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Natural Regulatory T Cells and De Novo-Induced Regulatory T Cells Contribute Independently to Tumor-Specific Tolerance[1]

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Thymus-derived, naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs) and Tregs induced in the periphery (iTregs) have both been implicated in regulating immune responses. However, the relationship between these populations in the same host, and their relative contribution to the overall Treg pool, has not been examined. Using a tumor-induced T cell tolerance model, we find that expansion of nTregs and de novo generation of iTregs both contribute to tumor-specific T cell tolerance. In this system in which the number of tumor-specific nTregs can be controlled, the efficiency of nTreg expansion significantly exceeds that of the induction of Tregs from uncommitted progenitors in the tumor-bearing host. However, pre-existing nTregs are neither required for the induction of Tregs nor measurably impact on the extent of their accumulation. Instead, induction of Ag-specific regulatory cells from naive cells is intrinsically influenced by the tumor microenvironment and the presence of tumor Ag. ***The Journal of Immunology,*** **2007, 178: 2155–2162.**

Accumulating evidence in recent years has identified active suppression mediated by regulatory T cells (Tregs)[3] to be one of the major obstacles to effective antitumor immunotherapy (1, 2). The prevalence of $CD4^+CD25^+$ T cells with regulatory activities is significantly increased in the peripheral blood and malignant effusions of patients with multiple types of cancers (2). In some cases, these $CD4^+$ Tregs have been shown to be specifically recruited to the tumor sites and effectively block antitumor CTL responses (3). It has been shown in animal models that targeted removal or inactivation of the $CD4^+CD25^+$ Tregs can lead to improved tumor immunosurveillance, better vaccine efficacy, and enhanced antitumor immunity (4–6).

The origin, Ag specificity and effector mechanism vary greatly among different subtypes of regulatory cells (7, 8). Naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs (nTregs) are shown to arise as a committed lineage in the thymus (9, 10), and bear a diverse TCR repertoire against a broad range of self Ags (11). Because many tumor-associated Ags are aberrantly expressed self-Ags (12), it is likely that nTregs play a role in suppressing antitumor immunity. Additionally, Tregs can be induced under particular conditions of antigenic stimulation both in vitro and in vivo, and are collectively termed induced Tregs (iTregs) (7, 13). Of particular interest, recent studies demonstrate the development of Ag-specific, inducible Tregs as the consequence of Ag recognition in vivo, with the Ags in the form of self-Ag (14, 15), tumor-associated Ags (12, 16), or Ags exogenously delivered through various routes (17–19). In cancer patients and animal tumor models, it is conceivable that the regulatory network includes both nTregs and iTregs. However, the relationship between these two populations and their relative contribution to peripheral T cell tolerance has not been examined (8, 20). From the perspective of cancer immunotherapy, a clear delineation of these issues will be informative for designing strategies to disrupt the regulatory network. Using a model in which Ag-specific T cell tolerance arises during tumor progression, we report that induction of tumor Ag-specific Tregs requires antigenic encounter in a tumor microenvironment. We show for the first time that the expansion of nTregs and the de novo induction of Tregs are two independent mechanisms contributing to tumor-specific T cell tolerance. In our adoptive transfer system, in which the percentage of Ag-specific nTregs can be controlled at the time of transfer, expansion of pre-existing nTregs following therapeutic vaccination predominantly contributes to the overall Treg pool in a tumor-bearing host. However, the extent to which naive, uncommitted tumor-specific $CD4^+$ T cells acquire regulatory function is not measurably influenced by the presence of nTregs specific for the same Ag.

Materials and Methods

Mice

BALB/c ($Thy1.2^{+/+}$) mice 5- to 7-wk-old were purchased from Harlan. TCR transgenic (Tg) mice (6.5 Tg mice) on a BALB/c background expressing an $\alpha\beta$ TCR specific for aa 110–120 from hemagglutinin (HA) were a gift from Dr. H. von Boehmer (Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA). The 6.5 Tg mice on $Thy1.1^{+/+}$, $Thy1.1^+/1.2^+$, or $Rag2^{-/-}$ background were used in experiments as specified. BALB/c $Rag2^{-/-}$ mice were purchased from The Jackson Laboratory. Experiments using mice were conducted in accordance with protocols approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Abs and flow cytometry

Abs for flow cytometry were anti-CD4 (allophycocyanin and PerCP), Thy1.1 (PerCP and PE), Thy1.2-allophycocyanin, CD25 (allophycocyanin and PE), CTLA-4 PE, Foxp3 PE (eBioscience), and glucocorticoid-induced TNFR (GITR) PE (R&D Systems). All Abs were purchased from BD Biosciences unless otherwise specified. For intracellular staining of CTLA-4 and Foxp3, cells were permeablized before adding these two Abs.

