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Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival

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Regulatory T (T_{reg}) cells mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. Failure of host antitumor immunity may be caused by exaggerated suppression of tumor-associated antigen–reactive lymphocytes mediated by T_{reg} cells; however, definitive evidence that T_{reg} cells have an immunopathological role in human cancer is lacking. Here we show, in detailed studies of $CD4^+CD25^+FOXP3^+$ T_{reg} cells in 104 individuals affected with ovarian carcinoma, that human tumor T_{reg} cells suppress tumor-specific T cell immunity and contribute to growth of human tumors *in vivo*. We also show that tumor T_{reg} cells are associated with a high death hazard and reduced survival. Human T_{reg} cells preferentially move to and accumulate in tumors and ascites, but rarely enter draining lymph nodes in later cancer stages. Tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates trafficking of T_{reg} cells to the tumor. This specific recruitment of T_{reg} cells represents a mechanism by which tumors may foster immune privilege. Thus, blocking T_{reg} cell migration or function may help to defeat human cancer.

T cells targeted at tumor-associated antigens (TAAs) are readily detectable in blood, tumors and draining lymph nodes of individuals with cancer, even at late stages of disease. These tumor-specific T cells[1–4] can be used to establish functional TAA-specific T cell lines, which kill autologous tumor cells *in vitro* and *in vivo*[5]. The spontaneous clearance of established tumors by endogenous immune mechanisms is, however, rare. In addition, vaccine-induced increases in TAA-specific T cells do not always coincide with tumor regression[1,6–8]. Thus, it is clear that established tumors induce immune tolerance to escape destruction[9–13], although the underlying mechanisms are not well defined.

Indirect evidence suggests that $CD4^+CD25^+$ T cells (T_{regs}) are important in suppressing TAA-specific immunity[14–21]. $CD4^+CD25^+$ T cells are present in malignant effusions[22,23] and blood[24] of individuals with various types of cancer, and they suppress nonspecific T cell responses *in vitro*. However, direct evidence supporting a role for T_{reg} cells in the immunopathogenesis of human cancers is lacking, as is specific knowledge of the trafficking mechanisms for T_{reg} cells.

RESULTS

$CD4^+CD25^+$ T cells in malignant ascites

In individuals with untreated malignant ovarian epithelial cancers ($n = 45$), we identified a substantial population of $CD4^+CD25^+CD3^+$ T cells (10–17% of all T cells) by flow cytometry (FACS analysis) in malignant ascites (**Fig. 1a**,**b**). $CD4^+CD25^+CD3^+$ T cells were more abundant in ascites of individuals with stage III ($n = 30$, $P < 0.01$) and stage IV ($n = 15$, $P < 0.01$) tumors than in their blood (**Fig. 1a**,**b**). We observed a small fraction of $CD4^+CD25^+CD3^+$ T cells (0.7–5.0%) in nonmalignant ascites from individuals with idiopathic cirrhosis ($n = 6$) or who had undergone a liver transplant ($n = 4$; **Fig. 1a**,**b**). There were fewer $CD4^+CD25^+$ T cells in nonmalignant ascites ($P < 0.01$) than in malignant ascites, however, supporting the idea that $CD4^+CD25^+$ T cells accumulate in a tumor-specific manner in malignant ascites. Malignant ascites in stage I and II ovarian tumors is uncommon and was not available for study.

$CD4^+CD25^+$ T cells in the solid tumor mass

To confirm our results in malignant ascites, we studied 104 tumor tissues from individuals with untreated ovarian epithelial cancers, including the 45 individuals described above. Consistent with results from malignant ascites, we identified a substantial accumulation of $CD4^+CD25^+CD3^+$ T cells by multicolor confocal microscopic analysis in the tumor mass. Tumor-infiltrating $CD4^+CD25^+$ T cells represented $23 \pm 11\%$ (mean ± s.e.m.) of tumor-infiltrating $CD4^+$ T cells (**Fig. 1c**). The percentage of $CD4^+CD25^+CD3^+$ T cells in $CD4^+CD3^+$ T cells was

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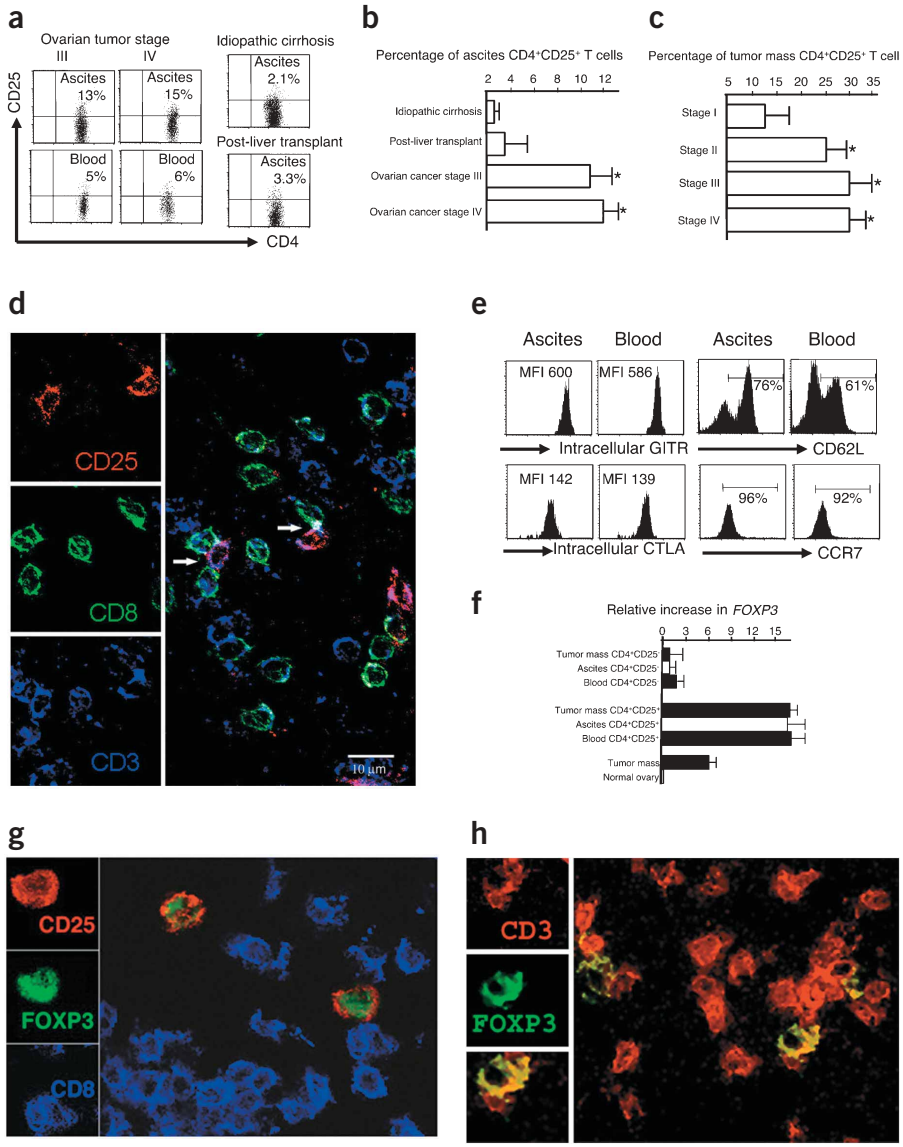

