Introduction

The goal of anti-tumor immunotherapy is to activate cytolytic immune responses, primarily mediated by CD8+ T cells, toward tumor-specific antigens. This can be achieved clinically by therapeutic vaccination against individual tumor antigens (such as prostatic acid phosphatase) or by blockade of immune inhibitory pathways including CTLA-4. In fact, many tumors may require that both strategies are deployed in combination because although tumorigenesis frequently leads to expression of embryonic or abnormal self-antigens, tolerance to these antigens is often established within the tumor microenvironment by both passive and active mechanisms. Two of these mechanisms, which may be interlinked, are the presence of high frequencies of FoxP3+ regulatory T cells (Treg) and defective antigen presentation within the tumor microenvironment. Tumors derive from cells to which our immune systems are tolerant, a state which is non-redundantly maintained by FoxP3+ expressing Treg cells. Thus, the majority of antigens expressed by tumor cells are germ-line encoded, may be expressed in the thymus and are proposed to be recognized by thymic-derived "natural" Treg (nTreg). Because nTreg are generated in the thymus they are thought to express T-cell receptors (TCR) specific for endogenous self-antigens. The opportunity for immunotherapy however arises via the expression of mutated or deregulated tumor protein fragments, which may be recognized by Tconv cells that survive negative selection in the thymus and differentiate into tumor antigen reactive effector T cells (Teff). However, the same population of Tconv may also differentiate into tumor antigen specific "induced" Treg (iTreg) and represent a potential barrier to tumor immunotherapy. Several studies have approximated the balance of nTreg and iTreg using either TCR spectratyping or adoptive transfer models in chemically induced or hematogenous tumors. These studies agree that the majority of Tregs accumulating in tumors are nTreg, but provide inconclusive data regarding the specific importance of iTreg cells in spontaneous tumors or tumors not expressing a pre-defined tumor antigen. Here we provide a systematic comparison of the balance of tumor-infiltrating nTreg and iTreg in both an antigen-specific and polyclonal context. Both polyclonal and antigen-specific iTreg were spontaneously induced in vivo within the microenvironment of chemically induced sarcomas and ovalbumin-expressing E.G7 tumors. The intratumoral balance of iTreg and nTreg was found to approximate that found in peripheral lymphoid tissues, but to consist of a population with a TCR repertoire distinct from peripheral Treg. Treg mediated suppression of vaccine-induced immunity in vivo is tissue-restricted and
antigen-specific, indicating that the specific elimination of intratumoral iTreg may release an important inhibitory checkpoint in anti-tumor immunity. These studies also indicate that antigen-specific suppression by Treg in vivo is specific to CD4+ cells, and that systemic suppression of CD8+ responses by established E.G7 specific suppression by Treg in vivo is specific to CD4+ cells, and tumoral iTreg may release an important inhibitory checkpoint in relative accumulation of Treg within the tumor microenvironment.

To investigate the specific tracking of iTreg and nTreg in vivo.

Specific tracking of iTreg and nTreg in vivo. To investigate the relative accumulation of Treg within the tumor microenvironment, an adoptive transfer model of differentially labeled nTreg (expressing FoxP3-GFP) and iTreg (expressing FoxP3-RFP) was developed. For all experiments, nTreg were purified from spleenocytes of FoxP3-GFP transgenic mice by flow-cytometry was developed. For all experiments, nTreg were purified from (expressing FoxP3-GFP) and iTreg (expressing FoxP3-RFP) transgenic mice and then cultured in vitro to induce FoxP3-RFP transgenic mice and then cultured in vitro to induce expression of FoxP3 as described. These iTreg were subsequently re-sorted on the basis of CD4 and FoxP3-RFP expression to over 99% purity. In some experiments, Tconv cells were adaptively transferred without in vitro induction of FoxP3, and spontaneous generation of iTreg in vivo was observed.