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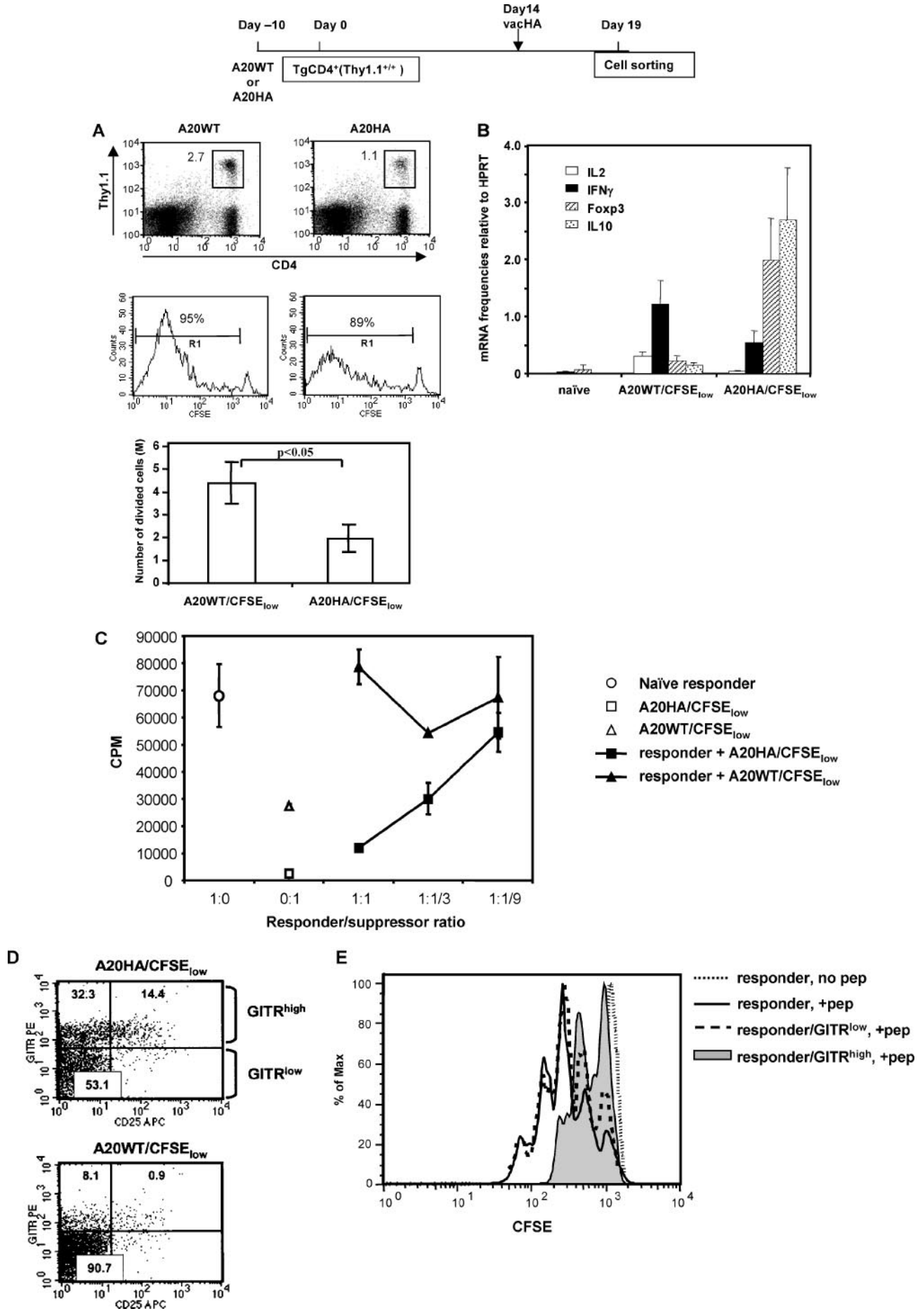
[3] Abbreviations used in this paper: Treg, regulatory T cell; nTreg, naturally occurring $CD4^+CD25^+Foxp3^+$ Treg; iTreg, induced Treg; GITR, glucocorticoid-induced TNFR; HA, hemagglutinin; Tg, transgenic; vacHA, vaccinia virus encoding HA; DC, dendritic cell.



The 30,000 gated events were collected on a FACSCalibur (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

Tumor cells and adoptive transfer

The generation and maintenance of A20HA B cell lymphoma cells was previously described (21). A total of 1×10^6 tumor cells were injected into each mouse via tail vein. For adoptive transfer using whole $CD4^+$ T cells, single cell suspensions obtained from lymph nodes and spleens of 6.5 Tg donor mice were enriched for $CD4^+$ cells using a $CD4^+$ isolation kit from Miltenyi Biotec. The purity of $CD4^+$ T cells was typically 92–96%. For experiments using $CD4^+CD25^-GITR^{low}$ or $CD4^+CD25^+GITR^{high}$ Tg cells, pre-enriched $CD4^+$ T cells were stained with CD25 and GITR and further fractionated by FACS sorting. The percentage of lymphocytes positive for CD4 and the clonotypic TCR (mAb 6.5) was determined by flow cytometry. A total of 2.5×10^6 $CD4^+$ 6.5 TCR^+ T cells were i.v. injected into each recipient. CFSE (Molecular Probes) labeling of purified $CD4^+$ T cells was previously described (16).

Cell sorting and quantitative real-time PCR

Pre-enriched $CD4^+$ cells were stained with specified mAbs, and sorted on FACSAria (BD Biosciences). The purity of the sorted cells was typically >98%. RNA was extracted using RNAeasy kit (Qiagen). Quantitative real-time PCR analysis was conducted as previously described (16).

In vitro proliferation

A total of 20,000 sorted or magnetically enriched $CD4^+$ T cells were incubated with 100,000 irradiated (3000 rad) BALB/c splenocytes in the presence of $HA_{110-120}$ peptide. For in vitro suppression assays, $CD4^+$ T cells from 6.5 $Rag2^{-/-}$ Tg mice were purified by Miltenyi Biotec beads and used as responder cells. Responder cells were incubated with indicated number of sorted cells and irradiated BALB/c splenocytes pulsed with HA peptide. At 72 h after incubation, cells were pulsed with [^{3}H]thymidine (1 μCi/well) and cultured for 12 h before harvesting and measuring scintillation counts. Alternatively, responder cells were labeled with 1 μM CFSE, and cultured either alone or together with sorted population in the presence of peptide-pulsed splenocytes. Cell division of the responder cells was monitored by the dilution of the intensity of CFSE.

In vivo priming with vaccinia virus encoding HA (vacHA)

A recombinant vacHA from the 1934 PR8 strain of influenza was previously described (22). On the days indicated, mice were primed by i.p. inoculation with 1×10^7 PFU of vacHA suspended in 0.1 ml of HBSS.

Statistical analysis

The significance of the results was determined using the Student's *t* test. A value for $p < 0.05$ was considered statistically significance.

