T Cell Trafficking Facilitated by High Endothelial Venules is Required for Tumor Control after Regulatory T Cell Depletion

Running title: HEV neogenesis facilitates tumor rejection

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Abstract

The evolution of immune blockades in tumors limits successful anti-tumor immunity, but the mechanisms underlying this process are not fully understood. Depletion of regulatory T cells (Tregs), a T cell subset that dampens excessive inflammatory and autoreactive responses, can allow activation of tumor-specific T cells. However, cancer immunotherapy studies have demonstrated that a persistent failure of activated lymphocytes to infiltrate tumors remains a fundamental problem. In evaluating this issue, we found that despite an increase in T cell activation and proliferation following Treg depletion there was no significant association with tumor growth rate. In contrast, there was a highly significant association between low tumor growth rate and the extent of T cell infiltration. Further analyses revealed a total concordance between low tumor growth rate, high T cell infiltration and the presence of high endothelial venules (HEV). HEV are blood vessels normally found in secondary lymphoid tissue where they are specialized for lymphocyte recruitment. Thus, our findings suggest that Treg depletion may promote HEV neogenesis, facilitating increased lymphocyte infiltration and destruction of the tumor tissue. These findings are important as they point to a hitherto unidentified role of Tregs, the manipulation of which may refine strategies for more effective cancer immunotherapy.
Introduction

Several laboratories have shown that CD4+ CD25+ Foxp3+ regulatory T cells (Tregs), which normally serve to control autoimmunity and inflammation, also inhibit immune responses to tumor antigens (1). Thus, strategies aimed at boosting anti-tumor responses, through manipulation of Tregs, are being intensely investigated. In mice, prophylactic depletion of Tregs using CD25-specific antibodies (Abs) can limit and even prevent tumor development and/or progression (2-4). The development of transgenic mice expressing the primate diphtheria toxin receptor (DTR) on Foxp3+ cells has allowed specific and complete depletion of Tregs by administration of diphtheria toxin (DT) (5). Using the melanoma cell-line B16, it has been demonstrated that selective Treg depletion using anti-CD25 Abs (6) or DT (7) results in activation of a tumor-specific CD8+ T cell response and slower tumor growth. Use of the DTR-Foxp3 transgenic mice has also shown that Treg depletion can prevent development and even limit the progression of tumors induced by the carcinogen methylcholanthrene (MCA) in a manner that is dependent on CD8+ T cells and IFNγ (8).

The success of these interventions however remains sub-optimal. Treg-depletion, even when combined with vaccination, does not readily result in the elimination of established tumours as tumor rejection is often observed in only a proportion of treated mice. Several factors, including the extent of T cell activation and/or tumor-infiltration, may account for this failure (6). In this study, we set out to identify factors limiting the success of anti-tumor immune responses. For this purpose, we utilised the MCA chemical carcinogenesis model in combination with Foxp3DTR mice (5), to identify the key features that distinguish progressing tumors from those that are controlled after Treg-depletion. Our findings clearly indicate that T cell infiltration and not the extent of activation, is a critical bottleneck to tumor destruction in Treg-depleted animals. Moreover, our extensive
immunohistochemical analyses of MCA-induced tumors from Treg-replete and Treg-depleted mice revealed that control of tumour growth, observed in Treg-depleted mice, was determined by development of high endothelial venules (HEV), specialised blood vessels that alter both the composition and size of the T cell infiltrate (9, 10). Overall, these data provide a new perspective on the impact of Treg depletion, demonstrating that Tregs control immune responses, not only through limiting immune activation, but also through influencing blood vessel differentiation.
Materials and Methods

Mice

We are grateful to Professor Alexander Rudensky for supplying Foxp3\textsuperscript{DTR} mice. These were backcrossed with C57BL/6 mice for $\geq 5$ generations and housed in accordance with UK Home Office regulations.

