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T helper type 17 cells contribute to anti-tumour immunity and promote the recruitment of T helper type 1 cells to the tumour

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Summary

T helper type 17 (Th17) lymphocytes are found in high frequency in tumour-burdened animals and cancer patients. These lymphocytes, characterized by the production of interleukin-17 and other pro-inflammatory cytokines, have a well-defined role in the development of inflammatory and autoimmune pathologies; however, their function in tumour immunity is less clear. We explored possible opposing anti-tumour and tumour-promoting functions of Th17 cells by evaluating tumour growth and the ability to promote tumour infiltration of myeloid-derived suppressor cells (MDSC), regulatory T cells and $CD4^+$ interferon-γ^+ cells in a retinoic acid-like orphan receptor γt (RORγt) -deficient mouse model. A reduced percentage of Th17 cells in the tumour microenvironment in RORγt-deficient mice led to enhanced tumour growth, that could be reverted by adoptive transfer of Th17 cells. Differences in tumour growth were not associated with changes in the accumulation or suppressive function of MDSC and regulatory T cells but were related to a decrease in the proportion of $CD4^+$ T cells in the tumour. Our results suggest that Th17 cells do not affect the recruitment of immunosuppressive populations but favour the recruitment of effector Th1 cells to the tumour, thereby promoting anti-tumour responses.

Keywords: anti-tumour immunity; Rorγt; T helper type 1 cells; T helper type 17 cells.

Introduction

Following their characterization as a functionally distinct T helper lineage, T helper type 17 (Th17) cells have become an important focus of cancer immunology in light of several studies that have reported their high frequency in various cancers, including ovarian, breast, colon, prostate and melanoma tumours.[1–4] Moreover, it has been demonstrated that tumour-secreted chemokines support the mobilization and expansion of Th17 cells. [4] These findings have sparked tremendous curiosity and efforts to characterize the function of Th17 cells in cancer development. In haematopoietic tumours, the expression of IL-17 was reported to reduce tumour growth rate by promoting the generation of tumour-specific $CD8^+$ cytotoxic T cells.[5] In contrast, interleukin-17 (IL-17) over-expression in fibrosarcoma and colon carcinoma promotes tumour growth both in immunocompetent and immunoablated mice, and correlates with increased microvascularity in the tumour, suggesting that IL-17 contributes to angiogenesis.[6] By addressing the function of Th17 cells using *Il17*$^{-/-}$ mice, it has been shown that the absence of this cytokine increases tumour growth and colonization of melanoma and colon carcinoma loci in the lungs and correlates with decreased infiltration of $CD4^+$ and $CD8^+$ T cells.[7,8] In contrast, reduced growth of solid melanoma and bladder carcinoma tumours has been observed in *Il17*$^{-/-}$ mice along with an increased number

Abbreviations: IFN, interferon; IL-17, interleukin-17; MDSC, myeloid-derived suppressor cell; Rorγt, retinoic acid-like orphan receptor γt; Th, T helper; Treg, regulatory T; WT, wild-type

of interferon-γ^+ (IFN-γ^+) $CD4^+$ and $CD8^+$ T cells.[9] The basis for these contradictory functions associated with IL-17 has not been elucidated. Treatment of mice with tumour-specific Th17 polarized cells has been reported to revert the growth of established solid and lung melanoma, and this is associated with enhanced antigen presentation and activation of tumour-specific $CD8^+$ cytotoxic T cells. However, since this effect depends on IFN-γ, it could be mediated by reprogramming the transferred Th17 cells into Th1 cells.[8,10] When Th17 cells are specifically induced at the tumour site with IL-6-transduced pancreatic cancer cells, the enhanced generation of Th17 cells has protective activity against pancreatic tumour growth and improved survival.[11] Altogether, the exact functions of Th17 cells and IL-17 in the tumour environment remain to be fully defined.