Results

Tumor Ag is essential for the development of Ag-specific $CD4^+$ Tregs

It has been reported that a variety of tumor-derived factors, including IL-10, TGF-β, vascular endothelial growth factor, PGE_2, and soluble phosphatidylserine contribute to the formation of an extensive immunosuppressive microenvironment, which promotes T cell tolerance and tumor immune evasion (23). We recently reported that uncommitted, naive $CD4^+$ T cells can differentiate into Ag-specific regulatory cells in tumor-bearing hosts (16). However, it was not clear whether the tumor microenvironment alone was sufficient for the induction of tumor-specific Tregs. Using the same model system (Fig. 1, schema), we set out to examine the requirement for tumor Ag in the tumor microenvironment for the induction of Ag-specific Tregs and their amplification in response to therapeutic vaccination. To this end, mice with established, systemic wild-type A20 tumor (A20WT) or its transfectant expressing influenza HA (A20HA) as surrogate tumor Ag received HA-specific Tg $CD4^+$ T cells, and were subsequently immunized with vacHA. Both tumors progressed in mice with similar growth kinetics and organ distributions as reported previously (22). As shown in Fig. 1*A*, vaccination led to extensive cell division and accumulation of HA-specific $CD4^+$ T cells in mice bearing wild-type tumor. With a comparable level of cell division, however, the overall accumulation of HA-specific $CD4^+$ cells was significantly reduced in vaccinated A20HA-bearing mice. Quantitative analysis of gene expression revealed that the divided donor cells from A20HA-bearing hosts ($A20HA/CFSE^{low}$) had decreased levels of IL-2 and IFN-γ, but highly elevated Foxp3 and IL-10, compared with their counterparts from A20WT-bearing mice ($A20WT/CFSE^{low}$) (Fig. 1*B*). In line with their gene expression profiles, $A20HA/CFSE^{low}$ cells were hyporesponsive when restimulated in vitro, barely producing IL-2 or IFN-γ (data not shown), and were capable of suppressing the proliferation of HA-specific naive cells (Fig. 1*C*). In contrast, $A20WT/CFSE^{low}$ cells responded to peptide challenge (although less vigorously than naive cells), produced some IL-2 and a large amount of IFN-γ (data not shown) and were not suppressive to naive cells (Fig. 1*C*). These data indicate that the donor T cells in A20WT-bearing mice have been effectively primed against HA Ag and differentiated into Th1 effector cells after vaccination, even though there was overwhelming tumor growth. In contrast, HA-specific suppressor cells with elevated levels of Foxp3 and IL-10 message emerged in response to the therapeutic vaccination when nominal Ag was present in the tumor microenvironment.

To further dissect the composition of the population with suppressive activities, the divided cells were costained for expression of CD25 and GITR, two molecules shown to be associated with natural Treg functions. As shown in Fig. 1*D*, $A20HA/CFSE^{low}$ cells had a significantly higher percentage of $GITR^{high}$ cells than $A20WT/CFSE^{low}$ cells. Moreover, the $GITR^{high}$ subset, containing

FIGURE 1. Tumor Ag is essential for the development of tumor-specific $CD4^+$ Tregs. A total of 2.5×10^6 CFSE-labeled HA-specific $CD4^+Thy1.1^+$ T cells were adoptively transferred to BALB/c recipients (Thy1.2 background) inoculated with either 1×10^6 wild-type (A20WT) or HA-expressing (A20HA) tumor cells 10 days earlier. Two weeks after T cell transfer, mice were immunized with vacHA and analyzed 5 days later. *A*, The frequency, cell division status, and accumulation of the transferred $Thy1.1^+CD4^+$ cells in the spleen. The cell frequency was measured by FACS analysis. The number in dot plots represents percentage of the gated population in total spleen cells. CFSE profiles of the gated cells are shown as histograms. Percentage of the divided cells in the gated population is indicated in each histogram. The absolute number of divided cells per spleen is calculated as (total number of spleen cells $\times$ percentage of donor cells in spleen $\times$ percentage of divided cells in donor cells). Results are shown as mean $\pm$ SE of three mice per group. The data shown are representative of two independent experiments. *B*, Quantitative real-time PCR analysis of divided cells. The divided donor cells (>2 cell divisions) in A20WT-bearing mice ($A20WT/CFSE^{low}$) or A20HA-bearing mice ($A20HA/CFSE^{low}$) were FACS-sorted and subjected to analysis. mRNA frequency of the indicated genes is normalized to HPRT. Freshly sorted $CD4^+$ T cells from 6.5 Tg mice were used as naive control. Results are shown as mean $\pm$ SE of triplicate reactions. *C*, In vitro suppression assay. $Rag2^{-/-}$ 6.5 $CD4^+$ responder cells, sorted cells were either cultured alone or cocultured together as specified at the indicated ratio in the presence of 10 μg/ml HA peptide and irradiated BALB/c splenocytes. Proliferation of the culture in the absence of peptide was <1000 cpm. Results are shown as mean $\pm$ SE of triplicate cultures. *D*, Costaining of CD25 and GITR on the divided cells. The number in each plot represents the percentage of the corresponding subset in the divided population. The plots are representative of two separate experiments. *E*, $GITR^{high}$ subset of the divided cells from A20HA-bearing mice exclusively suppresses responder cells. The divided donor cells from vaccinated A20HA-bearing mice were further fractionated into $GITR^{high}$ and $GITR^{low}$ subsets. CFSE-labeled $Rag2^{-/-}$ 6.5 $CD4^+$ responder cells were cultured with irradiated BALB/c ($Thy1.1^{+/+}$ background) splenocytes either alone or with an equal number of GITR-fractionated cells ($Thy1.1^{+/+}$). Three days later, cells were stained with anti-Thy1.2 and anti-CD4 mAbs. Plots shown are gated on $Thy1.2^+$ responder cells. The data shown are representative of two separate experiments.