Tumor induction, DT administration and tumor monitoring

Foxp3\textsuperscript{DTR} mice were injected subcutaneously in the left hind leg with 400 µg of 3-methylcholanthrene (MCA; Sigma-Aldrich) in 100 µl of olive oil under general anesthetic as described previously (11). Mice were monitored for tumor development weekly up to 2 months and daily thereafter. Tumor-bearing mice were culled before their tumors reached 1.7 cm in diameter, typically 80-150 days after injection or if tumors caused discomfort. Diphtheria toxin (DT; Sigma) diluted in PBS was administered intraperitoneally (i.p.) every other day after tumor detection.

Tumor growth rate (k, days\textsuperscript{-1}) was determined using a statistical software package Prism 5 (GraphPad) with the following equation for exponential growth: $Y = Y_0 \times \exp(k \times X)$. Tumor diameter (X, mm) was measured every 2 days using calipers. On average, measurements were taken for 12 days for PBS-treated mice and 17 days for DT-treated mice. Therefore, on average 6-8 measurements were used to calculate tumor growth rate.

Flow Cytometry
Single cell suspensions of spleens, inguinal lymph nodes and tumors were prepared. The inguinal lymph node from the tumor (left) side was taken as the tumor-draining lymph node (D) and the contralateral inguinal lymph node as a non-draining lymph node (ND).

For analysis of intracellular cytokines by flow cytometry, single cell suspensions were stimulated with 20 nM phorbol myristate acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich). Cells were incubated at 37°C for a total of 4 hours. After 1 hour 1 μl/ml of GolgiStop (containing monensin; BD) was added to each well.

To identify dead cells a fixable dead cell staining kit (LIVE/DEAD Aqua; Invitrogen) was used. Cells were washed twice in PBS and 3-6 μl of diluted (1:10; in PBS) dead cell stain was added to the cell pellet. Cells were stained at RT for 15 minutes in the dark then washed twice in FACS buffer. For surface-marker staining, fluorescently-labeled mAbs: anti-CD4 Pacific Blue (BD), anti-CD8 PerCP-Cy5.5 (eBioscience), anti-CD25 PE (eBioscience) were used. Then cells were fixed, permeabilized and stained for intracellular antigens using the Foxp3 Fix/Perm buffer set (eBioscience) and the following fluorescently labeled mAbs: anti-Foxp3 PE-Cy7 (eBioscience), anti-Ki67 Alexa Fluor 647 (BD), anti-IFNγ APC (eBioscience), anti-IL2 PE (eBioscience), anti-granzyme B Alexa Fluor 647 (eBioscience) and anti-CD107a PE (eBioscience). For flow cytometric analysis, samples were acquired using a FACS Canto II flow cytometer (BD) and were analyzed using FACSDiva software (BD).

**Immunohistochemistry**

**Paraffin sections**

Tumors were collected from Foxp3<sup>DTR</sup> mice and fixed in neutral buffered formalin and embedded in paraffin. Sections 5 μm in thickness, were cut and mounted on slides, dewaxed
in xylene and hydrated using graded alcohols to tap water. After performing antigen retrieval, by microwaving in 10 mM Tris, 1 mM EDTA buffer pH9 for 8 min, sections were cooled for 30 min and then equilibrated in PBS. Endogenous peroxidase activity was quenched with Peroxidase Suppressor (Thermo Scientific) for 15 min and non-specific antibody binding was blocked by incubating sections with 2.5% normal horse serum (VectorLabs) in PBS for 30 min. Sections were stained overnight at 4°C with the following primary antibodies diluted in 1% BSA in PBS: rat anti-peripheral node addressin (PNAd) antibody (clone MECA-79; Biolegend) and rat anti-Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) antibody (clone MECA-367; Biolegend). Primary antibodies were detected with anti-rat ImmPRESS (VectorLabs) for 30 min and visualized with impact DAB (VectorLabs). Sections were counterstained with haematoxylin, dehydrated in an ethanol series and mounted in DPEX. Photomicrographs were taken using a NIKON microscope and digital camera.

