Analysis of the T-Cell Receptor Repertoires of Tumor-Infiltrating Conventional and Regulatory T Cells Reveals No Evidence for Conversion in Carcinogen-Induced Tumors

James P. Hindley¹, Cristina Ferreira², Emma Jones³, Sarah N. Lauder¹, Kristin Ladell¹, Katherine K. Wynn¹, Gareth J. Betts¹, Yogesh Singh², David A. Price¹, Andrew J. Godkin¹, Julian Dyson², and Awen Gallimore¹

Abstract

A significant enrichment of CD4⁺ Foxp3⁺ T cells (regulatory T cells, Treg) is frequently observed in murine and human carcinomas. As Tregs can limit effective antitumor immune responses, thereby promoting tumor progression, it is important that the mechanisms underpinning intratumoral accumulation of Tregs are identified. Because of evidence gathered mostly in vitro, the conversion of conventional T cells (Tconv) into Tregs has been proposed as one such mechanism. We assessed the contribution of conversion in vivo by analyzing the TCR (T-cell receptor) repertoires of Tconvs and Tregs in carcinogen-induced tumors in mice. Our results indicate that the TCR repertoires of Tregs and Tconvs within tumor-infiltrating lymphocytes (TIL) are largely distinct. Indeed, the cell population with the greatest degree of repertoire similarity with tumor-infiltrating Tregs was the Treg population from the tumor-draining lymph node. These findings demonstrate that conversion of Tconvs does not contribute significantly to the accumulation of tumor-infiltrating Tregs; rather, Tconvs and Tregs arise from different populations with unique TCR repertoires. Enrichment of Tregs within TILs most likely, therefore, reflects differences in the way that Tregs and Tconvs are influenced by the tumor microenvironment. Elucidating the nature of these influences may indicate how the balance between tumor-infiltrating Tregs and Tconvs can be manipulated for therapeutic purposes.

Cancer Res; 71(3); 1–11. ©2010 AACR.

Introduction

There is evidence that T cells can infiltrate tumors and limit their progression (1). In turn, it is thought that successful tumor cells in patients with cancer are those that evolve strategies to avoid the attention of the immune response, either through downmodulation of recognition molecules or indirectly through immune subversion. Data from experiments performed using mouse models directly support this hypothesis (reviewed in ref. 2). One mechanism through which the immune response to tumors might be subverted is by enrichment of regulatory T cells (Tregs) within the tumor tissue and tumor-draining lymph nodes (DLN).

The normal functions of Tregs are to maintain immune homeostasis, prevent autoimmunity, and limit immunopathology (reviewed in ref. 3). However, many groups have reported, in both studies of mouse models and patients with cancer, that tumor development is often associated with an enrichment of Tregs in peripheral blood, local lymph nodes and tumor tissue (reviewed in ref. 4). Using the chemical carcinogen 3-methylcholanthrene (MCA), we previously examined the impact of Tregs on tumor immunosurveillance (5). We found that Tregs are significantly enriched in MCA-induced tumors (fibrosarcomas) compared with lymphoid tissue (~50% of CD4⁺ T cells in tumors express Foxp3 compared with ~15% in lymph nodes, P < 0.0001) and that even a partial and transient depletion of these cells results in a marked reduction in tumor incidence. Along with other studies, this observation supports the hypothesis that tumors can utilize Tregs for their own advantage by promoting Treg activity (6–9). As well as their potential for limiting the effectiveness of tumor immunosurveillance, it is likely that Tregs also represent a significant obstacle to successful immunotherapy (reviewed in ref. 10). It is important, therefore, to understand the factors that lead to the enrichment of Tregs during tumor development to enable the development of inhibitory strategies.