Figure 1 $CD4^+CD25^+$ T cells in distinct tumor microenvironments. (**a**,**b**) $CD4^+CD25^+CD3^+$ cells preferentially accumulate in malignant ascites. Cells were analyzed by FACS with multicolor staining and gating on $CD3^+CD4^+$ cells. (**a**) Two representative samples of malignant ascites from individuals with ovarian cancers ($n = 45$ analyzed), and two samples of non-tumor ascites from control individuals ($n = 10$). $^*P < 0.01$ versus nontumor ascites or blood. (**b**) Percentage of $CD4^+CD25^+$ T cells in $CD4^+CD3^+$ cells (mean ± s.e.m.). $^*P < 0.001$ versus nontumor ascites. (**c**,**d**) $CD4^+CD25^+CD3^+$ cells accumulate in tumor mass. (**c**) Percentage of tumor mass $CD4^+CD25^+CD3^+$ cells quantified by confocal microscopic analysis. Results are expressed as in **b**. $^*P < 0.001$ versus stage I. (**d**) $CD4^+CD25^+CD3^+$ cells are in close contact with $CD8^+CD3^+$ T cells in tumor tissues (arrows, stage III) *in vivo*. $CD4^+CD25^+CD3^+$ T cells were identified as $CD3^+CD8^-CD25^+$ T cells. White color indicates intimate association of $CD4^+CD25^+$ T cells and $CD8^+$ T cells. A representative sample is shown ($n = 53$). (**e–h**). Phenotype of tumor-associated $CD4^+CD25^+$ T cells. (**e**) All (100%) of tumor ascites and blood $CD4^+CD25^+$ T cells express intracellular GITR and CTLA-4, and most strongly express the lymphoid homing molecules CD62L and CCR7. Cells were gated on $CD3^+CD4^+CD25^+$ cells ($n = 5$ for each). MFI, mean fluorescence intensity. (**f**) Expression of *FOXP3* by human $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were sorted by FACS from peripheral blood and from infiltrating T cells in the tumor mass and ascites. Real-time PCR for *FOXP3* was done in triplicate and relative fold changes were normalized to *GAPDH*. $^*P < 0.001$, $CD4^+CD25^+$ T cells versus $CD4^+CD25^-$ T cells or normal ovary tissues. (**g**,**h**) $FOXP3^+$ T_{reg} cells in ovarian tumor mass. Tissues were stained with antibodies to human FOXP3, human CD25 and CD8 (**g**), or with antibodies to human FOXP3 and human CD3 (**h**) ($n = 5$ for each). $FOX3^+CD3^+$ (T_{reg}) cells are in close contact with $FOX3^-CD3^+$ cells (mostly $CD8^+$ T cells). Scale bars, 40 μm.

higher in later disease stages (stage II, $n = 11$; stage III, $n = 53$; stage IV, $n = 33$) than in early disease stages (stage I, $n = 7$; $P < 0.001$ for all). In addition, 75 ± 17% of $CD4^+CD25^+CD3^+$ T cells in the tumor mass were in proximity to infiltrating $CD8^+$ T cells ($n = 32$; **Fig. 1d**), suggesting that physical contact between $CD4^+CD25^+$ T cells and $CD8^+$ cytotoxic T cells mediates regulatory functions as proposed[14–17].

$CD4^+CD25^+$ T cells were undetectable in normal ovarian tissues from five control subjects without cancer (data not shown). Thus, these data show the accumulation of $CD4^+CD25^+$ T cells in malignant ascites and tumor tissue in individuals with ovarian cancers.

Phenotype of tumor-associated $CD4^+CD25^+$ T cells

Freshly isolated $CD4^+CD25^+$ T cells from tumor ascites and peripheral blood expressed similar levels of membrane glucocorticoid-induced tumor-necrosis factor (TNF) receptor family–related gene (GITR) and cytolytic T lymphocyte–associated antigen 4 protein (CTLA-4; data not shown). Because of their different localizations, we reasoned that tumor ascites and blood $CD4^+CD25^+$ T cells expressed different levels of intracellular GITR and CTLA-4. Tumor ascites $CD4^+CD25^+$ T cells expressed intracellular GITR and CTLA-4 (**Fig. 1e**), similar to circulating, $CD4^+CD25^+$ T cells[14–18]. The majority of $CD4^+CD25^+$ T cells in blood and malignant ascites expressed CD62L (>60%) and CCR7 (>90%; **Fig. 1e**).

Foxp3 is crucial for the differentiation and function of mouse $CD4^+CD25^+$ T_{reg} cells[25–27]. Real-time PCR showed strong and equivalent expression of *FOXP3* mRNA in $CD4^+CD25^+$ T cells isolated from malignant ascites ($n = 7$), the tumor mass ($n = 4$) and blood ($n = 6$) from individuals with ovarian cancers, as compared with their $CD4^+CD25^-$ T cells ($P < 0.001$ for each comparison; **Fig. 1f**). We also observed strong *FOXP3* expression in ovarian tumor tissues ($n = 10$, $P < 0.001$), but not in normal ovarian tissues from controls without cancer ($n = 5$; **Fig. 1f**). In support of this, we observed a substantial infiltration of $CD8^-$ $FOXP3^+CD25^+$ cells into the tumor mass. All (100%) of the tumor-infiltrating $FOXP3^+$ cells were $CD25^+$ T cells, whereas 90 ± 8% $CD25^+$ T cells were $FOXP3^+$ cells in the tumor mass ($n = 5$; **Fig. 1g**). Consistent with our above observation (**Fig. 1d**), many of the $FOXP3^+CD25^+$ cells (80 ± 17%) were in close contact with $CD8^+$ T cells (**Fig. 1g**).