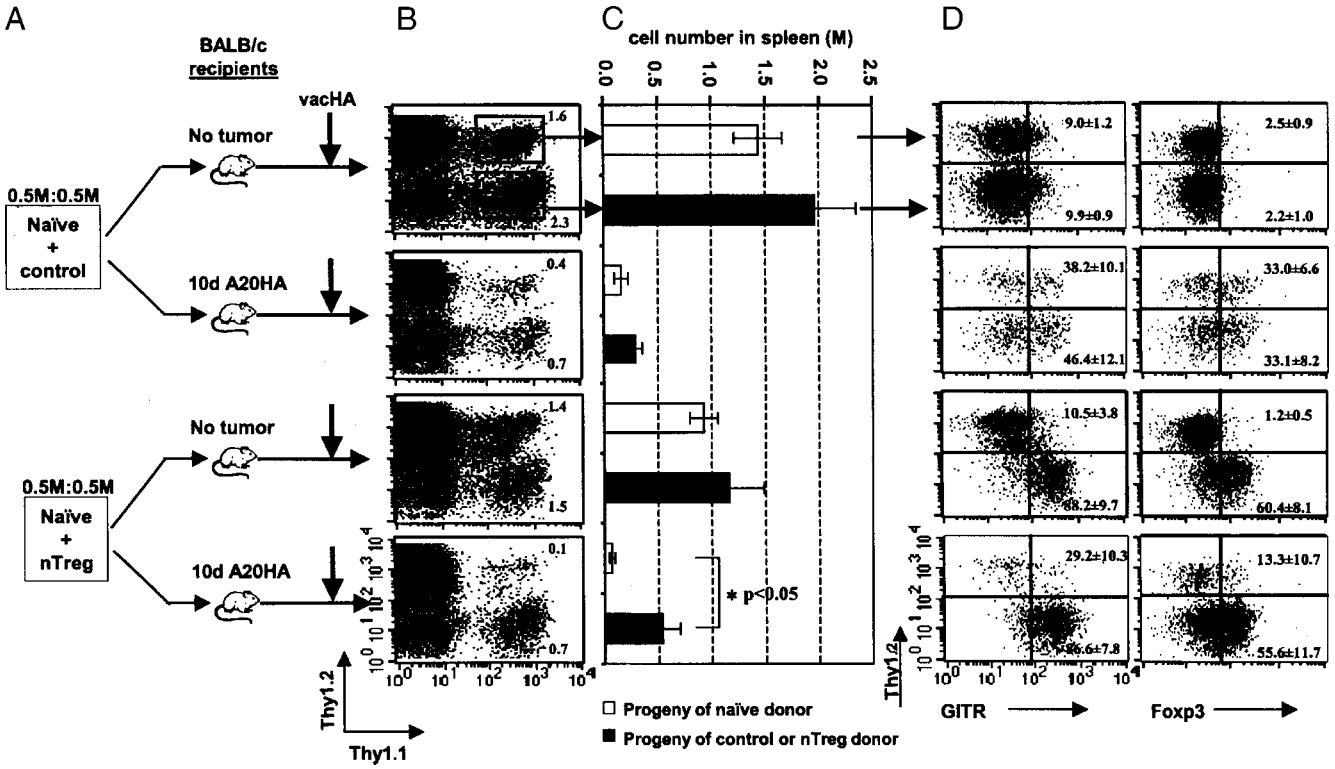


FIGURE 2. nTregs and de novo iTreg cells independently contribute to the Treg pool in periphery. *A*, A total of 0.5×10^6 sorted $CD4^+CD25^-GITR^{low}$ cells from 6.5 Tg mice ($Thy1.1^+/1.2^+$) were mixed with same number of control cells (sorted $CD4^+CD25^-GITR^{low}$ cells from $Thy1.1^+/1.2^-$ 6.5 Tg mice) or nTregs (sorted $CD4^+CD25^+GITR^{high}$ cells from $Thy1.1^+/1.2^-$ 6.5 Tg mice), and transferred into BALB/c recipients either tumor-free or bearing a 10-day A20HA tumor. The recipients received vacHA immunization 2 wk posttransfer T cell and were analyzed 5 days after vaccination. *B*, Splenocytes were stained with anti-Thy1.1 and anti-Thy1.2 mAbs. The number in each plot represents percentage of the gated population in total spleen cells. The data shown are representative of three mice per group. *C*, Absolute number of donor cells recovered from spleen are represented. The number of cells is calculated as (total splenocytes count × the percentage of $Thy1.1^+/1.2^+$) or (total splenocytes count × the percentage of $Thy1.1^+/1.2^-$). Results are shown as mean ± SE of three mice per group. *D*, GITR and Foxp3 staining on donor cells. Plots shown are gated on donor cells only. The numbers in each plot represent percentage of $GITR^{high}$ or $Foxp3^{high}$ cells in corresponding $Thy1.2^+$ or $Thy1.2^-$ donor cells, and are shown as mean ± SE of three mice per group. The arrows indicate the corresponding populations in analyses.

both $CD25^{high}$ and $CD25^{low}$ cells, was suppressive to naive cells (Fig. 1*E*), whereas the $GITR^{low}$ subset was nonsuppressive and able to proliferate and produce IFN-γ (data not shown). Therefore the in vitro hyporesponsiveness displayed by A20HA/$CFSE^{low}$ cells (Fig. 1*C*) was the result of dominant suppression of Th1 effector cells ($GITR^{low}$ subset) by the accompanying suppressor cells ($GITR^{high}$ subset).

Taken together, these results demonstrate that HA expression by tumor cells is essential for the development of HA-specific $CD4^+$ suppressor cells, and their amplification following therapeutic vaccination. The tumor environment per se (cellular components associated with tumor progression) is necessary but insufficient to generate iTregs from uncommitted precursors, nor significantly amplify pre-existing nTregs even when Ag is provided in the form of vaccination.

nTregs and de novo iTregs independently contribute to the Treg pool

One caveat of these experiments is that 5–10% of the donor HA-specific $CD4^+$ T cells are comprised of nTregs. We and others have previously shown that induction of Ag-specific Tregs can occur in the absence of pre-existing nTregs (15, 16). However, the influence of nTregs on the extent of iTreg conversion from uncommitted $CD4^+$ precursors, and the relative contribution of nTregs and iTregs to the total Ag-specific Treg population, were not examined.