The majority of Tregs that express the transcription factor Foxp3 (termed naturally occurring Tregs) are generated in the...
thymus as a distinct cell lineage (11–13). Foxp3+ Tregs may also be generated in the periphery through the conversion of conventional Foxp3− T cells (Tconvs) into Foxp3+ Tregs; these have been termed adaptive (or induced) Tregs (reviewed in ref. 14). Experiments using mice with restricted TCR (T-cell receptor) repertoires and limited studies in humans have suggested that the TCR repertoires of Tregs and Tconvs are largely distinct, overlapping by approximately 10% to 20% (11, 12, 15, 16). It has been suggested that Tconvs with TCRs overlapping with those of the Treg repertoire represent cells that recognize self-antigens (11). Adaptive Tregs that have arisen by conversion of Tconvs in the periphery may also contribute to the observed overlap in the TCR repertoires. The role and significance of adaptive Tregs in vivo, however, is unclear as the majority of the peripheral Treg repertoire is also represented within the thymic Treg repertoire (11). Furthermore, studies of diabetogenic NOD mice showed no overlap between the TCR repertoire of Tregs and Tconvs, indicating a lack of conversion in autoimmune disease (17). However, studies showing that tumors can facilitate conversion of Tconvs into Tregs in vitro (18) and, more recently, in vivo, imply that conversion contributes significantly to the accumulation of tumor-infiltrating Tregs (19, 20). Although informative, the tumor models described here often use tumor cell lines and track the fate of adoptively transferred T cells, but omit interactions between the immune system and the tumor during the early stages of tumor development; the reciprocal influences of the immune system and the tumor during these stages are likely to have a significant impact on the nature of their relationship during the period of tumor outgrowth. Here, we utilize the MCA tumor induction model described above, which takes these early interactions into account, to analyze the TCR repertoires of Tconvs and Tregs to assess definitively the contribution of conversion to Treg enrichment in tumors.

Materials and Methods

Mice

Mice expressing green fluorescent protein (GFP) under the control of the Foxp3 promoter (Foxp3-GFP mice) were obtained from Prof. Alexander Rudensky (University of Washington, Seattle, WA) and have been described previously (21). Mice were housed in accordance with UK Home Office regulations under specific pathogen-free conditions.

Tumor induction

Foxp3-GFP mice were injected subcutaneously in the left hind leg with 400 μg of MCA (Sigma-Aldrich) in 100 μL of olive oil under general anesthetic. Mice were monitored for tumor development weekly for up to 6 months. Tumor-bearing mice were culled when their tumors were between 1 and 2 cm in diameter, typically within 100 to 150 days after MCA injection.

Antibody staining and cell sorting

Single-cell suspensions of tumor, spleen, and inguinal lymph nodes were prepared by filtering tissues through 70-μm cell strainers (BD). The inguinal lymph node from the tumor (left) side of the mouse was taken as the tumor DLN and the contralateral inguinal lymph node was considered to be a nondonor lymph node (NDLN). For cell sorting, cells were stained with the following fluorescently labeled monoclonal antibodies: anti-CD4 Pacific Blue (BD), anti-CD25 PE (eBioscience), anti-CD44 APC (BD), and anti-CD62L PE-Cy7 (eBioscience). Cell sorting was performed using a MoFlo cell sorter (Dako Cytometry) or a customized 20-parameter FACSAria II flow cytometer (BD). CD4+ GFP+ lymphocytes were sorted as Tregs and CD4+ GFP− lymphocytes were sorted as Tconvs. Where indicated, Tconvs were further purified into antigen-experienced (CD44hi/CD62Llo) Tconvs and naive (CD44hi/CD62Lhi) Tconvs. Postsort purity was greater than 98% in all cases.