We also showed that 100% of the tumor-infiltrating $FOXP3^+$ cells were $CD3^+$ T cells ($n = 5$; **Fig. 1h**). Therefore, tumor-infiltrating $CD4^+CD25^+CD3^+$ T cells express FOXP3 and are often in close contact

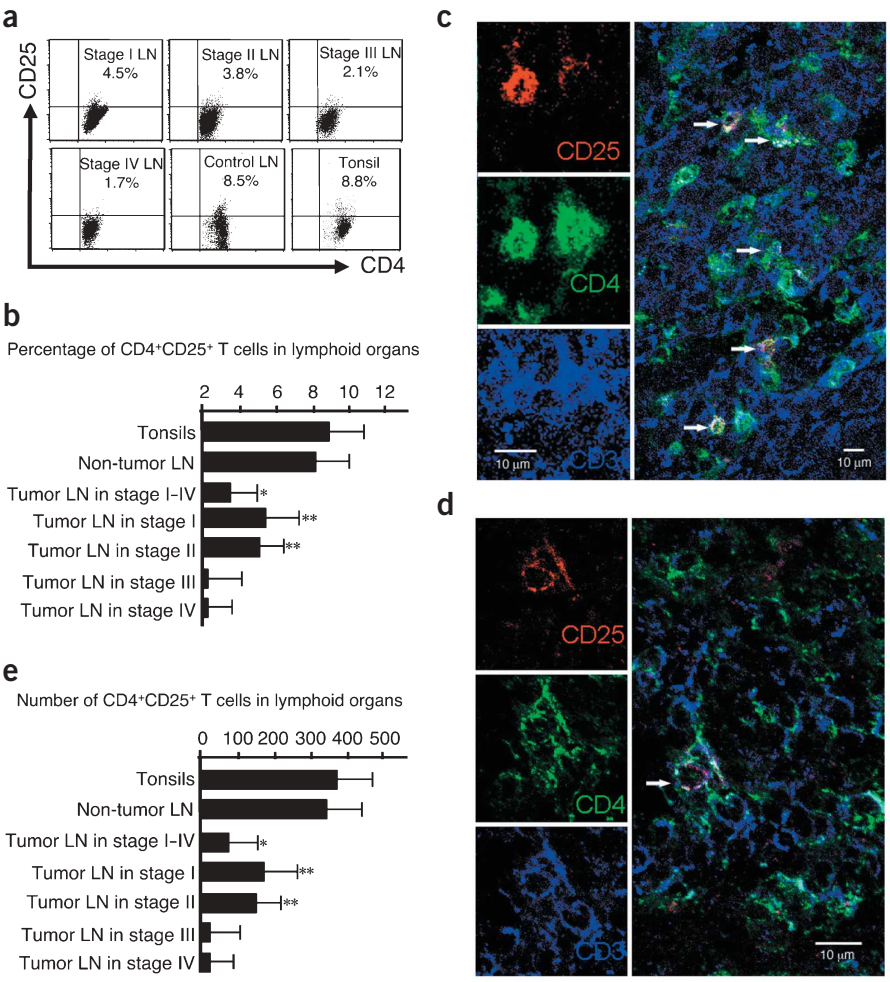

Figure 2 $CD4^+CD25^+$ T cells in lymph nodes. (**a**) Representative FACS analysis of samples from tumor-draining lymph nodes (LN) from 36 affected individuals. (**b**) Percentage of $CD4^+CD25^+$ T cells in $CD4^+CD3^+$ cells (mean ± s.e.m.). Lymphoid organ cells were analyzed by FACS with multicolor staining and gating on $CD3^+CD4^+$ cells. $^*P < 0.01$ versus nontumor lymph nodes ($n = 6$) or tonsils ($n = 8$). $^{**}P < 0.001$ versus stage III or stage IV. (**c**,**d**) Large numbers of $CD3^+CD4^+CD25^+$ cells are present in control lymphoid organs (**c**; $n = 8$), but few are present in tumor-draining lymph nodes (**d**; $n = 8$). Arrow and white color indicate triple-positive ($CD3^+CD4^+CD25^+$) cells. (**e**) Number of $CD4^+CD25^+$ T cells in draining lymph nodes of tumors at various stages of disease. $CD4^+CD25^+CD3^+$ T cells were quantified by confocal microscopy. $^*P < 0.001$ versus nontumor lymph nodes or tonsils. $^{**}P < 0.001$ versus stage III or stage IV. Data show the number of $CD4^+CD25^+CD3^+$ cells per ten HPF. Scale bars, 10 μm.

with $CD8^+$ T cells. Taken together, the $CD4^+CD25^+CD3^+GITR^+CTLA\text{-}4^+CCR7^+FOXP3^{hi}$ phenotype of tumor-associated $CD4^+CD25^+$ T cells suggests that they have characteristics of T_{reg} cells[14,25–27].

$CD4^+CD25^+$ T cells in tumor-draining lymph nodes

CD62L and CCR7 direct migrating cells to lymph nodes, where homeostatic T_{reg} cells readily accumulate[14,16,28]. We therefore studied the content of T_{reg} cells in tumor-draining lymph nodes from individuals with ovarian cancer ($n = 36$). We first compared the accumulation of $CD4^+CD25^+$ T cells in tumor-draining lymph nodes to that in lymph nodes from individuals without cancer. Less than 5% of $CD4^+$ T cells in tumor-draining lymph nodes were $CD4^+CD25^+$, as assessed by FACS. This accumulation of $CD4^+CD25^+$ T cells in tumor-draining lymph nodes was lower than in control lymph nodes ($n = 6$, $P < 0.01$) and tonsils ($n = 8$, $P < 0.01$; **Fig. 2a**,**b**). We were unable to obtain lymph nodes that did not drain the tumor in individuals with ovarian cancer.

We also examined accumulation of $CD4^+CD25^+$ T cells in tumor-draining lymph nodes according to tumor stage. $CD4^+CD25^+$ T cells in tumor-draining lymph nodes were less frequent in stage III ($n = 14$) or IV ($n = 11$) than in stage II ($n = 5$) or I ($n = 6$; $P < 0.01$ for III plus IV versus I plus II; **Fig. 2a**,**b**). We further quantified the absolute amount of $CD4^+CD25^+CD3^+$ cells in tumor-draining

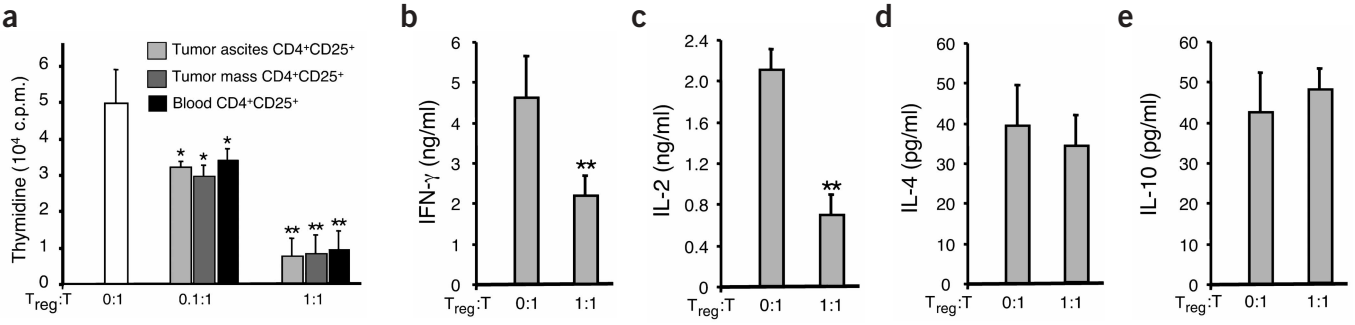

Figure 3 Tumor T_{reg} cells suppress T cell activation *in vitro*. Tumor ascites $CD3^+CD25^-$ T cells were stimulated with soluble antibody to CD3 plus monoclonal antibody to CD28 for 6 d. $CD4^+CD25^+$ T cells from tumor ascites, the tumor mass or blood were added to the culture at the indicated ratio of regulatory (T_{reg}) to responder (T) cells. (**a**) Tumor ascites and tumor mass $CD4^+CD25^+$ T cells are as efficient as blood $CD4^+CD25^+$ T cells at inhibiting T cell proliferation. White bar, no T_{reg}.(**b–e**) Tumor ascites $CD4^+CD25^+$ T cells inhibit T cell production of IFN-γ (**b**) and IL-2 (**c**), but have no effect on IL-4 (**d**) or IL-10 (**e**) production ($n = 5$, $^*P < 0.05$, $^{**}P < 0.01$).