A mixed population of RFP negative Tconv (from FoxP3-RFP reporter mice) and GFP positive nTreg (FoxP3-GFP reporter mice) were adaptively transferred to CD4−/− recipients (Fig. 1A). CD4+ mice were selected as recipients for these initial experiments to maximize engraftment of transferred CD4+ T cell populations, to minimize competition of adaptively transferred Tconv cells for FoxP3 induction in response to exogenous antigen and to maximize the sensitivity of analysis in the absence of background CD4+ T cell populations. Ten days after adoptive transfer, nTreg and spontaneous iTreg were analyzed by flow cytometry in the spleen and mesenteric lymph nodes (mLN). This analysis revealed that both Treg populations were detectable and that nTreg (FoxP3-GFP positive) represented 7.9 ± 1.0% of all CD4+ cells in the spleen and 4.2 ± 0.5% of all CD4+ cells in the mLN (Fig. 1B). Spontaneous polyclonal Treg induction (FoxP3-RFP positive) occurred in 2.4 ± 0.3% of all CD4+ cells in the spleen and 4.2 ± 1.6% of all CD4+ cells in the mLN (Fig. 1B). The absolute numbers of both nTreg and iTreg reflected the ratios indicated on a percentage basis (Fig. 1C). Thus, the balance on a cellular level was 5.9 ± 0.4 nTreg to every iTreg detected in the spleen and only 0.3 ± 1.0 nTreg for every iTreg in the mLN, indicating that roughly equal numbers of nTreg and iTreg were present in the gut draining lymph nodes. These results support the hypothesis that abundant levels of endogenous microbial and food antigens contribute to maintaining high levels of iTreg specific to those antigens to maintain tolerance within the gut. Thus, the Tconv/nTreg adoptive transfer model provides an appropriate system to investigate the relative balance of nTreg and iTreg as reporter for the presence of foreign or mutated self-antigens in other tissues, including the tumor microenvironment.

Results

Specific tracking of iTreg and nTreg in vivo. To investigate the relative accumulation of Treg within the tumor microenvironment, an adoptive transfer model of differentially labeled nTreg (expressing FoxP3-GFP) and iTreg (expressing FoxP3-RFP) was developed. For all experiments, nTreg were purified from spleenocytes of FoxP3-GFP transgenic mice by flow-cytometry was developed. For all experiments, nTreg were purified from (expressing FoxP3-GFP) and iTreg was calculated in the spleen and mLN. Data illustrate the mean ± SEM from four independent experiments, N = 12 mice per group.
frequencies of OT-II iTreg were found in the TDLN relative to nTreg (Fig. 2B), while in the spleen nTreg remained the predominant Treg population. These data indicate that when a known tumor antigen and cognate specific iTreg population are both present, those iTreg accumulate at higher relative frequencies in the TDLN than do polyclonal iTreg. This experimental setting is artificial in the sense that highly-expressed foreign antigens are not easily engineered into spontaneous human tumors, however it serves to highlight the biological property that specific tumor antigens may serve an instructive role in the recruitment of potentially suppressive Treg to the tumor microenvironment.

Unique TCR repertoire of intratumoral iTreg. The observation that the absolute frequencies of iTreg retrieved from TDLN in chemically induced sarcomas are similar to those observed in the spleen, when contrasted to the specific recruitment of OT-II iTreg to ova-expressing tumors, raises the question as to whether tumor infiltrating iTreg recognize unique tumor antigens that are not found in other peripheral tissues. To investigate this question, the TCR Vβ repertoire of Treg isolated from TDLN were compared to splenic Treg of the same mice. In these studies, the total number of retrievable Treg from TDLN (to ≥ 99% purity by flow cytometry based cell sorting) was on the order of 2 × 10^4 Treg cells. To control for artificial differences in TCR diversity created by the low numbers of retrievable Treg, the diversity of TDLN Treg was compared to cell-count matched populations of splenic Treg, and the diversity (D) scores of both of these Treg populations compared to a ‘control’ spectratype of 5 × 10^6 Treg cells. To examine antigen-specific suppression in vivo, FIR mice were adoptively transferred with a mixed population of ovalbumin-specific TCR transgenic CD4+ (OT-II) and CD8+ (OT-I) cells, together with either polyclonal nTreg, in vitro induced polyclonal iTreg or in vitro induced OT-II iTreg. Recipient mice were subsequently vaccinated by intra-peritoneal injection with aluminum salt precipitated ovalbumin (ova/alum). These experiments show reported that suppression of effector T cell immunity by Treg is more potent when the Treg and T effector cells are either tumor-specific than when either population is TCR mismatched or polyclonal.8,10 These data imply that antigen-matched suppression of effector T cell immunity by Treg may also be important for anti-tumor immunity, particularly in the context of vaccination. To examine antigen-specific suppression in vivo, FIR mice were adoptedively transferred with a mixed population of ovalbumin-specific TCR transgenic CD4+ (OT-II) and CD8+ (OT-I) cells, together with either polyclonal nTreg, in vitro induced polyclonal iTreg or in vitro induced OT-II iTreg. Recipient mice were subsequently vaccinated by intra-peritoneal injection with aluminum salt precipitated ovalbumin (ova/alum). These experiments show
that in the mLN, vaccination with ova/alum leads to proliferation of both OT-II and OT-I cells and that the proliferation of OT-II cells is inhibited in the presence of OT-II iTreg but not polyclonal iTreg or nTreg (Fig. 4A). Interestingly, inhibition of OT-II proliferation was observed in the mLN but not in the spleen (Fig. 4B), indicating that suppression occurs only locally. This may be explained by the observation that ova/alum mediated upregulation of CD98, and to a lesser extent CD86, expression was inhibited on peritoneal CD11c positive cells in animals adoptively transferred with OT-II iTreg but not polyclonal iTreg or nTreg (Fig. 4C). These data are in accord with previous in vitro studies demonstrating reduced expression of CD80 and CD86 by antigen presenting cells.20-22 The proliferation of OT-I cells was not inhibited in any tissue using any of the Treg populations indicated. As was observed in the TDNL, this suggests that intra-peritoneal vaccination with ova/alum leads to increased concentrations of ovalbumin in the mLN as compared to the spleen, which supports the local recruitment and accumulation of ovalbumin-specific OT-II iTreg.