Frozen sections

Tumors and lymph nodes were collected from Foxp3DTR mice, embedded in optimum cutting temperature compound (OCT; RA Lamb) and snap frozen in liquid nitrogen. Sections, 5 µm in thickness, were fixed for 10 min in ice-cold acetone and left to dry at room temperature. Slides were washed in PBS and blocked with Avidin/Biotin Blocking Kit (VectorLabs) and then 2.5% normal horse serum (VectorLabs) in PBS for 30 min. Sections were stained overnight at 4°C with the following primary antibodies diluted in 1% BSA in PBS: rat anti-PNAd antibody (clone MECA-79; Biolegend), Alexa Fluor 488 rat anti-MAdCAM-1 antibody (clone MECA-367; BioLegend), biotin rat anti-PNAd antibody (clone MECA-79; BioLegend), rat anti-CD4 (clone RM4-5; eBioscience), rat anti-CD8 (clone 53-6.7; eBioscience), FITC rat anti-CD31 (clone 390; eBioscience), biotin rat anti-CD45R/B220 (clone RA3-6B2; eBioscience), rabbit anti-CD3 (DAKO) and rat anti-CD35 (clone 8C12; BD
Pharmingen). Primary antibodies were detected with Alexa Fluor 488 goat anti-rat, Alexa Fluor 488 goat anti-rabbit and streptavidin-Alexa Fluor 555 (all from Invitrogen Life Sciences). When sections were double-stained with two unconjugated rat primary antibodies the first rat primary antibody (anti-CD4 or CD8) was detected with Alexa Fluor 488 anti-rat then fixed in 1% paraformaldehyde for 10 mins before using a biotinylated second rat primary antibody (anti-PNAd), which was detected by streptavidin-Alexa Fluor 555. All sections were counter-stained with TOTO-3 iodide (Invitrogen) before fixing with 1% paraformaldehyde for 10 mins and mounting with Vectashield, containing DAPI (4,6-diamidino-2-phenylindole; Vectorlabs). Images were collected with a Zeiss LSM5 Pascal confocal microscope. Images recorded in the far-red channel were pseudocolored blue. Images were assembled in Adobe Photoshop software.

Gene expression analysis

Samples were cut in 10 µM sections from OCT embedded tissue and RNA extracted using TRIZol reagent (Invitrogen). Gene expression profiling was carried out using MouseRef-8v2.0 whole genome expression bead chip (Illumina) as recommended by the manufacturer. Probe intensity values were corrected by background subtraction in GenomeStudio software and subsequently log-2 and baseline (median) transformed using Genespring software (Agilent) before analysis of genes.

Statistical analyses

All statistical analyses were performed using Prism 5 (GraphPad). Unless stated otherwise, data groups were compared using an unpaired, non-parametric Mann-Whitney test and displayed as median. Where stated, data groups were compared by an unpaired t test following confirmation that the set was normally distributed using the D’Agostino and
Pearson omnibus normality test. In this instance the mean was graphed. Correlation analyses were performed using the Pearson method and Pearson correlation coefficients ($r^2$) are displayed.
Results

Activation of tumor-infiltrating lymphocytes (TILs) after Treg depletion and impact on tumor progression

Previously we have found that Foxp3⁺ Tregs are significantly enriched in all MCA-induced fibrosarcomas (2, 11). Tumors were induced using the chemical carcinogen MCA in transgenic mice expressing the primate diphtheria toxin (DT) receptor on Foxp3⁻ cells (Foxp3DTR mice, (5)). Optimal Treg depletion in Foxp3DTR mice bearing MCA-induced tumors was determined by injecting increasing doses of diphtheria toxin (DT, 0.1 – 5 µg/kg) every other day. Successful depletion of Foxp3⁺ T cells was obtained in all mice after injecting 5 µg/kg DT (< 0.5% CD4+ cells expressing Foxp3 remain, Supplemental Figure 1 and (5)); concomitant activation of CD4⁺ FoxP3⁻ and CD8⁺ T cells was confirmed by examining expression of Foxp3, CD25 and Ki67 (Supplemental Figure 2 and (5)). A highly significant reduction in tumor growth rate was observed in DT treated (Treg⁻; n=43) compared to control treated (Treg⁺; n=24) mice (P=0.0008; Figure 1) and tumor regression was observed in 12% (n=5) of mice (tumor growth rate <0; Figure1). However, despite the overall reduction in tumor growth rate, no difference in growth rate was observed in many of the treated mice.