lymph nodes by multicolor confocal microscopic analysis. Consistent with FACS data, we observed numerous $CD4^+CD25^+CD3^+$ cells in control lymph nodes ($n = 7$; **Fig. 2c,e**), but significantly fewer in tumor-draining lymph nodes ($n = 36$; $P < 0.001$; **Fig. 2d,e**), suggestive of limited movement of $CD4^+CD25^+CD3^+$ cells into tumor-draining lymph nodes, which further declined in later tumor stages. By contrast, the frequency of $CD4^+CD25^+$ T cells increased in ascites and the solid tumor mass in later tumor stages (**Fig. 1**).

These data suggest that, in contrast to homeostatic $CD4^+CD25^+$ T cells, which preferentially home to lymphoid tissue, $CD4^+CD25^+$ T cells in individuals with cancer are preferentially recruited to the tumor mass and associated ascites, but not to locally draining lymph nodes.

Tumor $CD4^+CD25^+$ T cells suppress T cell activation

To determine whether the $CD4^+CD25^+$ T cells in tumor ascites and tumor tissue were functional T_{reg} cells, we used an *in vitro* cellular culture system[23,29]. When activated with soluble monoclonal antibody to CD3 plus monoclonal antibody to CD28, tumor ascites $CD3^+CD25^-$ T cells responded with robust proliferation (**Fig. 3a**). Tumor $CD4^+CD25^+$ T cells from malignant ascites and the tumor mass significantly inhibited this T cell proliferation in a dose-dependent manner ($n = 5$, $P < 0.05$; **Fig. 3a**).

We further observed that tumor ascites $CD4^+CD25^+$ T cells suppressed the production of interferon-γ (IFN-γ; **Fig. 3b**) and interleukin-2 (IL-2; **Fig. 3c**) by T cells. We observed little production of IL-4 (**Fig. 3d**) or IL-10 (**Fig. 3e**) by T cells in culture, and tumor ascites $CD4^+CD25^+$ T cells had no significant effect on either (**Fig. 3d,e**). Tumor-associated $CD4^+CD25^+$ T cells (from the tumor mass or ascites) and peripheral circulating $CD4^+CD25^+$ T cells comparably affected T cell suppression (**Fig. 3a**). Tumor-associated and circulating $CD4^+CD25^+$ T cells themselves proliferated poorly after activation with monoclonal antibody to CD3 plus monoclonal antibody to CD28 (<2,000 counts per min). Thus, tumor-associated $CD4^+CD25^+$ T cells (from malignant ascites or the tumor mass) are functional T_{reg} cells. Hereafter we refer to them as 'tumor T_{reg} cells.'

CCL22-mediated trafficking of T_{reg} cells *in vitro*

We next studied mechanisms by which T_{reg} cells might migrate into tumors. Malignant ascites induced significant migration (**Fig. 4a**) and transmigration (**Fig. 4b**) of T_{reg} cells *in vitro* ($P < 0.01$ for each). Ovarian cancers produce CXCL12, which mediates the trafficking of plasmacytoid dendritic cells to tumors[30]; however, a neutralizing monoclonal antibody to human CXCR4, which blocks CXCL12-mediated chemotaxis[30], did not block ascites-induced T_{reg} cell migration (**Fig. 4a**). A saturated concentration of monoclonal antibody-to–human CCL22, but not monoclonal antibody to CCL17, significantly blocked ascites-induced T_{reg} cell migration and transmigration (**Fig. 4a,b**). In support of this, recombinant CCL22 induced significant T_{reg} cell migration ($P < 0.01$ versus control), which was efficiently blocked by a saturated concentration of antibody to CCL22 (**Fig. 4a,b**).

All tumor T_{reg} cells expressed CCR4 (mean fluorescence intensity 116 ± 21, $n = 8$; **Fig. 4c**), a receptor for CCL22 and CCL17. Although tumor-associated T_{reg} cells expressed CCR7 (**Fig. 1f**), recombinant CCL19 and CCL21, which are ligands of CCR7, were significantly less efficient than CCL22 in mediating the migration of tumor-associated T_{reg} cells ($P < 0.001$ versus CCL22 or ascites; **Fig. 4a**). Thus, CCL22 mediates T_{reg} cells trafficking *in vitro* and may recruit T_{reg} cells to the tumor.

Tumor cells and environmental macrophages produce CCL22

Consistent with the *in vitro* migration data (**Fig. 4a,b**), we detected large amounts of CCL22 but not CCL17 in tumor ascites ($n = 18$) by specific enzyme-linked immunosorbent assay (ELISA; **Fig. 4d**). Tumor tissue ($n = 16$) and ascites cells ($n = 8$), but not peripheral blood mononuclear cells (PBMCs; $n = 8$) or normal ovaries ($n = 5$), strongly expressed *CCL22* mRNA ($P < 0.001$ for all versus PBMCs or normal ovaries; **Fig. 4e**). Primary tumor ascites macrophages ($n = 8$) were uniformly positive for intracellular CCL22 (**Fig. 4f**) and secreted CCL22 into culture supernatants (275 ± 121 pg/ml, $n = 6$). Thus, ovarian tumors and microenvironmental macrophages are major sources of CCL22 and may induce T_{reg} cells trafficking to tumors.