Immunohistochemistry

After removal, tumors were fixed in 10% neutral-buffered formalin (NBF) and embedded in paraffin wax. Five-micrometer thick sections were dewaxed and antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6). Sections were equilibrated in PBS before blocking peroxidase activity with Peroxidase Suppressor (ThermoScientific). Nonspecific antibody binding was blocked by incubating sections with 2.5% horse serum (Vector). Transforming growth factor (TGF) β was detected using rabbit anti-mouse TGFβ (sc-146; Santa Cruz Biotechnology) followed by anti-mouse/rabbit ImmPRESS and visualized using Vector SG. Foxp3 was detected subsequently using rat anti-Foxp3 (FJK-16; eBioscience) followed by anti-rat ImmPRESS and visualized using Impact DAB (VectorLabs). Equivalent concentrations of rabbit IgG and rat IgG2a were used as control antibodies. Sections were dehydrated and mounted in DPEX. Photomicrographs were taken using a Nikon microscope and digital camera.

TCR clonotyping

For analysis of specific segments of the TCR repertoire, RNA was extracted from sorted cells using a column-based RNA extraction kit (RNeasy micro, Qiagen). cDNA was reverse transcribed from 30 to 200 ng of total RNA using Superscript III (Invitrogen) with random hexamers. TCR sequences were amplified by PCR using either a TRBV13-2 specific primer (5'-GGTGACATTGAGCTGTAAT-3') paired with a common (TRBC) primer (5'-CAGAC-3') or a TRBV13-2 specific primer (5'-GGTGACATTGAGCTGTAAT-3') paired with a TRBJ2-5 specific primer (5'-TAA-GGAGGGAGGAGGG-3'). Agarose gel–purified PCR products were cloned into pCR2.1-TOPO plasmid vector (Invitrogen). The PCR products were then transformed into chemically competent Escherichia coli (TOP10; Invitrogen) and grown on selective LB (Luria–Bertani) plates [100 μg/mL ampicillin with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-gal for blue/white screening]. Up to 96 colonies per sort were picked and DNA sequenced (Beckman Coulter Genomics). The TRB gene usage and CDR3 amino acid composition was established using IMGT/V-QUEST software. For analysis of the total TCR repertoire, an unbiased template-switch–anchored reverse transcriptase (RT) PCR was used as described previously (22).
Statistical analysis

The level of similarity between the different TCR repertoires was measured using the Morisita–Horn (MH) similarity index. This unitless index ranging from 0 to 1 takes into account the number of shared sequences between 2 repertoires as well as the contribution of those shared sequences to each repertoire. The EstimateS software package was used to calculate the MH values (23).

Results

Enrichment of CD4\(^+\) Foxp3\(^+\) T cells in tumor and tumor DLN

We have reported that Foxp3\(^+\) cells are enriched (40%–50% of CD4\(^+\) T cells in tumors express Foxp3) within the CD4\(^+\) tumor-infiltrating lymphocytes (TIL) isolated from MCA-induced fibrosarcomas (5). This preliminary observation was replicated and extended in this study, confirming that there is a significant accumulation of Tregs in the tumor (P = 0.0002; Fig. 1A). Strikingly, mice partially depleted of Tregs showed a significant reduction in tumor incidence (P = 0.0004; Fig. 1B). Given the clear relevance of Tregs in this model, we utilized MCA-induced tumors to determine whether conversion of conventional CD4\(^+\) Foxp3\(^-/\) cells into CD4\(^+\) Foxp3\(^+\) cells accounted for Treg enrichment within TILs. This possibility was considered likely, as TGF\(b\) is readily detectable in MCA-induced tumors (Fig. 1C) and has been shown to induce Foxp3 expression in CD4\(^+\)CD25\(^-/\) cells (24, 25). We surmised that if Treg enrichment in tumors is due to the conversion of CD4\(^+\) Foxp3\(^-/\) cells into CD4\(^+\) Foxp3\(^+\) cells, then the degree of overlap between their TCR repertoires would be significantly higher in the tumor compared with other lymphoid tissues where Treg enrichment is not observed. Thus, we compared the extent of TCR repertoire overlap in Treg and Tconv populations isolated from tumor, spleen, inguinal NDLN, and inguinal DLN. For this purpose, we purified the CD4\(^+\) T cells from tumor-bearing Foxp3-GFP TCR transgenic mice. This was important as the Tregs and Tconvs could not be distinguished by CD25 expression. Although around 80% to 95% of tumor-infiltrating CD4\(^+\) Foxp3\(^+\) cells express CD25, CD25 expression is also observed on approximately 15% of the corresponding CD4\(^+\) Foxp3\(^-/\) population (Fig. 1D).