As T cells have been shown to control MCA-induced tumors following Treg depletion (7), we postulated that control of tumor growth would correlate with the extent of T cell activation following depletion of Tregs. Indeed, analysis by flow cytometry revealed a highly significant increase in the percentage of both CD8⁺ and CD4⁺ T cells in the spleen and tumors of Treg-depleted compared to control mice (Supplemental Figure 3). However, when we examined CD4⁺ and CD8⁺ TILs for expression of the activation markers CD25 and Ki67 and also for functional markers, IFNγ, IL-2, granzyme-B and CD107a we found no
significant correlation between tumour growth rate and any of these markers (Figure 2 and Supplemental Figure 4), indicating that following Treg-depletion, the extent of CD8+ or CD4+ T cell activation is not a critical bottleneck to successful control of tumor growth. We also evaluated whether tumor size at the start of treatment correlated with treatment success. The data, shown in Figure 3, clearly indicate that this is not the case, as the impact of Treg-depletion on tumor growth kinetics was not influenced by the size of the tumor at the start of treatment.

**Tumor control is associated with accumulation of TILs**

Next, we addressed whether the extent of T cell infiltration following Treg-depletion was associated with control of tumor growth. To enumerate tumor-infiltrating T cells, we performed extensive immunohistochemical analyses of 14 Treg- and 8 control, Treg+ tumors with representative examples of CD8- and CD4-specific staining shown in Figures 4A and 4B. A significant increase in the number of CD8+ cells (P=0.0140) and an overall increase in the number of CD4+ cells (P=0.0818) was observed in the tumors of Treg- versus control Treg+ mice (Figures 4C and 4D). However, the accumulation of tumor infiltrating T cells was not universal amongst treated mice, as many of these did not differ significantly from the control group (Figures 4C and 4D). Indeed the Treg- mice could clearly be split into two subgroups: those with less than 100 (TILlo) and those with greater than 100 (TILhi) CD4+ or CD8+ cells per high power field (Figures 4A–D). The correlation between the level of CD8+ and CD4+ cell infiltration and tumor growth rate in Treg- mice was striking (P=0.0238 and P=0.0047 respectively; Figures 4E and 4F) whereas no correlation was observed in control Treg+ mice (Figure 4G and H). Whilst we do not rule out a role for T cells in controlling tumors in any Treg+ tumor (particularly those with lower growth rates), these data indicate
that following Treg-depletion, T cell infiltration determines successful control of tumor growth.