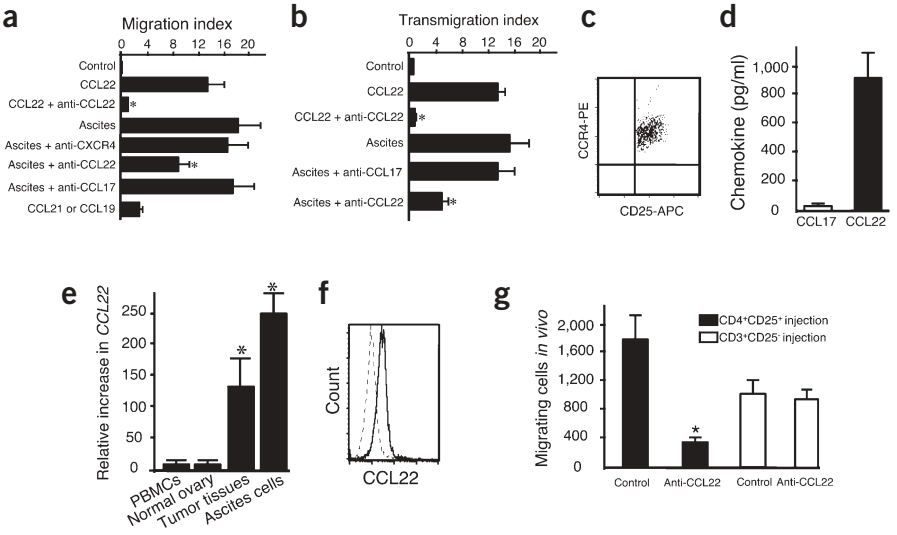

Figure 4 A CCL22-CCR4 signal mediates $CD4^+CD25^+$ T_{reg} cells migration. (**a**) $CD4^+CD25^+$ T_{reg} cells migrate in response to malignant ascites or recombinant CCL22. A specific antibody to CCL22 significantly inhibits $CD4^+CD25^+$ T_{reg} cell migration. Results are the mean ± s.d. (**b**) Similar results are observed for $CD4^+CD25^+$ T_{reg} cell adhesion or transmigration ($n = 4$ independent experiments; $^*P < 0.001$ versus control in **a** and **b**). (**c**) Tumor $CD4^+CD25^+$ T cells express high levels of CCR4. FACS analysis was gated on $CD3^+CD4^+CD25^+$ malignant ascites cells ($n = 8$). (**d**) CCL22 is detected in malignant ascites by ELISA ($n = 18$). (**e**) Real-time RT-PCR shows significant *CCL22* expression in tumor tissues and tumor ascites cells ($^*P < 0.001$ versus PBMCs or normal ovary). (**f**) Primary tumor ascites macrophages express intracellular CCL22 protein. Dotted line, isotype staining; solid line, CCL22 staining (one representative analysis is shown; $n = 8$). (**g**) CCL22-CCR4 signals mediate trafficking of $CD4^+CD25^+$ T_{reg} cells to the tumor *in vivo*. Human T cells and monoclonal antibody to CCL22 were injected into mice and tumors were extracted after 48 h to detect human T cell trafficking by FACS. Monoclonal antibody to CCL22 significantly inhibits trafficking of $CD4^+CD25^+$ T_{reg} cells but not $CD3^+CD25^-$ T cells to tumors ($n = 7$–10 per group, $^*P < 0.01$ versus control). Data show the total recovery of T_{reg} cells in 10^8 cells analyzed.

CCL22 induces migration of T_{reg} cells into tumor *in vivo*

We reconstituted human nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice with primary human ovarian tumors as described[5], and adoptively transferred human T_{reg} cells into these tumor-bearing mice. We observed numerous xenotransplanted human T_{reg} cells migrating into tumor tissues (**Fig. 4g**). *In vivo* treatment with monoclonal antibody to CCL22, but not monoclonal antibody to CCL17, significantly ($P < 0.01$, $n = 7$–10 per group) decreased T_{reg} cell migration into tumors, indicating that CCL22 has a role in tumor T_{reg} cell migration. Identical treatment did not inhibit the migration of $CD3^+CD25^-$ T cells into tumors (**Fig. 4g**). Therefore, CCL22 signal mediate the trafficking of T_{reg} cells to tumors *in vivo*.

Tumor T_{reg} cells suppress Her2-specific immunity

To determine the pathological importance of tumor T_{reg} cells, we tested their TAA-specific suppressive functions *in vitro* and *in vivo*. We first used an *in vitro* system[5,30]. We obtained myeloid dendritic cells (MDCs) from HLA $A2^+$ individuals with tumors expressing Her2 (also known as Neu or ErbB2) and loaded them with Her2 peptides[31] to produce TAA-MDCs, which induced specific T cell activation as expected (**Fig. 5**). This induction of T cell proliferation was inhibited by autologous tumor ascites T_{reg} cells ($n = 5$, $P < 0.05$; **Fig. 5a**).

Consistent with reports that strong T cell activation overcomes T_{reg}-mediated suppression[29,32], suppression was significantly more efficient when responder T cells were stimulated with fewer MDCs (**Fig. 5a**). Tumor T_{reg} cells also blocked the production of IFN-γ (**Fig. 5b,c**) and IL-2 (**Fig. 5b,d**) by effector T cells and Her2-specific cytotoxicity (**Fig. 5e**; $P < 0.05$ for all). We observed similar and limited production of IL-4 and IL-10 with or without tumor T_{reg} cells. TAA-MDCs stimulated poor proliferation of tumor T_{reg} cells (<2,000 counts per min). MDCs without peptides stimulated poor T cell proliferation (<2,000 counts per min) and cytotoxicity (<10%).

To test the function of tumor T_{reg} cells function *in vivo*, we stimulated tumor-associated T cells with autologous TAA-MDCs in