Inflammatory responses play an important role in different stages of cancer development. Cross-talk between the tumour stroma and immune cells can subvert the protective anti-tumour response into signals that enable tumour growth, angiogenesis and metastasis.[12] Myeloid-derived suppressor cells (MDSC) are a heterogeneous population composed of myeloid progenitor cells and immature myeloid cells that strongly suppress T-cell proliferation and are greatly expanded in human and mouse tumours.[13] Recent studies have suggested that chronic inflammation promotes tumour progression by down-regulating anti-tumour responses through the induction of MDSC by pro-inflammatory cytokines IL-6 and IL-1β present in the tumour environment[14] as well as other inflammatory mediators induced in various cell types by IL-17.[15–20] These MDSC can in turn promote the development of regulatory T (Treg) cells *in vivo* in tumour-bearing mice.[21] Moreover, two studies reported an effect of IL-17 in promoting the recruitment of immunosuppressive populations to the tumour microenvironment. The first study showed that the development of tumours was inhibited in IL-17R-deficient mice or after IL-17 neutralization using antibodies, whereas systemic administration of IL-17 promoted tumour growth. This study also demonstrated that IL-17 promotes tumour progression by recruiting MDSC and by reducing $CD8^+$ T-cell infiltration in the tumour microenvironment.[22] A second study showed that IL-17 mediates Treg cell infiltration by up-regulating CCL17 and CCL22 chemokines and Treg cell activity by up-regulating CD39 and CD73 expression on these cells.[23]

In the current study, we examined the effect of endogenous Th17 cells in the development of intradermal B16.F10 melanoma using the Ror$\gamma t^{gfp/gfp}$ mouse model, which has impaired Th17 differentiation. We confirmed that Ror$\gamma t^{gfp/gfp}$ mice have a reduced frequency of Th17 cells in the tumour as well as undetectable levels of IL-17. Importantly, Ror$\gamma t^{gfp/gfp}$ mice show enhanced tumour growth compared with wild-type (WT) mice. Further comparison of different populations in the tumour environment revealed that the enhanced tumour growth did not correlate with a higher frequency of immunosuppressive MDSC and Treg cells; however, retinoic acid-like orphan receptor γt (RORγt) -deficient mice show a significant decrease in the percentage of total $CD4^+$ T cells, and an increase in the percentage of $CD8^+$ T cells.

Our results support the idea that Th17 cells may provide protection against tumour growth by promoting the recruitment of Th1 cells into the tumour.

Materials and methods

Mice

C57BL/6 and Ror$\gamma t^{gfp/gfp}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and kept in an animal facility under standard housing guidelines. The animal work was carried out under institutional regulations of Fundacion Ciencia y Vida and the Facultad de Ciencias, Universidad de Chile, and was locally approved by an ethics review committee. Before conducting experiments, the genotype of 21-day-old Ror$\gamma t^{gfp/gfp}$ and Ror$\gamma t^{+/gfp}$ pups was tested with DNA isolated from the tail through conventional PCR, using Platinum PCR Supermix (Invitrogen, Carlsbad, CA) and the following primers (Invitrogen): forward 5′-CCC CCT GCC CAG AAA CAC T-3′; reverse 5′-GGA TGC CCC CAT TCA CTT ACT TCT-3′; mutant reverse 5′-CGG ACA CGC TGA ACT TGT GG-3′. PCR products were 174 bp in WT mice, 241 bp in Ror$\gamma t^{gfp/gfp}$ homozygous mice, and 174 and 241 bp in Ror$\gamma t^{gfp/+}$ heterozygous mice.

Tumour cells

B16.F10 murine melanoma cells were obtained from the American Type Culture Collection (Manassas, VA). B16.OVA murine melanoma cells were kindly provided by Dr Randolph Noelle (King's College London, UK). Hepa 1-6 cells and MB49 cells were kindly provided by Dr Luis Burzio (Fundacion Ciencia & Vida) and Dr Maria Ines Becker (Biosonda, Santiago, Chile), respectively. A total of $0{\cdot}24 \times 10^6$ cells (B16.F10 and B16.OVA), $0{\cdot}3 \times 10^6$ cells (MB49) and 2×10^6 cells (Hepa 1-6) (> 95% viability assessed by Trypan blue exclusion) were injected in 50 μl PBS in the intradermal layer of the right flank of mice that were previously shaved and anaesthetized with sevofluorane (Abbott Laboratories, Buenos Aires, Argentina). Tumours became visible at day 10 and the size was measured every 2–3 days. Two perpendicular measurements were made with a caliper and the tumour area was estimated as the product of both measurements.