**HEV neogenesis in Treg-depleted, but not Treg-replete tumors**

We hypothesized that those tumors that are controlled after depleting Tregs would differ from those that continue to progress with respect to mechanisms governing T cell trafficking. Thus, tumors were examined by immunohistochemistry for markers associated with high endothelial venules (HEV), specialized post capillary venules found in secondary lymphoid tissues which facilitate extravasation of naïve and central memory T cells from blood to lymphoid organs (9). For this purpose, tumors were stained using antibody clone MECA-79 which recognizes peripheral node addressin (PNAd), a carbohydrate epitope expressed on a variety of L-selectin ligands which is specifically expressed by HEV (12). No PNAd staining was observed in any of the tumors recovered from Treg+ control animals (Figures 5A, n = 14). However, PNAd staining was identified in 50% of tumors recovered from animals depleted of Treg (Figures 5B, C, D, and E, n = 14). The cuboidal morphology of the cells (black arrowheads) expressing PNAd in the tumors was consistent with HEV morphology (Figure 5D). Furthermore, PNAd expression often co-localised with Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), an HEV-associated molecule, normally expressed on immature HEV, adult mucosal lymphoid tissue and lamina propria (Figure 5F). To determine whether PNAd was expressed on endothelial cells, we co-stained tumor sections with the endothelial cell marker CD31 (PECAM-1), and PNAd. Whilst many CD31+ cells do not express either MAdCAM-1 or PNAd, our data clearly show co-expression of CD31 on all MAdCAM-1 or PNAd+ cells (Figure 5G). Collectively, these data indicate the presence of HEV within tumor tissue. Under non-pathological conditions, HEVs are confined to secondary lymphoid tissues. HEV develop as an integral part of the stromal component of...
lymph nodes during lymphoid neogenesis in embryonic life. Lymphotoxins α (LTα) and β (LTβ) are required for the development of all HEV-containing lymph nodes in mice and the lymphoid chemokines, CCL21, CCL19 and CXCL13 segregate incoming lymphocytes into discrete T- and B-cell containing areas (9, 13-15). To determine whether the same mechanisms contribute to HEV neogenesis in tumors, expression of these molecules, as well as the PNAad scaffold protein GlyCAM-1, was compared in tumors containing HEVs (Treg-, HEV+) and in tumors with no detectable HEVs (Treg+, HEV- and Treg-, HEV-). Expression of each molecule was increased in the HEV+ tumors (Figure 6A) indicating that mechanisms facilitating development of HEV-containing lymph nodes also exist during HEV neogenesis in tumors (reviewed in (9)). HEV neogenesis has also been reported under conditions of chronic inflammation and in autoimmune lesions, where their presence is often associated with the development of tertiary lymphoid organs (TLO) (9, 16, 17). The events that govern HEV neogenesis and TLO development at these sites are undefined. However, TLOs, like secondary lymph nodes are characterised by discrete T- and B- cell areas with a follicular dendritic cell (FDC) network forming the centre of the B cell area. We therefore performed extensive immunohistochemical staining for T and B cells and FDCs but found that in contrast to the organisation found in lymph nodes, T and B cells were intermingled throughout the tumor mass and there was no FDC network (Figures 6B and C). Furthermore, CD4⁺CD3⁻IL7R⁺,RORγ T lymphoid tissue inducer (LTi) cells, which are required for secondary lymph node development were not detected in HEV+ tumours (Supplemental Figure 5) (18-20). Collectively, these data indicate that although HEV neogenesis is accompanied by expression of the lymphoid chemokines CCL19, CCL21 and CXCL13, it takes place without the need for LTi cells or for lymphoid neo-organogenesis. It is however possible, that HEV development and expression of lymphoid chemokines represent the early stages of this process, preceding development of TLO.
**HEV neogenesis promotes T cell infiltration and tumor control**

HEV neogenesis has been reported in the context of chronic inflammation and autoimmune disease in both mouse models and human disease (9, 21, 22). Consistent with their ability to facilitate increased lymphocyte extravasation, HEV are found in regions of extensive lymphocytic infiltrate. Thus, we compared the number of tumor-infiltrating T cells in HEV+ and HEV- tumors. A clear association was observed between presence of HEV within tumor tissue and a high number of CD8+ T cells (Figure 7A; P=0.0034). When we compared the presence of HEV with tumor growth rate, HEV+ tumors showed significantly reduced tumor growth rate compared to HEV- tumors (Figure 7B; P=0.0082). Interestingly, no correlation was observed between the extent of CD31 staining and numbers of CD8+ T cells, indicating that the development of HEV and not blood vessel development *per se*, is central to the increase in T cell infiltration and control of tumor growth (Figure 7C). In support of the premise that tumor HEV allow the infiltration of T cells into the tissue, we frequently found T cells attached to or extravasating through the luminal wall of the vessel (Figure 7D and E, white arrowheads). Moreover, when expression of CD3, CCR7, L-Selectin and the transcription factor, KLF2 were assessed and compared between HEV- and HEV+ tumors, there was a clear increase in the expression of these molecules in the HEV+ tumors (Figure 7F). Thus, these data suggest that development of HEV in tumors, allows infiltration of L-selectinhiCCR7+ T cells, which includes both central memory and naïve T cells. In support of this, we have observed clonal expansion within tumor-infiltrating T cells, suggesting that an antigen driven response is generated intra-tumorally (Supplemental Figure 6). Overall, these data strongly support the premise that following Treg depletion, the formation of HEV shapes the size and composition of the T cell infiltrate resulting in tumor control.
Discussion