Figure 1. CD4\(^+\) Foxp3\(^+\)/CD25\(^-\) Tregs are enriched within TGF\(b\) containing MCA-induced tumors and tumor DLN. Spleen, tumor, NDLN and inguinal DLN were excised from mice with MCA-induced tumors. Cells were analyzed for CD4, CD25, and Foxp3 expression by flow cytometry. A, the percentages of CD4\(^+\) lymphocytes expressing Foxp3 are shown as mean ± SD. Statistical significance was assessed using the paired t test (tumor and spleen n = 23; lymph nodes n = 11; 5 independent experiments). B, mice (n = 41) were given 500 µg of anti-CD25 (PC61) or control antibody prior to MCA injection and monitored for tumor development. Statistical analysis was performed using the log-rank method. C, serial sections from MCA-induced tumors were stained with antibodies specific for TGF\(b\) (gray) and Foxp3 (brown). The stain shown is representative of 6 MCA tumors from 3 independent experiments. D, representative flow cytometry plots showing CD4\(^+\)GFP/Foxp3\(^-\) T cells infiltrating an MCA tumor and the gating strategy used for sorting. Gated cells were analyzed for CD25 expression. The percent of gated cells expressing CD25 is shown.
TCR repertoires of CD4$^{+}$Foxp3$^{-}$ and CD4$^{+}$Foxp3$^{+}$ T cells in tumor-bearing mice

We aimed to analyze the repertoire of CD4$^{+}$Foxp3$^{-}$ cells and CD4$^{+}$Foxp3$^{+}$ by sequencing individual TCRs expressed by T cells present within the different anatomic locations described above. For this purpose, we focused our analysis on a representative V$\beta$-chain subset. Initially, CD4$^{+}$Foxp3$^{-}$ and CD4$^{+}$Foxp3$^{+}$ cells from MCA tumor-bearing mice were screened for TCR $\beta$-chain variable domain (TRBV) subset usage with antibodies specific for TRBV 2, 13-1/2, 13-3, 15, and 16 (Supplementary Figs. 1 and 2). We found no statistically significant difference in V$\beta$ subset usage between Tregs and Tconvs sorted from MCA tumors. Both cell populations appeared to have a broad range of TRBV gene usage with no skewing toward any particular subset within the tumor or the spleen of tumor-bearing mice. These findings were confirmed at the molecular level in tumor, spleen, and inguinal NDLN using an unbiased template-switch–anchored RT-PCR (Supplementary Fig. 3). Given that many thousands of unique clonotypes can comprise a single TRBV family, we expanded the investigation to include high-resolution clonotypic analysis of one V$\beta$ subset. For this detailed analysis, the TRBV13-2 subset was selected as it was well represented (up to 30%) within the total V$\beta$ TCR repertoire.