Bone marrow (BM) chimeras

C57BL/6 mice were lethally irradiated (900 cGy) and reconstituted with 20×10^6 total BM cells through tail

vein injection. Donor bone marrow cells were obtained from the femur and tibia of C57BL/6 and Rorγt$^{gfp/gfp}$ mice. Irradiated mice were allowed to recover for 5 weeks before inoculation with B16.F10 cells.

Isolation of mononuclear cells from solid tumours

To examine tumour-infiltrating cells, whole tumours were dissected and disaggregated mechanically. Minced tissues were resuspended in 5 ml Hanks' balanced salt solution + 5% fetal calf serum (FCS) and digested in the presence of 1 mg/ml Collagenase D (Roche, Mannheim, Germany) and 25 μg/ml DNase I (Roche) for 30 min at 37° with constant agitation. The cell suspension was filtered with a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ). Red blood cells were lysed in a hypotonic ammonium chloride solution. Leucocytes were resuspended in 40% Percoll (GE Healthcare, Uppsala, Sweden) and gently layered over 70% Percoll. The gradient was centrifuged at 750 ***g*** for 20 min at room temperature. Mononuclear cells were collected from the interphase and were washed and resuspended in RPMI + 10% FCS.

Flow cytometry and antibodies

The following antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ): CD4 (clone L3T4), CD8 (clone 53-6.7), H2-Kb (clone AF6-88.5), H2-Kd (clone SF1-1.1), CD11c, IAb (clone 25-9-17), CD19 (clone 1D3), CTLA-4 (clone UC10-4F10-11), PD-L1 (MIH5) and CD49b (clone DX5). The following antibodies were purchased from eBioscience (San Diego, CA): CD16/32 (clone 93), CD4 (clone GK1.5), CD25 (clone PC61.5), CD11c (clone N418), CD39 (clone 24DMS1), CD45.2 (clone 104), CD62L (clone MEL-14), GARP (clone YGIC86), Gr-1 (clone RB6-8C5), Foxp3 (clone FJK-16s), IFN-γ (XMG1.2) and IL-17 (clone eBio17B7). The following antibodies were purchased from Biolegend (San Diego, CA): CD3 (clone 17A2), NK1.1 (clone PK136), CD49d (clone 9C10), CD11b (clone M1/70), CD45.1 (clone A20) and B220 (clone RA3-6B2).

For intracellular detection of cytokines, cells were stimulated for 4 hr at 37° and 5% CO_2 with 0·25 μM PMA (Sigma-Aldrich, St Louis, MO) and 1 μg/ml Ionomycin (Sigma-Aldrich) in the presence of Golgi Plug (BD Biosciences). Cells were surface-stained with anti-CD3 and anti-CD4 antibodies in PBS + 2% FCS for 20 min at 4°. Cells were resuspended in a fixation/permeabilization solution (Cytofix/Cytoperm; BD Pharmingen) and incubated with anti-IFN-γ and anti-IL-17 antibodies for 30 min at 4°. For Foxp3 detection, unstimulated cells were surface stained with antibodies against CD4 and CD25 and permeabilized with Foxp3 Staining Buffer (eBioscience) and incubated with anti-Foxp3 antibody or isotype control for 30 min at 4°. Cells were then washed with permeabilization buffer and resuspended in PBS + 2% FCS for FACS analysis (FACSCanto II; BD Bioscience). In some cases, propidium iodide (Calbiochem, La Jolla, CA) was used to discard dead cells from the analysis. Analysis of FACS data was performed using the FLOWJO software (Tree Star Inc., Ashland, OR).

Generation of Th17 and Th1-polarized cells

Antigen presenting cells (APC) were purified from spleens of WT and Rorγt$^{gfp/gfp}$ mice. The spleen was mechanically disaggregated and resuspended in 5 ml RPMI + 10% FCS. Tissues were digested in the presence of Collagenase D (1 mg/ml) and DNase I (25 μg/ml) for 45 min at 37° with constant agitation. Cell suspensions were filtered with a 70-μm cell strainer (BD Falcon). Red blood cells were lysed in a hypotonic ammonium chloride solution. The APC were positively selected using anti-CD11c MACS (clone n418; Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's instructions. The purity of APC in the positive fraction was confirmed by FACS analysis, which contained CD11c$^+$ dendritic cells (70%) and CD19$^+$ B220$^+$ B cells (30%).