To address these questions, highly purified $CD25^-GITR^{low}$ naive $CD4^+$ T cells were cotransferred with purified $CD25^+GITR^{high}$ $CD4^+$ nTregs, which were distinguishable by a congenic marker, or with similarly marked control cells that were also $CD25^-GITR^{low}$ naive $CD4^+$ T cells (Fig. 2*A*). Equal numbers of naive cells and control vs nTregs were mixed and injected into either tumor-free BALB/c recipients or mice with a 10-day A20HA tumor burden. A subset of the recipients was immunized with vacHA 2 wk after T cell transfer. Cell expansion in spleens was analyzed 5 days after vaccination (Fig. 2*B*). Donor cells in unimmunized recipients were barely detectable in the spleen (<0.05%; data not shown). Vaccination of tumor-free recipients led to robust expansion of both the naive and the cotransferred populations (Fig. 2*B*, *top* and *third plots*). In contrast, expansions of both the naive donor cells and the cotransferred naive control or nTregs were significantly reduced in vaccinated tumor-bearing mice (Fig. 2*B*, the *second* and *bottom plots*). As expected, the relative expansion of naive donor cells and the control cells was roughly equivalent in tumor-free mice (1.6 vs 2.3%) and tumor-bearing mice (0.4 vs 0.7%). However, although naive donor cells and coinjected nTregs also expanded comparably in vaccinated tumor-free mice (1.4 and 1.5%, respectively), there was a predominant expansion of the nTregs over the naive donor cells (0.7 vs 0.1%) in vaccinated tumor-bearing mice.

This expansion pattern was also mirrored by the actual numbers of the corresponding donor cells in spleens (Fig. 2*C*). There was an

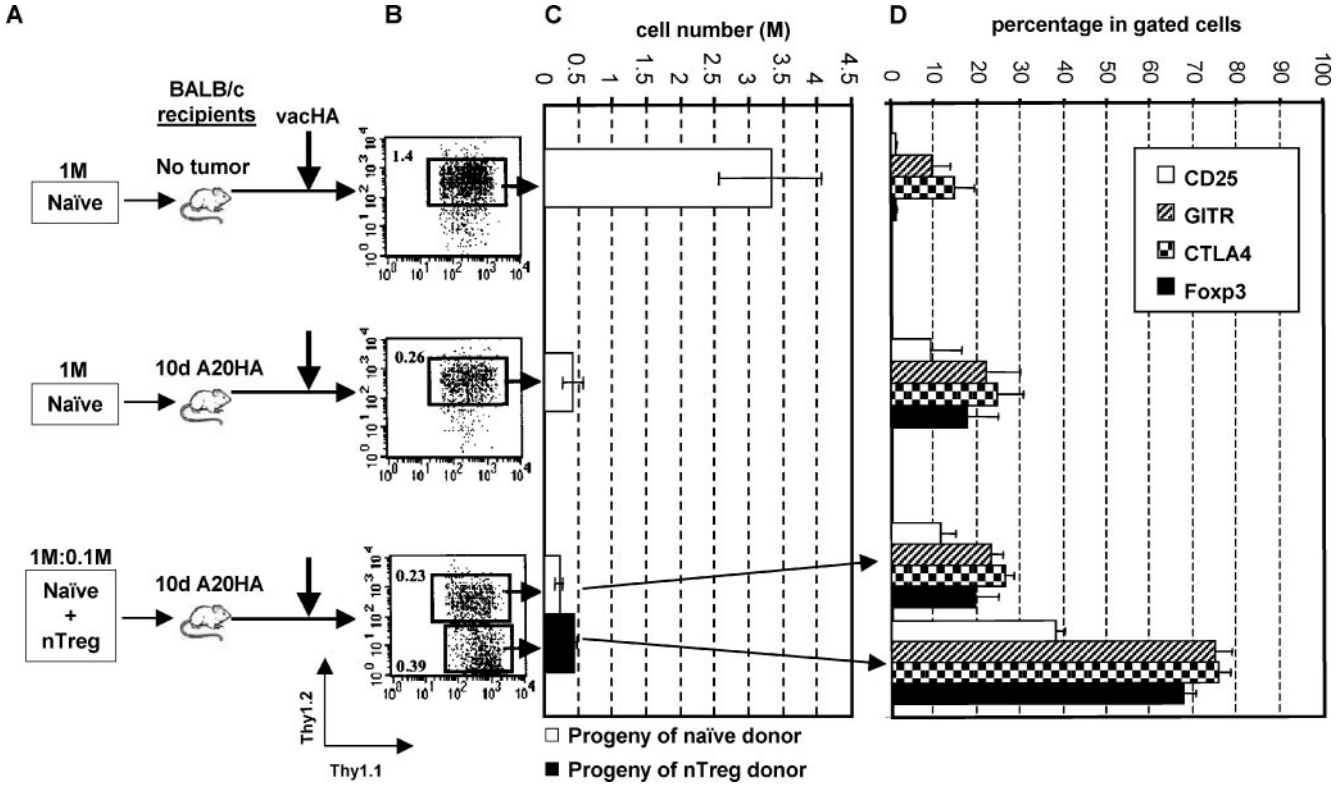


FIGURE 3. Expanded nTregs predominantly contribute to the total Treg pool. *A*, A total of 1×10^6 sorted $CD4^+CD25^-GITR^{low}$ cells from 6.5 Tg mice ($Thy1.1^+/1.2^+$) were transferred alone or together with 0.1×10^6 nTregs (sorted $CD4^+CD25^+GITR^{high}$ cells from $Thy1.1^+/1.2^-$ 6.5 Tg mice) to tumor-free or 10-day A20HA tumor-bearing recipients. The recipients received vacHA immunization 2 wk posttransfer T cell and were analyzed 5 days after vaccination. *B*, Splenocytes were stained with anti-Thy1.1 and anti-Thy1.2 mAbs. Plots shown were gated on donor cells. The number in each plot represents the percentage of the gated population in total spleen cells. The data shown are representative of three mice per group. *C*, Absolute number of donor cells recovered from spleen. The number of cells is calculated as described in Fig. 2*C*. Results are shown as mean ± SE of three mice per group. *D*, Summary of FACS analysis. Splenocytes were stained with anti-Thy1.1, Thy1.2, CD4 mAbs, together with one of CD25, GITR, CTLA-4, or Foxp3 mAbs. The data shown are the percentages of $CD25^{high}$, $GITR^{high}$, $CTLA\text{-}4^{high}$, or $Foxp3^{high}$ cells in corresponding $Thy1.2^+$ or $Thy1.2^-$ donor cells. Results are shown as mean ± SE of three mice per group. The arrows indicate the corresponding populations in analyses.