In agreement with previous reports (11, 12), we observed that the TCR repertoires of Treg and Tconv in the spleen and the tumor NDLN of tumor-bearing mice were largely distinct (Fig. 2A and B). In the spleen, of 68 different TRBV13-2 Treg clonotypes, only 1 overlapped with the Tconv repertoire; this accounted for only 1.3% of the Treg TCR repertoire. Similarly, none of the 73 different Treg clonotypes overlapped in the NDLN. Thus, the TCR repertoires of Tconvs and Tregs are nonoverlapping in tumor-bearing mice. Tconv-sorted (CD4$^{+}$Foxp3$^{-}$) and Treg-sorted (CD4$^{+}$Foxp3$^{+}$) cells were analyzed by TCR clonotyping. CDR3 amino acid sequences of the TRBV13-2 subset were determined and used to identify individual TCRs for each T-cell subset from the (A) spleen, (B) NDLN, (C) DLN, and (D) tumor of mice with MCA-induced tumors. Each graph displays the different TCR sequences observed (x-axis) within the Treg (filled & above) and Tconv (open & below) repertoires, and the frequency (y-axis) of each sequence within that subset. The total number of TCR sequences analyzed is displayed as n, with the number of unique TCR sequences observed in parentheses. The proportion of the Treg repertoire that shared sequences with the Tconv repertoire is also shown. The data are representative of 2 independent experiments in 2 mice.
generally distinct. Interestingly, the lack of overlap between the Tconv and Treg TCR repertoires was also reflected in the tumor DLN (Fig. 2C), where less than 2.5% of the Treg TCR repertoire overlapped with the Tconv TCR repertoire, as well as in the tumor (Fig. 2D), where only 2 of 77 different Treg clonotypes overlapped with Tconv TCRs. These 2 clonotypes only accounted for around 3% of the Treg TCR repertoire; thus, more than 95% of the tumor-infiltrating Treg TCR repertoire was distinct compared with the colocalized Tconv TCR repertoire. Similar patterns were also observed in analyses of other mice, indicating no statistically significant increase in overlap between the two T-cell repertoires in tumors compared with the other sites (Supplementary Figs. 4 and 6).

To quantify the similarity between the TCR repertoires from the CD4 Foxp3 and CD4 Foxp3 subsets, we used the MH similarity index. The levels of similarity between the TRBV13-2 TCR repertoires of Tconv and Treg were consistently low (MH < 0.04) throughout all four tissues measured (Fig. 3A). The highest level of similarity was actually observed between the Treg repertoires from DLN and tumor (Fig. 3B). The Treg repertoire from the tumor was on average approximately 20% (MH = 0.19) similar to the tumor DLN Treg repertoire and approximately 12% similar to the spleen Treg repertoire. In contrast, the Tconv and Treg repertoires from the tumor were only 3.8% (MH = 0.038) similar. This suggests that the Treg cells within the tumor are more likely to have derived from the DLN Treg pool or vice versa than the tumor-infiltrating Tconv population.

**TRBV13-2/TRBJ2-5 TCR repertoires in tumor-bearing mice**

Because of the high clonotypic diversity observed within the total TRBV13-2 repertoire across all tissues, it is possible that some TCR sequences may have been missed in the initial TRBV13-2 analysis. To focus our clonotypic examination in more detail, we performed TCR sequencing on a single TCR β-chain joining (TRBJ) segment within the TRBV13-2 population. As for the Vβ usage analysis, we observed no significant skewing toward any particular Jβ subset within the Treg repertoire from the spleen, tumor, or lymph nodes using either an unbiased template-switch–anchored RT-PCR (Supplementary Fig. 3) or a specific TRBV13-2/TRBC PCR (Supplementary Fig. 5). Therefore, as above, we selected the relatively common TRBJ2-5 gene as a representative subset for the deeper analysis.