CD4$^+$ T cells were purified from spleens of WT, Rorγt$^{gfp/gfp}$ or OT-II mice. The spleen was perfused with RPMI + 10% FCS. Red blood cells were lysed in a hypotonic ammonium chloride solution. CD4$^+$ T cells were positively selected using anti-CD4 MACS (clone L3T4; Miltenyi Biotec) following the manufacturer's instructions. The purity of CD4$^+$ T cells contained in the positive fraction was confirmed by FACS analysis (> 80%). CD4$^+$ T cells and APC were co-cultured in a 5 : 1 ratio ($0{\cdot}12 \times 10^6$ CD4$^+$ T cells/well) in a 96-well round bottom microplate (Orange Scientific, Braine-l'Alleud, Belgium). T cells were stimulated with 1 μg/ml α-CD3 antibody (eBioscience) in the presence or absence of Th17 polarizing conditions (Th17 PC): 20 ng/ml IL-6 (R&D Systems, Minneapolis, MN), 5 ng/ml transforming growth factor-β, 10 ng/ml IL-1β (eBioscience) and 5 μg/ml α-IFN-γ (Biolegend); or Th1 polarizing conditions (Th1 PC): 10 ng/ml IL-2 and 10 ng/ml IL-12 (R&D Systems). After 7 days of culture, intracellular cytokines were analysed as described in the Flow Cytometry section.

Cytokine secretion measurement

To determine cytokine secretion by splenocytes and tumour-infiltrating lymphocytes, cells were activated for 4 hr with 0·25 μM PMA (Sigma Aldrich) and 1 μg/ml ionomycin (Sigma Aldrich). Following activation the supernatants were harvested and analysed using the mouse Th1/Th2/Th17 CBA Kit (BD Biosciences), following the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM. Differences between WT and Rorγt$^{gfp/gfp}$ mice were determined with a Student's *t*-test. Where indicated, differences were analysed using one-way analysis of variance paired with Bonferroni post-tests. Statistical analysis and graphs were obtained with GRAPHPAD PRISM (GraphPad Software Inc., La Jolla, CA).

Results

Rorγt$^{gfp/gfp}$ mice present enhanced tumour growth and decreased percentage of tumour-infiltrating Th17 cells

To evaluate the effect of Th17 cells in tumour progression, we used a mouse model with impaired Th17 differentiation. Homozygous Rorγt$^{gfp/gfp}$ mice do not express the RORγt transcription factor required for the polarization of naive CD4$^+$ T cells into IL-17-producing CD4$^+$ T cells.[24] To address the effect of Th17 cells in tumour development, we challenged WT and Rorγt$^{gfp/gfp}$ with a solid melanoma tumour by an intradermal injection of B16.F10 cells. After 3 weeks of monitoring tumour growth, results showed that tumour growth was significantly enhanced in Th17-deficient Rorγt$^{gfp/gfp}$ mice (Fig. 1a). Similar results were obtained when we used MB-49 and Hepa 1-6 cells as other models of solid tumours (see Supplementary material, Fig. S1). To confirm that the reduction in the percentage of Th17 cells was the cause of the enhanced tumour growth, we compared the rate of tumour growth in Rorγt$^{gfp/gfp}$ mice that were adoptively transferred with Th17 cells generated *in vitro* versus untreated Rorγt$^{gfp/gfp}$ mice and WT mice. For this experiment, mice were challenged with ovalbumin (OVA) -expressing B16.F10 cells and then injected with 1×10^6 OVA-specific Th17 or Th1 cells generated from OT-II CD4$^+$ T cells. Our results show that tumour growth was significantly reduced in Rorγt$^{gfp/gfp}$ mice that received Th17 cells or Th1 cells compared with control Rorγt$^{gfp/gfp}$ mice; however, the transfer of Th17 cells had a more dramatic effect on tumour growth than the transfer of Th1 cells (Fig. 1b). Moreover, we observed that upon transfer to Rorγt$^{gfp/gfp}$ or C57BL/6 mice, almost 50% of *in vitro*-generated Th17 cells produce IFN-γ within the tumour (see Supplementary material, Fig. S2), a fact that suggests that the effect of transferred Th17 cells on tumour growth may be at least partially mediated by IFN-γ production. This result confirms that enhanced tumour growth observed in Rorγt$^{gfp/gfp}$ mice is the result of a reduced frequency of Th17 cells, suggesting that these cells may have important anti-tumour functions.