approximately equal number of expanded naive cells and the control cells or nTregs in the spleens of vaccinated tumor free mice, although the presence of nTregs led to a somewhat decreased accumulation of the total HA-specific T cell population, and in particular of the naive T cells as we previously reported (16). Strikingly, the presence of HA-expressing tumor was associated with a reduced accumulation of donor cells regardless of their origins (Fig. 2, *B* and *C*, *second* and *bottom plots*). Moreover, the progeny of nTregs significantly outcompeted the progeny of non-Tregs in vaccinated tumor-bearing mice, whereas there was no obvious difference in the absence of tumor.

We further examined the phenotypes of the expanded donor cells under each condition. In vaccinated tumor-free recipients, the percentages of naive and control donor cells that express Foxp3 (Fig. 2*D*, *top plot*) were not statistically different from the background. In contrast, Foxp3 expression on these cells was significantly increased when the recipients had tumor (Fig. 2*D*, *second plot*, 33.0 ± 6.6 and 33.1 ± 8.2% in naive and control donor cells, respectively). Surprisingly, the presence of nTregs did not lead to conversion of naive cells into $Foxp3^+$ cells in vaccinated tumor-free mice (Fig. 2*D*, *third row*, 1.2 ± 0.5% $Foxp3^+$ in naive donor cells). Even in tumor-bearing mice, Foxp3 level on the naive donor cells was not enhanced by coexisting nTregs (Fig. 2*D*, *bottom plot*, 13.3 ± 10.7 vs 33.0 ± 6.6% in *second plot*). GITR expression followed the same trend as Foxp3 but with somewhat higher percentages in all groups, consistent with the notion that GITR is not an exclusive Treg marker. These data clearly demonstrate that the emergence of $GITR^{high}$ and $Foxp3^+$ cells from uncommitted naive precursors is totally determined by the tumor context.

Next we mixed naive donor cells and nTregs at a more physiological ratio (10:1) before adoptive transfer (Fig. 3*A*). Again, there was greater expansion and accumulation of the nTregs than that of the naive donor cells in vaccinated tumor-bearing mice (Fig. 3, *B* and *C*), with Tregs increasing from 10% of the total HA-specific $CD4^+$ T cell population to over 60% after vaccination. Also, the results confirmed that the presence of tumor blunted the expansion of naive HA-specific T cells (Fig. 3*C*), and resulted in their conversion to $Foxp3^+$ iTregs (Fig. 3*D*). Moreover, the extent of this conversion was not influenced by the accompanying nTregs (Fig. 3*D*).

These data indicate that the de novo induced regulatory cells and expanded nTregs both contribute to the total Treg pool in periphery. Nevertheless, under these experimental conditions in tumor-bearing hosts, the progeny of the nTregs predominate in the total Treg pool, but they do not measurably impact on the conversion of uncommitted precursors into iTregs. The presence of nTregs, however, significantly influences the response of naive cells to vaccination, particularly in the tumor environment.

Induction of HA-specific regulatory cells is independent of the polyclonal nTregs in the host

The experiments described so far used nTregs bearing HA-specific TCRs for adoptive transfer, but did not examine the role of the endogenous, polyclonal nTregs in the host. To this end, $Rag2^{-/-}$