**Figure 3.** Low levels of similarity between the TRBV13-2 TCR repertoires of Tconv and Treg cell subsets from various tissues. The TRBV13-2 TCR sequences were determined by clonotyping and used to identify individual TCRs. A, similarity between each TCR repertoire was measured using the MH index. This index measures similarity based on the number of shared sequences between 2 populations as well as how well represented those overlapping sequences are within the 2 populations. A value between 0 (no similarity) and 1 (identical) was calculated by comparing the TCR repertoire from each population and colored according to the scale shown. Values used are mean similarity taken from 2 independent experiments in 2 mice. B, the TCR repertoires of Tregs from tumor and Tregs from DLN were compared. Different TCR sequences observed (x axis) within the tumor Treg (filled & above) and DLN Treg (open & below) repertoires, and the frequency (y axis) of each sequence within that subset, are displayed. The total number of TCR sequences analyzed is displayed as n, with the number of unique TCR sequences observed in parentheses. The proportion of the tumor Treg repertoire that shared sequences with the DLN Treg repertoire is shown.
In agreement with our findings from the TRBV13-2 TCR repertoire analysis, the TRBV13-2/TRA12-5 TCR repertoires of Tconv and Treg were generally nonoverlapping within all tissues including the tumor (Fig. 4). No shared TCR sequences were found within the tumor or the NDLN. Furthermore, only 1 overlapping sequence was found within the spleen and DLN repertoires. Therefore, the tumor-infiltrating Treg cells had a distinct TRBV13-2/TRA12-5 TCR repertoire compared with Tconvs. This further supports the notion that tumor-infiltrating Tregs do not arise through the conversion of Tconvs. Conversely, when the TRBV13-2/TRA12-5 TCR repertoires of Tregs from the DLN and Tregs from the tumor were compared, a high degree of overlap (33%) was clearly observed (Fig. 5A). This similarity between the Treg subsets was highlighted when analyzed using the MH similarity index (Fig. 5B). The high similarity (MH = 0.16) between the TCR repertoires of the tumor-infiltrating Tregs and DLN Tregs suggests, as previously, that the tumor Tregs and the DLN Tregs derive from the same population that is distinct from the Tconv pools residing in the same locations.

**TCR repertoires of antigen-experienced Tconvs and Tregs in tumor-bearing mice**

TCR engagement is necessary for the conversion of Tconvs into Tregs in vitro. Therefore, it is likely that Tconvs must similarly encounter antigen in vivo before they are...
converted into Tregs. Accordingly, we hypothesized that if conversion of Tconvs into Tregs was an ongoing process in tumors, an overlap between the TCR repertoires would most likely be observed within the antigen-experienced population. To test this hypothesis, CD4^{+}Foxp3^{-} cells were purified as an antigen-experienced (CD44^{hi}CD62L^{lo}) T conv population (Fig. 6A) and analyzed by TCR clonotyping. Similar to our previous analyses of the whole Tconv population, the TCR repertoires of antigen-experienced Tconvs and Tregs were nonoverlapping in all tissues including the tumor (Fig. 6B–D). In addition, we observed no overlap between CD4^{+}CD62L^{lo} (naive) Tconv cells and Tregs purified from the tumor (data not shown). Collectively, these data further support the premise that conversion of tumor-infiltrating Tconvs into Tregs is infrequent and does not account for the large enrichment of Tregs in tumors. The TCR repertoires of antigen-experienced Tconvs and Tregs from the spleen, DLN, and tumor were each compared using the MH similarity index (Fig. 6E). This analysis confirmed the previous results. No overlap was observed between Tregs and Tconvs, and the greatest degree of overlap was present between the same type of T cell, most notably the antigen-experienced Tconv populations, recovered from the tumor and the tumor DLN (Fig. 6E). Collectively, these data demonstrate that migration of T cells between the tumor and lymphoid tissue occurs, but that these cells remain largely unconverted.