These findings suggest that vaccination against a particular TSA would be strongly suppressed by TSA specific iTreg in the tumor microenvironment. To test this hypothesis, in vivo vaccination suppression studies were performed in FIR mice adaptively transferred with OT-I and OT-II Tconv prior to inoculation with ovalbumin expressing EG.7 tumors. This experimental setting is predicted to approximate autologous tumor development in an animal with pre-existing CD4+ Tconv that may share TCR specificity with a spontaneously generated TSA (in this case the artificial antigen ovalbumin). Our previous studies indicated that spontaneous induction of iTreg occurs in vivo in tissues where cognate foreign antigens are in abundance (Fig. 1) and that cognate-antigen specific iTreg preferentially accumulate in tissues where a known foreign antigen is present, including TDNL (Fig. 2). Thus, the presence of OT-II Tconv in mice subsequently inoculated with the ovalbumin expressing EG.7 tumor was predicted to lead to the spontaneous generation of OT-II iTreg and accumulation of these iTreg within the TDNL. After 14 days of tumor growth, we observed that OT-II iTreg represented 1.5 ± 0.3% of OT-II cells in the TDNL (data not shown). These data indicate that the microenvironmental conditions permissive for iTreg induction were present and sufficient in the TDNL and that the TDNL remained a site of accumulation of these OT-II iTreg.

Due to the local induction and accumulation of TSA-specific iTreg within the TDNL, we asked whether local suppression of vaccine-induced T cell immunity was similarly restricted to the TDNL as was previously observed in the mLN. These experiments indicated that following immunization with ova/alum, OT-II Tconv undergo significant expansion in the spleen, mLN and especially the peritoneal cavity (Fig. 5A). In contrast, OT-II proliferation was not observed in the TDNL. OT-I were observed to expand in the peritoneal cavity (PEC), but not in the spleen, mLN or TDNL (Fig. 5B). Immunization with ova/alum led to the proliferation of OT-II iTreg in all tissues analyzed (Fig. 5C).

