vivo.

Depletion of T reg cells ameliorates NK cell–mediated lysis of human tumor cells

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 expose TFG-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 $CD4^+CD25^{high}$ but not on $CD4^+CD25^{low}$ 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 $TGF\text{-}\beta^{-/-}$ 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 effectors. 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-nonspecific 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 (either 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-2Rγ 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 if stimulation of the NK cell response against malignant cell is the therapeutic goal and if the predictable proautoimmune side effects of such a manipulation are manageable.

MATERIALS AND METHODS

Patients

Patients enrolled in the French Phase II trial (BRF14; 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 (Etablissement 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 benefiting from surgical resection of the primary lesion and LNs gave their consent to the monitoring of NK cell activity in tumor-infiltrating mononuclear cells. PBMCs were isolated from blood by Ficoll density centrifugation (Ficoll-Paque; GE Healthcare).

Mice

C57BL/6 WT nude mice and TGF.$\beta^{-/-}$ mice were obtained from the Centre d'élevage Janvier, the Mollegaard 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.

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/Sn 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, NKG2D-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 AF-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 Biotec) 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 Biotec) 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 allogenic reaction and because they express Foxp3 mRNA using RT-PCR analysis (unpublished data). For adoptive transfer experiments, 10^6 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 IU/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 AF-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 NKG2D–PE (IgG1, clone 149810; 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 2×10^4 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 μg/ml soluble MICA, 15 μg/ml NKG2D-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 ^{51}Cr release assay at 37°C. Spontaneous release was assessed from wells that contained labeled target cells alone, and maximum ^{51}Cr release was assessed by addition of 2% cetrimide (Sigma-Aldrich). Specific cytotoxicity was calculated as follows: percent ^{51}Cr release = 100 × (cpm experimental − cpm spontaneous release) / (cpm maximum release − cpm spontaneous release).

Cytokine detection. 10^5 NK cells were cultured alone or with T reg cells or T conv at 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 allogeneic reactions. Allogeneic monocyte–derived DCs propagated in GM-CSF and IL-4 were obtained as previously described (20). 10^4 DCs were cultured with 10^5 T conv alone or with 10^5 $CD4^+CD25^{high}$ or $CD4^+CD25^{low}$ T cells (isolated by cell sorting or immunomagnetic beads with anti-CD25/CD4 mAb) for 5 d. 0.5 μCi/well of [^{3}H]thymidine was added during the final 18 h. [^{3}H]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) or LPS (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 × 10^6 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 of B16F10 melanoma cells (3 × 10^5 at day 0) consisted of oral feeding with 150 mg/kg STI571 twice a day for 10 d and 100 mg/kg of CTX at day 6 for a death at day 12 and enumeration of lung metastases.

Cytotoxicity against YAC-1 cells. Splenocytes were either subjected to a direct 4-h chromium release assay against YAC-1 cells, or NK cells were first purified using anti-Dx5 mAb magnetic beads.

Statistical analyses

Student's *t* test, Wilcoxon analysis, or Fisher's exact method were used as described in the figure legends to compare various groups in each experiment using PRISM software.

Online supplemental material

Fig. S1 shows that T reg cells directly inhibit NK cell lytic functions against K562. Fig. S2 depicts the dose response of NK cells to IL-2Rγ chain–dependent cytokines for cytolytic functions. Fig. S3 shows the role of membrane-bound TGF-β in mediating T reg cell inhibition on K562 NK cell target recognition. Fig. S4 A depicts the differential effects of soluble rhu–TGF-β and T reg cells on cell surface expression of NKp30 and p46 and NKG2D on human resting NK cells. K562 and GIST882 are NKG2D ligand–expressing targets (Fig. S4, B and C). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20051511/DC1.

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