Discussion

Our study was undertaken to understand further the mechanisms underlying the enrichment of Tregs in tumors. We assessed the contribution of conversion of Tconvs into Tregs in tumor-bearing mice through a detailed dissection of the TCR repertoires from CD4^{+}Foxp3^{-} cells in lymphoid tissue and TILs in mice expressing a normal TCR repertoire. The degree of overlap between the TCR repertoires expressed by both populations was lower than that reported by other groups using TCR transgenic mice (11, 12). This disparity is expected given the curtailed TCR repertoires found within such mice. In these models, a limited number of TCR structural combinations pass thymic selection resulting in restricted peripheral diversity and, accordingly, an increased chance of identifying recurring overlapping clonotypes. The T-cell dynamics during a natural tumor response in a TCR intact animal are considerably more complex considering the
full $2 \times 10^6$ ($25 \times 10^6$ in humans) receptor pool available in mice (26, 27). In this study, we used non-TCR transgenic mice to investigate whether the degree of repertoire overlap between the two T-cell populations was significantly different in cells isolated from the tumor compared with lymphoid tissue. In agreement with the findings of others, we observed that the TCR repertoires of Tconvs and Tregs are largely distinct in the lymphoid compartments. However, we also...
observed that this is the case for tumor-isolated Tconvs and Tregs, indicating that conversion of Tconvs does not make a major contribution to the accumulation of tumor-infiltrating Tregs, even in tumors where TGFβ is readily detected. In support of this conclusion, the greatest degree of repertoire similarity was observed between corresponding T-cell populations from the DLN and the tumor. These data imply that T cells may be primed in the DLN or enter the node via draining lymphatics (Fig. 6E).

Previous investigations, despite showing that conversion of conventional T cells into Tregs is possible, do not demonstrate the extent to which conversion might occur in a natural context and whether it accounts for the large enrichment of Tregs often seen in tumors (18–20). Exemplifying this, a more recent study of melanoma patients implied that peripheral conversion of CD4+CD25− T cells into Tregs in response to tumor antigens leads to Treg enrichment in these patients (28). Although this study demonstrates that conversion is possible, the extent to which this mechanism contributes to the enrichment of Tregs in tumors was not addressed. Our data do not exclude conversion of Tconvs as a mechanism contributing to Treg enrichment in tumors but do demonstrate, in the case of a spontaneously developing tumor, that the majority of tumor-infiltrating Tregs do not arise through conversion. We found no significant difference in similarity between the TCR repertoires of Tconvs and Tregs from tumor compared with normal lymphoid tissue (Supplementary Fig. 6). Importantly, on average, more than 96% of the Treg repertoire did not overlap with the TCR repertoire of Tconvs from the tumor or tumor DLN (Supplementary Fig. 6). Indeed, the cell population with the greatest degree of repertoire similarity with tumor-infiltrating Tregs was the Treg population from the tumor DLN. In addition, we observed a significant overlap in the repertoires of antigen-experienced Tconvs in the tumor and tumor DLN (Fig. 6E and Supplementary Fig. 7). These data strongly suggest that migration of both Tconvs and Tregs between these 2 sites occurs without conversion.

It could be argued that conversion of Tconvs into Tregs is instantaneous in the tumor environment. Should this be the case, it may not be possible to detect Tconvs and Tregs with overlapping repertoires in the tumor and an analysis such as ours would therefore fail to detect conversion. In vitro studies, however, argue against this scenario as it takes between 1 and 4 days for detectable levels of Foxp3 to be induced in Tconvs (29). Our own in vivo data also argue against the possibility of instant conversion. In this scenario, one would expect to observe a significant overlap not only between antigen-experienced Tconvs in the tumor DLN and Tconvs in the tumor (as shown in Fig. 6E and Supplementary Fig. 7A) but also between antigen-experienced Tconvs in the tumor DLN and Tregs in the tumor. No such overlap was observed (Fig. 6E and Supplementary Fig. 7B).

We selected the MCA tumor model for this work as several reports support a role for the immune system, including Tregs, in influencing disease progression (5, 30). In accordance with our findings, a recent study of T-cell responses in patients with colorectal carcinoma demonstrated that Tregs and Tconvs tend to recognize distinct antigens (31). Our findings are also similar to those from a study that analyzed the degree of conversion in self-antigen–specific T cells in the context of a mouse model of type I diabetes (17). The TCRs of Tregs and Tconvs isolated from the pancreas and pancreas DLN of BDC2.5/NOD TCR transgenic mice were distinct, thereby indicating no role for conversion in response to a pancreatic autoantigen. Similarly, a more recent study of the TCR repertoire and T-cell specificity in experimental allergic encephalomyelitis (EAE) concluded that, despite sharing autoantigen specificity, effector T cells and Tregs had largely distinct TCR repertoires, thereby similarly suggesting that conversion between these 2 populations was limited (32). Thus, the combined results of these studies imply that tolerance in the context of autoimmunity and tumor immunity is achieved without substantial conversion of conventional T cells into Tregs.