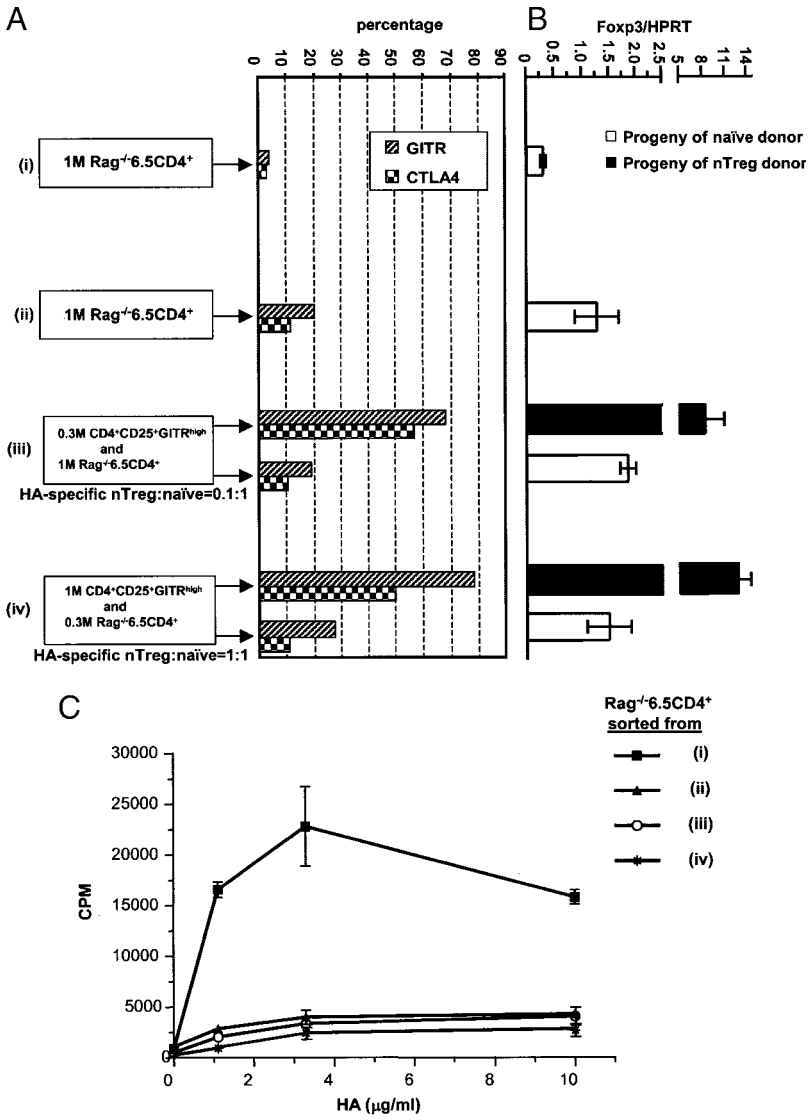


FIGURE 4. Induction of HA-specific regulatory cells in tumor-bearing lymphopenic host. Purified $CD4^+$ T cells from $Rag2^{-/-}$ 6.5 Tg mice ($Thy1.1^-/1.2^+$) were transferred alone or together with varied amounts of sorted $CD4^+CD25^+GITR^{high}$ nTreg cells from $Thy1.1^+/1.2^-$ 6.5 Tg mice, into tumor-free or A20HA tumor-bearing $Rag2^{-/-}$ recipients. When counting the ratio of naive vs nTreg cells, it should take into consideration that all $CD4^+$ T cells from $Rag2^{-/-}$ 6.5 Tg mice are HA-specific, whereas nearly one-third of the $CD4^+CD25^+GITR^{high}$ nTreg cells are HA-specific. All mice received vacHA immunization 2 wk posttransfer T cell and were analyzed 5 days after vaccination. Spleen cells of the same group (three mice per group) were pooled. Spleen cells were stained with anti-Thy1.1, Thy1.2, and CD4 mAbs. Analyses were gated on $CD4^+$ cells. *A*, Percentage of $GITR^{high}$ or CTLA-4^{high} cells in the progeny of naive or nTreg donor cells. *B*, Foxp3 quantitative real-time PCR analysis on progeny of donor cells. Progeny of the naive donor cells or nTregs were FACS-sorted and subjected to analysis. Results are shown as mean ± SE of triplicate reactions. *C*, Progeny of the naive donor cells from each group was subjected to in vitro proliferation assay. Sorted cells were stimulated with fresh APCs in the presence of a varied amount of cognate peptide. Results are shown as mean ± SE of duplicate cultures.

mice, which are T cell-deficient and thus devoid of any nTregs, were used as recipients. As shown in Fig. 4*A*, $CD4^+$ naive T cells from Tg mice on $Rag2^{-/-}$ background, which have been shown to be devoid of HA-specific nTregs (15), were transferred either alone or together with varied amounts of HA-specific nTregs from Tg mice. Interestingly, $GITR^{high}$ and CTLA-4^+ cells still arose from the naive donor cell population in tumor-bearing mice (Fig. 4*A*, group ii), even when both HA-specific and polyclonal nTregs were absent. These cells also had elevated levels of Foxp3 mRNA compared with expanded donor cells in tumor-free recipients (Fig. 4*B*). Furthermore, the presence of transferred HA-specific nTregs did not have significant impact on the expression levels of Treg-associated molecules on the progeny of naive donor cells (Fig. 4, *A* and *B*, group iii and iv). Correlating with the emergence of $GITR^{high}$ and Foxp3-elevated cells, cells derived from the naive donor cells in tumor-bearing mice were hyporesponsive when stimulated with cognate peptide in vitro (Fig. 4*C*), suggesting that the $GITR^{high}$ subset actively suppressed the $GITR^{low}$ subset. These results clearly demonstrate that induction of tumor-specific Tregs is independent of existing Ag-specific nTregs, as well as polyclonal nTregs in the host.

Discussion

It is well documented that tumor cells are able to induce immune tolerance through a number of mechanisms, including release of immunosuppressive cytokines, alteration of Ag processing and presentation pathways, and deletion of tumor-reactive T cells (24). In recent years accumulating evidence indicates that Tregs are enriched in the blood, malignant effusions, draining lymph nodes, and tumor tissues of various kinds of cancer patients, suggesting that the tumor microenvironment may foster immune tolerance by attracting and/or inducing dominant suppressor cells (25). Indeed, tumor-derived soluble factors such as vascular endothelial growth factor, IL-10, and TGF-β have been shown to be involved in the establishment of a complex regional immunosuppressive network (23). We reported previously the induction of tumor-specific T cell anergy using an adoptive transfer and murine B cell lymphoma model system (22). Recently, we further characterized the composition of the "anergic" T cell population, which contains a majority of naive cells and a small subset of tumor Ag-experienced cells that have acquired regulatory functions (16). In this model, T cell anergy, which is hyporesponsiveness upon antigenic restimulation, is actually a reflection of the dominant suppression of the former by the latter subset. Immunization of tumor-bearing hosts with vaccinia virus expressing the tumor Ag leads to expansion of the regulatory population, which potently suppresses the function of effector cells that arise concomitantly following vaccination. The molecular and cellular mechanisms for Treg induction during tumor progression are poorly understood. In particular, the role of