Discussion

It has historically been difficult to differentially track nTreg and iTreg populations in vivo because cell surface markers have not been described that reliably distinguish the two sub-sets. These experiments are now possible due...
to a variety of congenic and FoxP3-reporter transgenic mouse models. Such a model was utilized here to study the cellular distribution of nTreg and iTreg in the tumor microenvironment. In several autoreactivity models, the relative importance of cognate antigen specific iTreg was compared to polyclonal nTreg, and such questions are equally relevant to understanding and inducing therapeutic immunity to cancer. Specifically, we sought to address whether tumor specific antigens play an instructive role for the accumulation of iTreg in the tumor microenvironment, because iTreg are believed to be more potent suppressors of vaccine-stimulated T-cell immunity than antigen-mismatched Treg, and that this suppression is specific to the site where cognate antigen specific Treg accumulate and exerts via suppression of an antigen-presenting cell intermediate. In E.G7 tumor bearing mice, ovalbumin-specific OT-II iTreg were spontaneously induced from adoptively transferred OT-II Tconv and detected in the TDLN. When E.G7 tumor bearing mice were subsequently immunized with ovalbumin, the remaining OT-II Tconv underwent clonal expansion in the peritoneal cavity and distributed to the spleen and mLN but not in the TDLN. In contrast, the spontaneously induced OT-II iTreg expanded in all tissues. The mechanism behind this observation is unclear, however it appears that the local availability of antigen (ova) within the tumor and TDLN may provide local support for the immunosuppressive activity of OT-II iTreg, which interfere with OT-II Tconv trafficking into the tumor bed, as has been described.

Interestingly, in tumor naive mice, antigen-specific iTreg were incapable of suppressing the proliferation of vaccine-primed CD8+ T cells. In the presence of an established E.G7 tumor however, suppression of CD8+ T cells was observed to be more generalized than suppression of CD4+ cells and antigen-specific CD8+ T-cell proliferation was only observed at the site of vaccination. Together, these observations indicate a disconnect in the suppressive mechanisms utilized to suppress CD4+ and CD8+ T cell immunity. As is widely known, the suppressive mechanisms utilized by Treg differ based on the experimental conditions utilized and may differ in vivo from what is observed from in vitro suppression assays; these mechanisms remain under investigation.

Materials and Methods

Mice and cell lines. Wild type C57BL/6 mice were purchased from Charles River Laboratories. FoxP3-RFP reporter mice on a B6 background (FIR mice, generously provided by D. Richard Flavell), FoxP3-GFP (generously provided by Dr Alexander Rudensky), OT-II and CD4+ OT-II mice were bred in our animal facility. OT-II mice were crossed to OT-II mice to generate OT-II/FIR mice, screened by flow cytometry for expression of Vα8, Vβ5 and FoxP3-RFP in the CD3+CD4+ gate and bred in our animal facility. Mice were used at 6–12 weeks of age and were maintained in pathogen-free conditions at the UM animal facilities. All animal use procedures were approved by the University
of Miami Animal Care and Use Committee. The IC-1 sarcoma cell line was generated by cultured tumor explants generated in C57BL/6 mice by subcutaneous injection of 5-methylcholanthrene (Sigma, 50 μg dissolved in peanut oil) as described in reference 34. Tumor explants were minced, digested with trypsin-EDTA (Life Technologies) for 5 min and cultured in IMDM supplemented with 10% fetal calf serum (Life Technologies). Both IC-1 and E.G7 tumor cell lines were maintained in IMDM supplemented with 10% fetal calf serum (Life Technologies).

Reagents, antibodies and flow cytometry. Commercial antibodies for use in flow cytometry and in vivo studies were purchased from BD Pharmingen, eBioscience or BioLegend. For flow cytometry analysis, single cell suspensions were prepared from spleen and lymph nodes. 10^6 cells were pre-blocked with anti-mouse CD16/CD32 and stained with different antibody combinations. Intracellular staining was performed according to standard procedures. Flow cytometric analysis was performed on a Becton Dickinson Fortessa instrument and DIVA or Flowjo software. Cell sorting was done using a FACSAria II cell sorter (BD) after enrichment of splenocytes for CD4+ T cells using the EasySep Mouse CD4+ T cell Pre-Enrichment Kit (Stem Cell Technologies). Suspensions of ovalbumin (crystallized chicken egg ovalbumin Grade V, Sigma) and aluminum sulphate (Sigma) were prepared as previously described in reference 35, and administered (66 μg/mouse unless otherwise indicated) by intraperitoneal injection.

In vitro Treg induction. Sorted CD4+Foxp3-RFP cells (purity ≥ 99.5%) from Foxp3-RFP reporter mice were activated in vitro (10^6 cells/ml) with plate-bound α-CD3 (clone 2C11, 2 μg/ml), TGFβ (5 ng/ml), retinoic acid (RA; 100 nM) and IL-2 (100 U/ml) for 4 d to induce Treg cells (induction rate ≥ 60% iTreg cells). After 4 d, the iTreg cells were sorted and used for adoptive transfer into CD4-/- mice.