Selective depletion of Foxp3+ regulatory T cells (Tregs) can result in immune-mediated control of tumors. We examined the distinguishing features of controlled versus progressing tumors following therapeutic Treg depletion in mice bearing palpable carcinogen-induced tumors. Previously, using the tumor cell-line, B16, it was reported that whilst Treg-depletion promoted systemic T cell activation (as also shown above), control of tumor growth was limited by failure of the T cells to infiltrate the tumor (6). In this and previous studies, it was demonstrated that sensitising the tumor stroma through irradiation or inflammation, induced vascular remodelling and up-regulation of adhesion molecules facilitating T cell infiltration into tumours (6, 23). The data presented herein identify, for the first time, a previously unsuspected effect of Treg-depletion in facilitating neogenesis of HEV, which enable T cell recruitment into the tumor from blood. The significance of this effect is striking: HEV are not observed unless Tregs are depleted and there is an absolute concordance between presence of HEV, high numbers of tumor-infiltrating T lymphocytes and control of tumor growth.

As yet, we have not identified the mechanisms underpinning HEV neogenesis in tumors. Under non-pathological conditions, HEV development is confined to secondary lymphoid tissue. Ligation of the LTβ receptor (LTβR) on stromal-organiser cells by LTαβ expressing LTi cells represents a key event in the development of lymph nodes (18, 19). Although LTβR signalling is required to maintain fully differentiated HEV (24, 25), mechanisms underpinning HEV formation in lymph nodes and whether they are related to those driving stromal cell differentiation remain incompletely defined. HEV neogenesis within chronically inflamed tissue was first reported around 15 years ago and more recently in tumors (26-28). As in lymph nodes, the presence of HEV is almost always associated with the development of a stromal cell network that organises infiltrating lymphocytes into
discrete T and B-cell areas in so-called tertiary lymphoid organ (29-31). However, the mechanisms underlying HEV neogenesis and how it relates to the development of the stromal cell network of TLO are poorly defined. In the case of the HEV+ tumors described in this study, whilst we have found no evidence of LTi cells or TLOs, expression of inflammatory cytokines and lymphoid chemokines is increased. Collectively these data imply that HEV neogenesis in tumors occurs through mechanisms that promote inflammation (in this case, Treg depletion) and deviate from those operating in secondary lymph nodes, possibly in terms of the cellular source of LTαβ.

An unexplained aspect of our findings is that HEV-development, whilst tightly linked to Treg-depletion, is only observed in around half of Treg-depleted mice. Several explanations may account for this finding, an exploration of which may offer vital clues to defining the mechanisms underpinning HEV neogenesis in tumours. Firstly, the nature of the immune response stimulated following Treg depletion may affect whether HEV develop or not. Perhaps, following Treg-depletion, T cells and or B cells in some, but not all mice, produce important HEV inducing factors (e.g. LTαβ). Furthermore, the tumor may specifically impinge on the type of immune response induced following Treg depletion. Tumors that retain immunogenicity, perhaps as a result of Treg-induced immunosuppression, may activate tumor-specific T and B cells which, as described above, drive HEV development. On the other hand, tumors that have escaped the attention of the immune system through the loss of strong antigens (immunoediting), may fail, even after Treg-depletion, to induce a sufficiently robust, HEV-promoting immune response. Indeed, Matsushita et al. recently demonstrated that the outgrowth of MCA-induced tumors could result from T cell dependent selection of tumours lacking strong antigens (32). Alternatively, tumour-intrinsic features may influence HEV development in a stochastic fashion. For instance, tumors may produce factors that either actively inhibit or facilitate HEV
development. Gaining an understanding of these complex issues will uncover the mechanisms underpinning HEV neogenesis in tumors and facilitate a better understanding of whether a given immune response can promote tumor immunity through blood vessel differentiation.