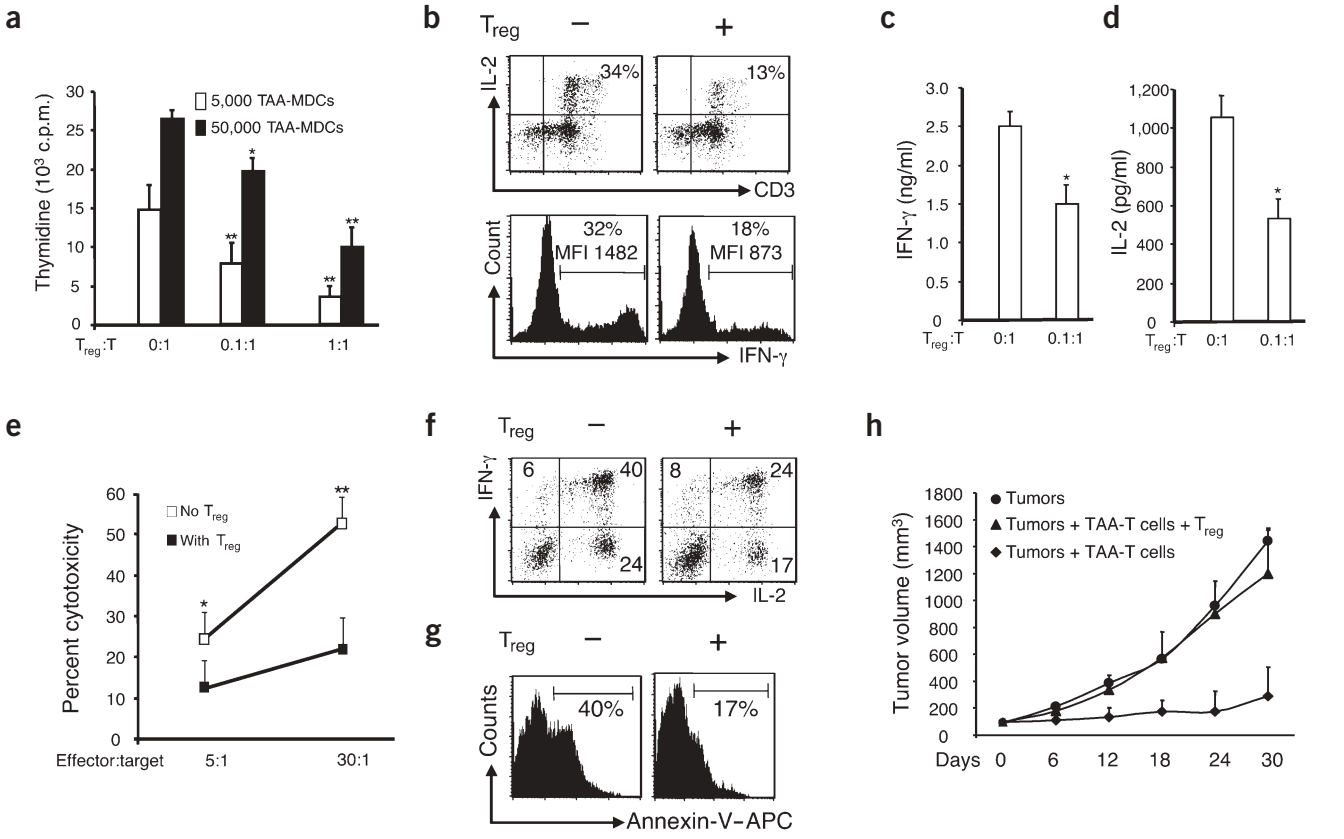

Figure 5 Tumor T_{reg} cells inhibit TAA-specific T cell immunity. (**a**) Tumor ascites T_{reg} cells inhibit TAA-specific T cell proliferation *in vitro*. Tumor ascites $CD3^+CD25^-$ T cells were stimulated with Her2 peptide–loaded MDCs (TAA-MDCs) for 6 d. Tumor T cell proliferation was detected by [^{3}H]thymidine incorporation. Dendritic cells alone did not stimulate significant T cell proliferation ($n = 5$, $^*P < 0.05$, $^{**}P < 0.01$). (**b**,**c**) Tumor T_{reg} cells inhibit TAA-specific T cell production of IL-2 and IFN-γ *in vitro*. Tumor T cells were stimulated with TAA-MDC in the presence of autologous tumor T_{reg} cells. T cell cytokines were detected by intracellular staining (**b**) and ELISA (**c**,**d**) on day 6. (**e**) Tumor T_{reg} cells inhibit Her2-specific T cell cytotoxicity *in vitro*. MDC-activated Her2-specific T cells were used as effector cells and Her2 peptide–loaded T2 cells were used as target cells. Her2-specific cytotoxicity was determined by FACS. Four HLA-$A2^+$ individuals were studied ($^*P < 0.05$, $^{**}P < 0.01$). (**f**,**g**) Tumor T_{reg} cells suppress TAA-specific T cell immunity *in vivo*. Tumor ascites $CD3^+$ T cells plus TAA-MDCs were transferred into NOD/SCID mice with or without autologous tumor T_{reg} cells. Three days after T cell transfer, either the peritoneal cells were collected and TAA-specific T cell IL-2 and IFN-γ production was determined by FACS gated on human $CD3^+$ T cells (**f**), or HLA-$A2^+$ $Her2^+$ T2 cells were additionally injected into mice and T2 cells were collected from the peritoneal cavity after 40 h and analyzed for *in vivo* cytotoxic T cell activity by FACS (**g**). A representative experiment is shown ($n = 4$, $^*P < 0.05$, $^{**}P < 0.01$). The $T_{reg}/CD3^+$ T cell ratio was 0.1/1 in **b**–**g**. $^*P < 0.05$ versus no T_{reg} cells in **f** and **g**. (**h**) Mice were injected with human primary ovarian tumors and treated with autologous tumor-specific T cells (group 2) or tumor-specific T cells plus tumor ascites T_{reg} cells (group 3). Controls received no additional injection (group 1). Tumor volumes (mean ± s.d.) were measured ($n = 5$–7 mice per group). The day of T_{reg} cell injection was taken as day 0. At all time points after T cell injection, $P < 0.05$ for group 3 or group 1 versus group 2.

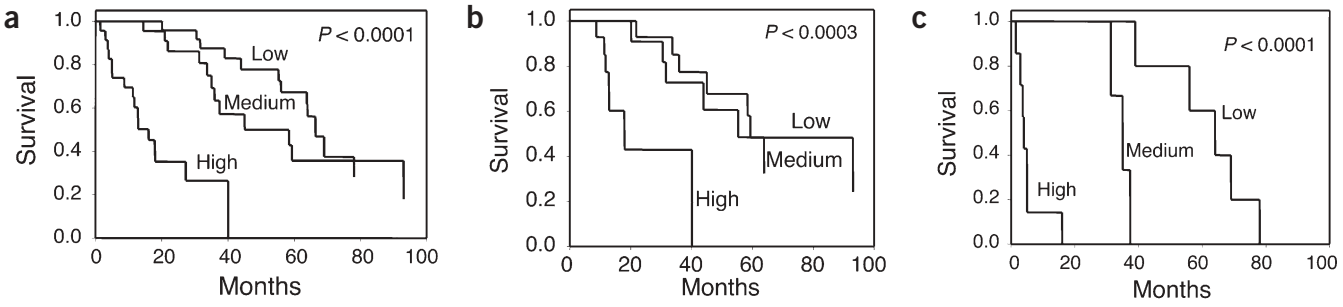

Figure 6 Accumulation of tumor T_{reg} cells predicts poor survival in individuals with ovarian carcinoma. A Kaplan-Meier curve for overall survival by number of tumor-infiltrating T_{reg} cells in 70 individuals with stages I–IV (**a**), stage III only (**b**) or stage IV only tumors (**c**). Samples were divided into three groups on the basis of the content of tumor-infiltrating T_{reg} cells. Survival significantly decreased as a function of T_{reg} cell content.

NOD/SCID mice using our xenotransplant model[5]. As expected and in support of the *in vitro* results, MDCs alone did not induce significant T cell activation (<5% IL-2$^+$ or IFN-γ^+ T cells), whereas TAA-MDCs induced potent TAA-specific T cell production of IL-2 and IFN-γ ($P < 0.001$; **Fig. 5f**), and cytotoxic T lymphocyte (CTL) activity *in vivo* ($P < 0.05$; **Fig. 5g**). We observed similar results when we used autologous primary ovarian tumor cells rather than Her2-pulsed T2 cells as the target cells. These TAA-specific immune responses were significantly inhibited *in vivo* by the adoptive transfer of autologous T_{reg} cells from tumor ascites (**Fig. 5f,g**).

Because tumor T_{reg} cells blocked tumor-specific T cell immunity *in vitro* and *in vivo* (**Fig. 5a–g**), we tested the hypothesis that tumor T_{reg} cells would disable TAA-specific T cell immunity *in vivo* and in turn allow tumor growth. Consistent with our previous observations[5], mice without tumor-specific T cell transfusion showed progressive tumor growth ($n = 7$), and mice with tumor-specific T cell transfusion showed significantly reduced tumor volume ($n = 7$). Adoptively transferring tumor T_{reg} cells alone had no effects on tumor growth ($n = 5$; data not shown). Mice receiving tumor-specific T cells plus tumor T_{reg} cells showed progressive tumor growth ($n = 6$), comparable to controls without tumor-specific T cell transfer (**Fig. 5h**), indicating that tumor T_{reg} cells blocked the protective effects mediated by tumor-specific T cells. Taken together, these data indicate that tumor T_{reg} cells may hamper tumor-specific effector T cell immunity in individuals with cancer.