Adoptive transfer models. For in vivo polyclonal iTreg cells experiments, Tconv from Foxp3-RFP reporter mice were high speed cells sorted by flow cytometry. Separately, nTreg cells (CD4+Foxp3+GFP- cells) were sorted by flow cytometry from Foxp3+GFP reporter mice. Tconv (2 × 10^6 cells) were mixed with nTreg cells expressing Foxp3+GFP isolated from Foxp3- GFP reporter mice (5 × 10^5 cells) and adoptively transferred to CD4+ recipient mice by tail vein injection in 100 μl HBSS. For in vivo TCR transgenic OVA(323-339) specific iTreg cell experiments, Tconv from OT-II/FJR mice were high speed cells sorted by flow cytometry on the basis of CD4+, CD8+, Foxp3+RFP phenotype. Separately, nTreg cells (CD4+Foxp3+GFP- cells) were sorted by flow cytometry from Foxp3-GFP reporter mice. OT-II Tconv (2 × 10^6 cells) were mixed with nTreg cells-Foxp3+GFP cells isolated from Foxp3-GFP reporter mice (5 × 10^6 cells) and adoptively transferred to CD4- recipient mice by tail vein injection in 100 μl HBSS.

In vivo suppression assays. For analysis of Treg suppressive activity in vivo, polyclonal iTreg or OT-II iTreg were generated in vitro as described above and purified by high-speed cell sorting. nTreg were purified directly from splenocytes as described above. The indicated Treg population (7.5 × 10^6 cells) was mixed with both OT-II Tconv (7.5 × 10^6 cells) and OT-I cells (5 × 10^6 cells) and adoptively transferred to FJR mice. 24 hours after adoptive transfer, recipient mice were immunized with oval/albumin. On the indicated day post-immunization, tissues were isolated, cells counted and analyzed by flow cytometry.

CDR3 size and sequence analysis. Primers for Vβ (Table S1) spectratyping and the determination of CDR3 lengths have been previously described in references 13, 14 and 36. Total RNA from highly purified CD4+Foxp3+ Treg cells was isolated using TRIzol reagent (Invitrogen) according to manufacturers instruction. Reverse transcription was performed using oligo-d(T)16 primer, Superscript III (Invitrogen) and buffer containing 5 mM DTT (Invitrogen) and 20 U RNasin (Promega). PCR conditions were: denaturation (1 min, 95°C), followed by 40 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. For Vβ spectratyping, 0.5 μl of the PCR reaction was subjected to a second round of PCR for 30 cycles using the reference described above. The Jβ1.1 primers used during the second PCR reaction were labeled with 5'-6-FAM and used together with the specific Vβ region primer described previously. After amplification, PCR products were diluted 1:20 in distilled water and 0.5 μl of the diluents were loaded to ABI Prism 3730x/l DNA analyzers (Applied Biosystems). Fragment analysis sample files were analyzed with Peak Scanner software (Applied Biosystems).

Spectratype data analysis. The method used to quantify skewing of the TCR repertoire has been described previously and is represented as diversity (D) scores.13,15 Briefly, fluorescent intensity (peak height) was measured using Peak Scanner software for each peak in an individual Vβ repertoire and transferred to Excel (Microsoft) spreadsheets. The representation of each individual peak was calculated as a percentage of the sum of all peak heights within each Vβ profile. These calculations were initially performed for a splenocyte-derived Treg (5 × 10^6 cells) “reference” profile that is estimated to exceed the total possible TCR diversity of all Treg in an individual mouse, and followed a Gaussian distribution characteristic of a highly diverse TCR repertoire. This was repeated for five individual mice and the results for each Vβ segment were individually pooled and averaged, the resulting spectratypes were subsequently considered the ‘reference’ sample for each Vβ. For the experimental samples, the absolute difference in the percent-representation of each peak within a given Vβ was compared to the reference sample, summed and then divided by two. This value represents the extent that the repertoire varied from the reference for an individual Vβ profile. The D score represents the mean of these values for each of the Vβ segments for each individual sample.

Statistical analysis. All graphing and statistical analysis were performed using the AIBI Prism program. Paired analysis was performed using the Student’s t-test. Analysis of conditions with more than two conditions was performed using one-way ANOVA with Tukey’s post-test. Significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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