The importance of understanding how HEV can develop in peripheral sites has clear implications for cancer immunotherapy. Recently, in a study of over 300 human tumors, Martinet and colleagues discovered a highly significant association between the presence of HEV and the frequency of tumor-infiltrating T cells (26). Moreover, when a cohort of around 150 breast cancer patients were investigated, the same study reported a significant correlation between HEV and a favorable prognosis. These data support a crucial role for HEV neogenesis in tumor rejection. Our study highlights one avenue towards achieving this goal, namely the depletion of Tregs. It would be extremely interesting therefore to examine whether in large cohorts of human tumors such as those used in the above studies, there is an inverse association between Tregs and the presence of HEV.

Although the precise mechanism of HEV induction in our study remains to be elucidated, the significant increase in expression of LTβ in HEV+ tumors and the previously described role for LTαβ in maintaining fully functional HEV, support an important role for this cytokine. A study by Schrama et al. showed that administration of an LTα fusion protein into B16 melanoma-bearing mice resulted in HEV neogenesis, which subsequently promoted entry of naïve T cells that were primed intra-tumorally and which contributed to tumor destruction (10). Similarly, Yu et al. reported the same effect in mice inoculated with LIGHT-expressing tumors; this effect was attributed to the ability of LIGHT, like LTα to facilitate generation of lymphoid structures which enabled entry of naïve T cells into the
tumor (33). In both cases, this resulted in efficient priming of tumor-specific T cells, presumably due to the high probability of these T cells meeting their cognate antigen within the tumor mass. We suggest that depleting Tregs can lead to HEV neogenesis; a process that is critical in determining the composition and size of the T cell infiltrate thus permitting tumor destruction. Whilst the mechanisms of HEV induction and the influence of Tregs on this process are poorly understood, it is clear that a better understanding of these mechanisms will present new opportunities for more effective anti-tumor therapy. Furthermore, HEV neogenesis may play deleterious role in autoimmunity and chronic inflammation (9). Our findings may therefore point to a hitherto unidentified mechanism through which a defect in Treg activity drives autoimmune disease and chronic inflammation by allowing HEV induction, hence sustained immune cell infiltration and destruction of the affected tissue. Harnessing this activity of Tregs therefore has implications for both cancer immunotherapy and the treatment of chronic inflammatory diseases.
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References


Figure legends

Figure 1 – Depletion of Foxp3+ Tregs reduces tumor growth rate

Tumor bearing Foxp3\textsuperscript{DTR} mice received either DT (n= 43) or control treatment (n=24). Tumors were measured every other day during treatment and tumor growth rates (k, days-1) were calculated. ***, P < 0.001; Mann-Whitney test. Data from at least 3 independent experiments.

Figure 2 – Reduced tumor growth rate following depletion of Foxp3+ Tregs does not correlate with the extent of T cell activation

The proportion of tumor infiltrating CD8\(^+\) T cells expressing CD25, Ki67, the cytokines IFN\(\gamma\) and IL-2 and the functional markers granzyme B and CD107a were correlated with tumor growth rate (A - F). Statistical significance was evaluated using the Pearson correlation method. Data from at least 3 independent experiments.

Figure 3 - Reduced tumor growth rate following depletion of Foxp3+ Tregs does not correlate with tumor size at start of treatment

Mice were monitored for tumor development weekly and injected with DT typcially 80 – 150 days after MCA injection. Tumor size was evaluated at the start of treatment and measured every 2 days thereafter as described in Materials and Methods. Statistical significance was evaluated using the Pearson correlation method. Data from at least 3 independent experiments.
Figure 4 – Tumor growth rate is associated with the extent of CD8+ and CD4+ T cell infiltration

Tumor sections were stained with anti-CD8 or -CD4 antibodies (green) and counterstained with TOTO-3 (blue). Representative images from control and Treg depleted mice with low or high T cell counts are shown (A, B). CD8+ and CD4+ T cells from Treg depleted (n = 14) and control mice (n = 8) and were compared (C, D) and correlated with individual tumor growth rates (E, F = DT-treated mice, G, H = control untreated mice).