In contrast, a role for conversion may exist in preventing immune responses to some foreign antigens such as those of commensal bacteria in the gut (induced tolerance). In the intestine, the immune system must resist infection from occasional pathogens while maintaining tolerance to commensal flora and dietary antigens. The generation of Tregs by conversion from Tconvs has been suggested as an important mechanism for establishing this tolerance to persistent antigens in the gut (33). Recent data suggest that the induction of Foxp3 in gut-associated lymphocytes involves the conserved noncoding DNA element CNS1 (34).

The mechanisms that underlie Treg cell enrichment in tumors remain unclear. Our data indicate that the TCR repertoires of tumor-infiltrating Tconvs and Tregs are largely distinct, implying that conversion of Tconvs is not a significant cause of Treg enrichment in tumors. It is possible that Treg enrichment reflects the type of antigens expressed by tumor cells. Evidence suggests that the Treg population contains a higher number of cells that recognize self-antigens compared with Tconvs (35–37). Thus, in the tumor environment, Tregs may receive stronger antigen-driven signals than conventional T cells. It also remains possible that Tregs are preferentially recruited or retained in the tumor environment and/or that the tumor microenvironment promotes Treg cell proliferation and survival. It is known that Tregs exhibit a higher turnover than Tconvs under homeostatic conditions and that tumors can license dendritic cells (DC) to promote the proliferation of Tregs in lymph nodes, possibly through the production of TGFβ (38). Thus, strong antigenic signals within the tumor microenvironment accompanied by the secretion of cytokines, such as IL-2 (interleukin-2) and TGFβ, may serve to further promote Treg cell proliferation and survival. In support of this, it has previously been found that the majority of CD25+CD4+ Tregs infiltrating transplanted MCA-derived cell lines emanate from the recruitment and proliferation of naturally occurring CD25+ Tregs (39).

Tregs clearly play a role in the progression of some types of tumors and, on this basis, targeting these cells...
for immunotherapeutic purposes is attractive. Conversion of Tconvs into Tregs has been proposed as a means of Treg cell enrichment within TILs and hence, as a mechanism that could be targeted in the immunotherapeutic setting. Our study, however, indicates that Tconvs entering the tumor remain largely unconverted. It is more likely, therefore, that the enrichment of Tregs within TILs reflects differences in the way Tregs and conventional T cells are influenced by the tumor microenvironment. Understanding the nature of these influences should reveal mechanisms through which the balance between tumor-infiltrating Tregs and Tconvs can be altered for the purpose of halting tumor progression.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

References


Acknowledgment

The authors thank Drs. John Miles and R. James Matthews for invaluable advice and discussion and Kathryn Smart, Heather Leach, and Hayley Brogan for support with data analysis.

Grant Support

This work was supported by a Medical Research Council Senior Fellowship (G117/488) awarded to A. Gallimore, a University Award from the Wellcome Trust (096983), and an MRC project grant G0801190. D.A. Price is a Medical Research Council senior clinical fellow. K.K. Wynn is funded by a Wellcome Trust Value In People Award. C. Ferreira, Y. Singh, and J. Dyson were supported by funding from the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 25, 2010; revised November 23, 2010; accepted November 24, 2010; published OnlineFirst December 14, 2010.


Analysis of the T-Cell Receptor Repertoires of Tumor-Infiltrating Conventional and Regulatory T Cells Reveals No Evidence for Conversion in Carcinogen-Induced Tumors

James P. Hindley, Cristina Ferreira, Emma Jones, et al.

Cancer Res  Published OnlineFirst December 13, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-10-1797

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/12/13/0008-5472.CAN-10-1797.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.