Increases in tumor T_{reg} cells predict poor survival

We predicted that tumor T_{reg} cells would adversely affect survival. To test this prediction, we analyzed all relevant clinical and pathological information, including tumor-associated survival (**Supplementary Tables 1** and **2** online), that was available on 70 of our 104 individuals with cancer and correlated the data with the number of T_{reg} cells in tumor tissues determined by confocal microscopy. There was a significant correlation between tumor T_{reg} cell content and survival in the group as a whole ($n = 70$, $P < 0.0001$ for all), and also for individuals in stage II ($P = 0.0362$), stage III ($P = 0.0003$) and stage IV ($P = 0.0001$; **Supplementary Table 2** online). Tumor T_{reg} cells were a significant predictor of death hazard ($P < 0.0001$), even after controlling for stage, surgical debulking and other factors known to affect survival, by using a Cox proportional hazards model.

In an alternative analysis, individuals were divided into three equal groups on the basis of numbers of tumor T_{reg} cells. The low T_{reg} cell group included all those with a tumor T_{reg} cell count of 131 or less per ten high-power field (HPF; $n = 24$), the high T_{reg} cells group included those with a tumor T_{reg} cell count of 346 or more per ten HPF ($n = 23$), and the medium tumor T_{reg} cell group included the remainder ($n = 23$). Survival functions were significantly different for the three groups ($P < 0.0001$; **Fig. 6a**).

T_{reg} cells were a significant predictor of death even after controlling for stage of disease and surgical debulking. Individuals in the highest T_{reg} cell group experienced a 25.1-fold higher death hazard as compared with those in the lowest T_{reg} cell group (95% confidence interval, 6.8–92.1). Individuals in the medium T_{reg} cell group experienced a 5.8-fold higher death hazard as compared with those in the lowest group (95% confidence interval, 1.9–17.5). Individuals in the highest T_{reg} cell group experienced a 4.2-fold reduction in survival, and those in the medium group experienced a 2.8-fold reduction in survival, as compared with those in the lowest T_{reg} cell group ($P < 0.0001$; **Fig. 6a**).

The Cox proportional hazards model accounts for tumor stage, which is a known survival factor in ovarian cancer[33]. Nonetheless, we further stratified stage III and IV individuals into three subgroups of low (≤131), medium (132–345) and high (≥346) tumor T_{reg} cells numbers. Survival functions were still significantly different for the three groups in stage III ($P < 0.0003$; **Fig. 6b**) and stage IV ($P < 0.0001$; **Fig. 6c**). Therefore, an increase in the number of tumor T_{reg} cells is a significant predictor of increased risk for death and for reduced survival in ovarian cancer. This relationship holds whether T_{reg} cells are treated as a continuous or categorical variable (data not shown), and even after adjusting for disease stage and other significant clinical effects.

DISCUSSION

Emerging evidence suggests that T_{reg} cells, particularly CD4$^+$CD25$^+$ T_{reg} cells, are key mediators of peripheral tolerance[15,16,28,34–36]. Engendering strong antitumor immunity may thus involve breaking T_{reg}-mediated peripheral tolerance to TAAs. Consistent with this concept, experimental depletion of T_{reg} cells in mice with tumors improves immune-mediated tumor clearance[37] and enhances the response to immune-based therapy[38].

Humans with cancer have increased numbers of peripherally circulating and tumor T_{reg} cells[22–24]. Previous studies have confirmed that these T_{reg} cells are functional through their inhibition of nonspecific T cell activation *in vitro*. Although this indirect evidence implicates T_{reg} cells in the immunopathogenesis of cancer, a definitive demonstration in humans is lacking. Our data show that T_{reg} cells in human ovarian cancers express intracellular CTLA-4, GITR and FOXP3, inhibit TAA-specific immunity *in vitro* and *in vivo*, and contribute to tumor growth. These data therefore provide direct *in vitro* and *in vivo* evidence that human T_{reg} cells have an important immunopathological role in human cancer by suppressing endogenous TAA-specific T cell immunity. We further linked this immunopathological role to clinical outcomes, by demonstrating that there is an inverse correlation between tumor T_{reg} cell content and patient survival.

Under homeostatic conditions, mouse and human T_{reg} cells are found primarily in lymphoid organs[14–17], a finding that our work confirms. On the basis of these observations, others have postulated that T_{reg} cells mediate their suppressive effects by inhibiting T cell priming, which occurs in lymph nodes[14,16,28]. Nonetheless, mouse T_{reg} cells also block antigen-specific T cell effector functions occurring outside lymph nodes[39,40]. In our studies of human ovarian carcinomas, there were significantly fewer T_{reg} cells in tumor-draining lymph nodes than in control lymph nodes and tonsils. T_{reg} cell numbers were also significantly lower in tumor-draining lymph nodes in late stage (III and IV) as compared with early stage (I and II) ovarian cancers. By contrast, there was a significant trend towards a higher accumulation of T_{reg} cells in ascites and the solid tumor mass in later tumor stages.

Thus, T_{reg} cells seem to migrate preferentially and predominantly to the tumor mass and the associated malignant ascites. This lack of apparent migration to lymph nodes suggests that tumor T_{reg} cells may primarily work by inhibiting extranodal effector cell function rather than by suppressing naive T cell priming in lymph nodes, at least in later stages of tumors. This supposition is supported by our demonstration that tumor T_{reg} cells inhibit effector T cell function *in vivo*, and this inhibition is associated with progressive tumor growth in the face of existing TAA-specific immunity.

Homeostatic and tumor T_{reg} cells expressed similar amounts of the lymphoid homing molecules CCR7 and CD62L. It is unknown whether these molecules are involved in homeostatic T_{reg} cell lymphoid homing. It is unclear why tumor T_{reg} cells are not more abundant in draining lymph nodes. We favor the concept that T_{reg} cell trafficking to tumor differs from the T_{reg} cell trafficking to lymph nodes as previously shown for tumor plasmacytoid dendritic cells[30]. Consistent with this concept, normal blood T_{reg} cells express CCR4 and CCR8, and migrate in response to CCL1, CCL17 or CCL22 on the basis of *in vitro* assays[41]. We have shown here, however, that tumor T_{reg} cells express functional CCR4, the receptor for CCL22, and can migrate to CCL22 present in the tumor microenvironment.

Evidence suggests that CCL22 preferentially attracts activated antigen-specific T cells to dendritic cells[42,43]. Tumors may have capitalized on this effect to attract T_{reg} cells to the microenvironment. Our data suggest that the source of this CCL22 is the ovarian tumor and associated macrophages. Functional tumor microenvironmental CCL22 has not been previously reported. Our studies show that blocking CCL22 *in vivo* reduces human T_{reg} cell tumor trafficking. The data suggest that, in addition to depleting T_{reg} cells, blocking T_{reg} cell tumor trafficking represents a potential strategy for treating human cancers.