Figure 5 – Staining of HEV associated molecules PNAd and MAdCAM-1 in tumors from Treg depleted mice

Paraffin embedded sections, from Treg replete (Treg+) and Treg depleted (Treg-) tumors, were stained with anti-PNAd antibodies (brown) and counterstained with haematoxylin (blue; A - D). Representative low power (A, B) and high power (C, D) fields of view are shown. Cells stained with anti-PNAd antibodies display a cuboidal morphology typical of HEV (black arrowheads). Frozen sections from Treg+ (n=6) and Treg- (n=14) tumors were stained with anti-PNAd antibodies and levels of PNAd expression were determined by quantitating the percentage of PNAd-positive pixels per area of tumor (E). Frozen sections, from controlled tumors, were stained with anti-PNAd (red) and anti-MAdCAM-1 (green) antibodies (F) and anti-PNAd (red) and anti-CD31(green) antibodies (G). Cells were counterstained with TOTO-3 (blue). Data from at least 2 independent experiments.

Figure 6 – HEV Neogenesis is associated with expression of lymphoid chemokines but not development of TLO
The relative expression of various HEV associated genes were determined and compared between (Treg+, HEV-), (Treg-, HEV-) and (Treg-, HEV+) tumors (A). Frozen sections, from lymph nodes (B) and Treg depleted (Treg-), HEV+ tumors (C), were stained with antibodies specific for B and T cells (red and green; anti-B220 and anti-CD3), follicular dendritic cells (green; anti-CD35) or T cells and PNAd (green and red; anti-CD3 and anti-PNAd). Cells were counterstained with TOTO-3 (blue). Data from at least 2 independent experiments.

**Figure 7 – Increased T cell infiltration and reduced tumor growth rate in HEV+ Treg depleted tumors**

The number of tumor infiltrating CD8+ cells (A) and the tumor growth rates (B) of (Treg+ HEV-, n = 8), (Treg- HEV-, n = 6), and (Treg- HEV+, n = 7) tumors were compared. ***, P < 0.01; Mann-Whitney test. Frozen sections were stained with CD31- and CD8-specific antibodies. Levels of CD31 expression were determined by quantitating the percentage of CD31-positive pixels per area of tumor and compared with numbers of CD8+ T cells (C). Frozen sections, from HEV+ tumors, were stained with anti-PNAd (red) and anti-CD8 (green) antibodies (D) or anti-PNAd (red) and anti-CD4 (green) antibodies (E). Cells were counterstained with TOTO-3 (blue). CD8+ and CD4+ cells are seen attached to and extravasating through the vessel wall (white arrowheads; D and E). Gene expression analysis of T cell associated genes was compared between tumor samples (F). Data from at least 2 independent experiments.
Figure 1.

Tumor growth rate $k$ (days$^{-1}$)

- Treg+
- Treg-

***P = 0.0008

Progression

Regression

Downloaded from cancerres.aacrjournals.org
Figure 2.

A. CD25

\[ R^2 = -0.222 \]
\[ \text{ns } P = 0.257 \]

B. Ki67

\[ R^2 = 0.040 \]
\[ \text{ns } P = 0.850 \]

C. IFN\( \gamma \)

\[ R^2 = -0.199 \]
\[ \text{ns } P = 0.363 \]

D. IL-2

\[ R^2 = 0.231 \]
\[ \text{ns } P = 0.228 \]

E. Granzyme B

\[ R^2 = -0.411 \]
\[ \text{ns } P = 0.091 \]

F. CD107a

\[ R^2 = 0.048 \]
\[ \text{ns } P = 0.806 \]
Figure 3.

Tumour growth rate ($k$, days$^{-1}$) vs. Tumour start size (mm).

$r^2 = 0.0001417$
$nsP = 0.9411$
Figure 4.

A. CD8+ T cells

B. CD4+ T cells

C. 

D. 

E. 

F. 

G. 

H. 

No of CD8+ cells (per high power field)

No of CD4+ cells (per high power field)

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Figure 5.

A. PNAd Treg+  B. PNAd Treg-

C. PNAd Treg-  D. PNAd Treg-

E. *P=0.0287

F. PNAd MAdCAM-1  G. PNAd CD31
T cell trafficking facilitated by high endothelial venules is required for tumor control after regulatory T cell depletion

James P. Hindley, Emma Jones, Kathryn Smart, et al.

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