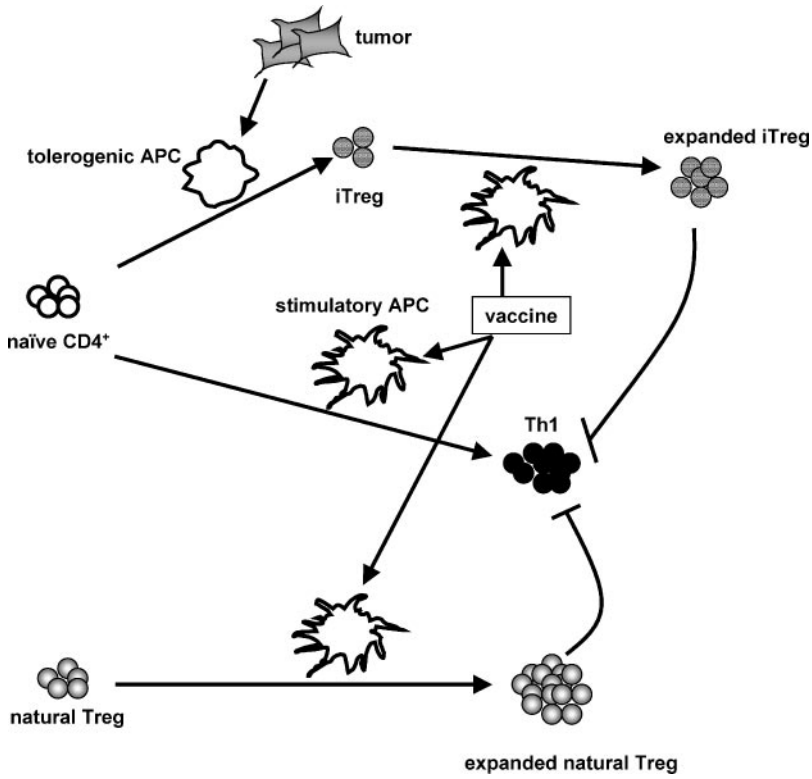


FIGURE 5. Representative model for de novo induction of Tregs and expansion of nTregs in a tumor environment. APCs conditioned by tumor cells, possibly through tumor-derived factors such as vascular endothelial growth factor, IL-10, and TGF-β, can acquire tumor Ags and present them to naive $CD4^+$ T cells in a tolerogenic fashion. This antigenic encounter converts some naive cells to iTregs. Subsequent vaccination expands the iTregs and amplifies preexisting nTregs. By unknown mechanism, expansion of nTregs seems to be more efficient than that of iTregs. Although some naive cells may differentiate into Th1 cells in response to vaccination, their effector functions are dominantly suppressed by the expanded Treg population, leading to tumor-specific T cell tolerance.

the tumor microenvironment in inducing and expanding Ag-specific Tregs has not been examined. In this report, we directly compared the efficiency of Ag-specific Treg development in mice harboring the identical tumor that either did or did not express the nominal Ag. The progression of A20WT neither led to induction of Ag-specific Tregs nor promoted the expansion of preexisting Ag-specific nTregs upon vaccination (Fig. 1). The requirement for tumor-expressing Ag suggests that Ag presentation as influenced by the tumor environment is essential for the development of Ag-specific Tregs. It is noteworthy that previous studies using this system have demonstrated that T cell anergy induction requires Ag presentation by nonmalignant, bone marrow-derived host APCs, rather than by the tumor cells themselves (26, 27), even though A20HA tumors are MHC class II-positive. It is conceivable that a subset of host APCs may be conditioned by tumor-derived immunosuppressive factors. These "tolerogenic" APCs acquire tumor Ags and present the Ags to naive T cells, leading to induction of Ag-specific Tregs. This notion is supported by a recent report showing that tumor progression selectively promotes the proliferation of natural Tregs via a subset of dendritic cells (DC) exhibiting a myeloid immature phenotype (28). Nonetheless, whether the parallel development of effector cells and suppressor cells demonstrated in our model is mediated by specialized APC subtypes, respectively, or by APCs at different maturation stages requires further investigation.

We showed previously that conversion of uncommitted precursors to Tregs does not require pre-existing nTregs (16). However, the presence of nTregs did result in higher overall frequency of $CD25^+GITR^{high}Foxp3^+$ cells than when nTregs were absent. This increase could be due to the direct expansion of nTregs or the enhanced conversion of iTregs from naive cells in the presence of nTregs. It has been debated whether iTregs substantially contribute to the total Treg population or whether they are functionally different from the thymically generated nTregs (20). It is difficult to address these questions by looking at the unmanipulated endogenous T cell repertoire because it is currently impossible to distinguish these two populations. In our adoptive transfer system, Ag-specific nTregs and naive cells with different cogenic markers were mixed at the time of transfer so that both populations could be monitored simultaneously for the changes in their expansion, phenotype, and function. Our data clearly show that conversion of naive cells to iTregs is intrinsic to the presence of tumor. Preexisting nTregs do not influence the extent of iTreg conversion, but significantly limit the response of naive cells in tumor-bearing hosts. Regardless the initial ratio of nTreg to naive cells (1:1 or 0.1:1), the expansion of nTregs consistently outcompetes naive cells in response to vaccination in tumor-bearing hosts (Figs. 2 and 3). Accordingly, expanded nTregs outnumber iTregs in the total regulatory population. This finding could be due to the differences in kinetics of conversion of naive cells to iTregs vs direct amplification of pre-existing nTregs, which have been previously activated against self-Ags. Whether iTregs once formed have the same expansion capacity as nTregs upon vaccination is presently unknown; however, it is clear that these iTregs do manifest suppressive properties (Fig. 4).

It has been suggested that a small number of nTregs may maintain tolerance by amplifying suppression through "infectious tolerance" (29, 30), converting uncommitted precursors into cells with regulatory properties. In our system, this type of infectious tolerance is not observed either under tolerizing (tumor) or nontolerizing (tumor-free and vaccination) conditions. Notably, nTregs and naive cells expand equivalently upon vaccination in the absence of tumor (Fig. 2). It is clear that this form of vaccination does not lead to conversion of iTregs from naive cells but indiscriminately expands both effector cells and existing nTregs. Thus, approaches that can preferentially prime and expand effector cells, and adjuvants that can specifically block Treg expansion, should be taken into consideration in efforts to improve tumor vaccine efficacy.

In conclusion, we propose that induction of tumor-specific Tregs is intrinsic to the context of antigenic encounter in the tumor microenvironment. As shown in Fig. 5, tumor-conditioned tolerogenic APCs, such as immature myeloid DC (28) or other types of tolerizing DC (31), convert naive T cells into regulatory cells. Vaccinations prime and expand Th1 effector cells, but also indiscriminately amplify both nTregs and iTregs. The combined Ag-specific Treg population dominantly suppresses the antitumor effects of Th1 effector cells. Although the relative abundance of tolerogenic vs immunogenic APCs may be influenced by existing nTregs through "educating" network (32), tumors clearly have acquired mechanisms to alter APC stimulatory properties independent of such pathways. Our data imply that a combination of nTreg depletion and stimulatory conditioning of host APCs may effectively remove the regulatory barrier tempering successful cancer immunotherapy.

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Disclosures

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