The accumulation of T_{reg} cells in tumor predicts a marked reduction in patient survival, providing the 'smoking gun' that links T_{reg} cells and the immunopathogenesis of human cancer. Taken together, these data provide the basis for developing novel immune-boosting strategies based on ridding the cancer patient of this cell population.

METHODS

Human samples. We studied previously untreated individuals with epithelial ovarian carcinomas, classified as stage I to IV according to the International Federation of Gynecology and Obstetrics. Individuals gave written, informed consent. Specimens were collected at the Tulane University, New Orleans, Louisiana, USA, and the University of Turin, Turin, Italy. We obtained normal human tissues from the Cooperative Human Tissue Network. Histopathological findings were independently confirmed at the Tulane University, New Orleans, Louisiana, USA[5] and the University of Pennsylvania, Philadelphia, USA[44]. The study was approved by local institutional review boards.

Cells and tissue biopsies. Cells and tissues were obtained from ascites, blood, lymph nodes and tumors as described[5,30,44]. $CD4^+$ T cells were purified with Untouched kits (Miltenyi). $CD25^+$ cells were purified with paramagnetic beads (Miltenyi) and sorted with phycoerythrin (PE)-conjugated antibody to CD25 (PharMingen)[21]. Cell populations were assessed to be more than 90% pure by FACS.

FACS and PCR. Details of FACS and PCR[44–46] are given in **Supplementary Methods** online.

$CD4^+CD25^+$ T cells and $FOXP3^+$ T cells in human tissues. Detailed information is given in **Supplementary Methods** online.

***In vitro* non-antigen-specific immunosuppression.** Tumor ascites $CD3^+CD25^-$ T cells (10^5 per ml) were stimulated with soluble monoclonal antibody to human CD3 (5 μg/ml, UCHT, IgG1; PharMingen) and monoclonal antibody to human CD28 (2.5 μg/ml, cd28.2, IgG1; PharMingen) for 6 d. The added autologous $CD4^+CD25^+$ T cell/$CD3^+CD25^-$ T cell ratio was 0/1, 0.1/1 or 1/1. On day 6, T cell proliferation and cytokines were detected as described[5,30].

Migration and adhesion or transmigration. Migration and adhesion or transmigration were assessed as described[30] using $5–20 \times 10^4$ $CD4^+CD25^+$ T_{reg} cells. Human chemokines (CXCL12, CCL17, CCL19, CCL21 and CCL22, 100 ng/ml of each; all from R&D Systems), or cell-free tumor ascites were added to the lower chamber. Antibodies to CCL17 (540.26, IgG1, 500 ng/ml), CCL22 (57226.11, IgG2b, 500 ng/ml) and CXCR4 (44717, IgG2b, 500 ng/ml) were from R&D Systems. We confirmed the identity of migrating T_{reg} cells by FACS analysis for CD4 and CD25.

CCL22 protein. CCL22 protein was detected by ELISA (R&D Systems). Intracellular human CCL22 was detected by FACS with monoclonal antibody to CCL22 (57226.11, IgG2b, R&D Systems), counterstained with fluorescein isothiocyanate (FITC)-conjugated goat antibody to mouse IgG (PharMingen).

***In vivo* migration.** Human primary ovarian epithelial carcinomas were established in female NOD.CB17/SCID[5] mice aged 6–8 weeks (Jackson Laboratories). On day 12 after tumor inoculation, human $CD4^+CD25^+$ T_{reg} cells or $CD3^+CD25^-$ T cells (2.5×10^6) were injected in a 100-μl volume into tail veins. Some mice received intraperitoneal mouse antibody to human CCL22 (500 ng per 200 μl, 57226.11, IgG2b: R&D Systems), or control IgG (PharMingen), 48 h before and 24 h after T cell injection. After 48 h, some mice were killed and the extracted tumors were mechanically disrupted. Human $CD3^+$ T cells were identified by FACS with antibody to human CD3 and are expressed as human $CD3^+$ T cells per 10^8 tumor cells.

***In vitro* TAA-specific immunosuppression.** Tumor MDCs were differentiated from tumor macrophages as described[47]. Dendritic cells were loaded with 5 μg/ml of three distinct HLA-A2-binding Her2 peptides[31], p369-384 (KIFGSLAFLPESFDGDPA), p688-703 (RRLLQETELVEPLTPS) and p971-984 (ELVSEFSRMARDPQ), or with an HLA-A2 influenza virus matrix control peptide GILGFVFTL (Multiple Peptide System). Peptide-loaded MDCs (TAA-MDCs, $5–50 \times 10^3$ per ml) were used to activate autologous tumor $CD3^+CD25^-$ cells (10^5 per ml). The added T_{reg}/$CD3^+CD25^-$ T ratio was 0/1, 0.1/1 or 1/1. On day 6, we detected TAA-specific T cell proliferation and cytokines as described[5,30]. T2 cells ($HLA\text{-}A2^+$ lymphoblastoid cell lines; 5×10^6 per ml, American Type Culture Collection) were labeled with 10 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 10 min in the dark at 37 °C. Cytotoxicity was assessed by using CFSE-labeled T2 cells bearing all three Her2 peptides or the matrix control with annexin V and 7-AAD staining[48].

***In vivo* TAA-specific T cell immunosuppression.** We used cells from ascites of $HLA\text{-}A2^+$ individuals affected with $Her2^+$ ovarian cancer. TAA-MDCs (10^6 per mouse) and autologous tumor ascites $CD3^+CD25^-$ T cells (10^7 per mouse) were injected into NOD/SCID mice with or without autologous tumor T_{reg} cells (10^6 per mouse). After 3 d, we collected the peritoneal cells and assayed them for intracellular IL-2 and IFN-γ gated on human $CD3^+$ T cells as described[5,30]. In some experiments, 3 d after the first injection the mice were injected intraperitoneally with $Her2^+$, $HLA\text{-}A2^+$, CFSE-labeled T2 cells or autologous primary ovarian

tumor cells (10^8 per mouse). Forty hours after this second injection, we collected the T2 cells and stained them with annexin V and 7-AAD to determine cytotoxicity[48]. All studies were approved by the Tulane Animal Use Committee.

***In vivo* tumor regression assay.** Primary ovarian tumor cells (1×10^7) in 200 μl of buffered saline were injected into dorsal subcutaneous tissues[5]. Autologous tumor ascites $CD4^+CD25^+CD3^+$ cells (2.5×10^6) were injected intravenously into mice in 100 μl of buffered saline on day 12 after human tumor inoculation. In some cases, 12 h after injecting tumor ascites $CD4^+CD25^+CD3^+$ cells, tumor-specific T cells (5×10^6) were injected intravenously into mice. Tumor size was measured twice weekly using calipers fitted with a Vernier scale. Tumor volume was calculated on the basis of three perpendicular measurements[5].

Statistical analysis. Details of all statistical analyses are given in **Supplementary Methods** online.

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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