Since it has been described that Th17 cells are expanded in tumour-bearing mice and humans and are enriched in tumour tissues compared with blood and lymphoid organs,[1–4] we confirmed the expansion of Th17 cells upon a challenge with B16.F10 melanoma cells in Rorγt$^{gfp/+}$ heterozygous mice. Indeed, we found an increase in the CD4$^+$ Rorγt-GFP$^+$ population in tumour-bearing mice compared with mice without a tumour (Fig. 1c). Next, we compared the percentage of Th17 cells found in the spleen, tumour and lamina propria of Rorγt$^{gfp/gfp}$ mice and WT mice. Intracellular staining of IL-17 in splenocytes from WT and Rorγt$^{gfp/gfp}$ mice was not significantly detected as compared with isotype control staining (data not shown). Notably, a significant decrease in the percentage of tumour-infiltrating Th17 cells was found in Rorγt$^{gfp/gfp}$ mice compared with WT mice (Fig. 1d–e). The percentage of Th17 cells in the lamina propria, a site where these cells are particularly enriched in WT mice, was dramatically diminished in Rorγt$^{gfp/gfp}$ mice (Fig. 1d). Accordingly, upon stimulation with PMA plus ionomycin, the levels of IL-17 produced by tumour-infiltrating lymphocytes in Rorγt$^{gfp/gfp}$ mice were consistently below detection levels (Fig. 1f), indicating that both tumour-infiltrating Th17 cells as well as IL-17 production are compromised in Rorγt$^{gfp/gfp}$ mice. With the exception of an increase in the level of IL-6 in Rorγt$^{gfp/gfp}$ tumour tissue, no other changes in cytokine profile were observed between WT and Rorγt$^{gfp/gfp}$ mice (Fig. 1f). To confirm a defect in the differentiation of Th17 cells in Rorγt$^{gfp/gfp}$ mice we also tested whether we could induce the *in vitro* Th17 differentiation of CD4$^+$ T cells isolated from Rorγt$^{gfp/gfp}$ mice. Our results indicate that the percentage of naive CD4$^+$ T cells that differentiate into IL-17-producing cells in Rorγt$^{gfp/gfp}$ mice was dramatically reduced compared with those from WT mice whereas Th1 differentiation was not affected (See Supplementary material, Fig. S3), confirming that CD4$^+$ T cells from Rorγt$^{gfp/gfp}$ mice cannot differentiate to Th17 cells but can differentiate into other Th cell subsets.

In addition to its expression in Th17 cells, the RORγt transcription factor is expressed during fetal life and is required for the generation of lymphoid tissue inducer cells. Therefore, lymphoid tissue inducer cells as well as lymph nodes and Peyer's patches are absent in Rorγt$^{gfp/gfp}$ mice.[25] To demonstrate that the enhanced tumour growth observed in Rorγt$^{gfp/gfp}$ mice is a result of the lack of Th17 cells and not of the lack of secondary lymphoid organs, we performed bone marrow transplants from WT or Rorγt$^{gfp/gfp}$ donors to WT mice and tumour progression was evaluated after B16.F10 injection. In agreement with our previous findings that suggest that Th17 cells are essential in mediating anti-tumour responses, mice that received bone marrow from Rorγt$^{gfp/gfp}$ donors showed enhanced tumour growth compared with the group that received bone marrow from WT donors (Fig. 2a). Maximum tumour growth 3 weeks after injection observed in mice transplanted with Rorγt$^{gfp/gfp}$

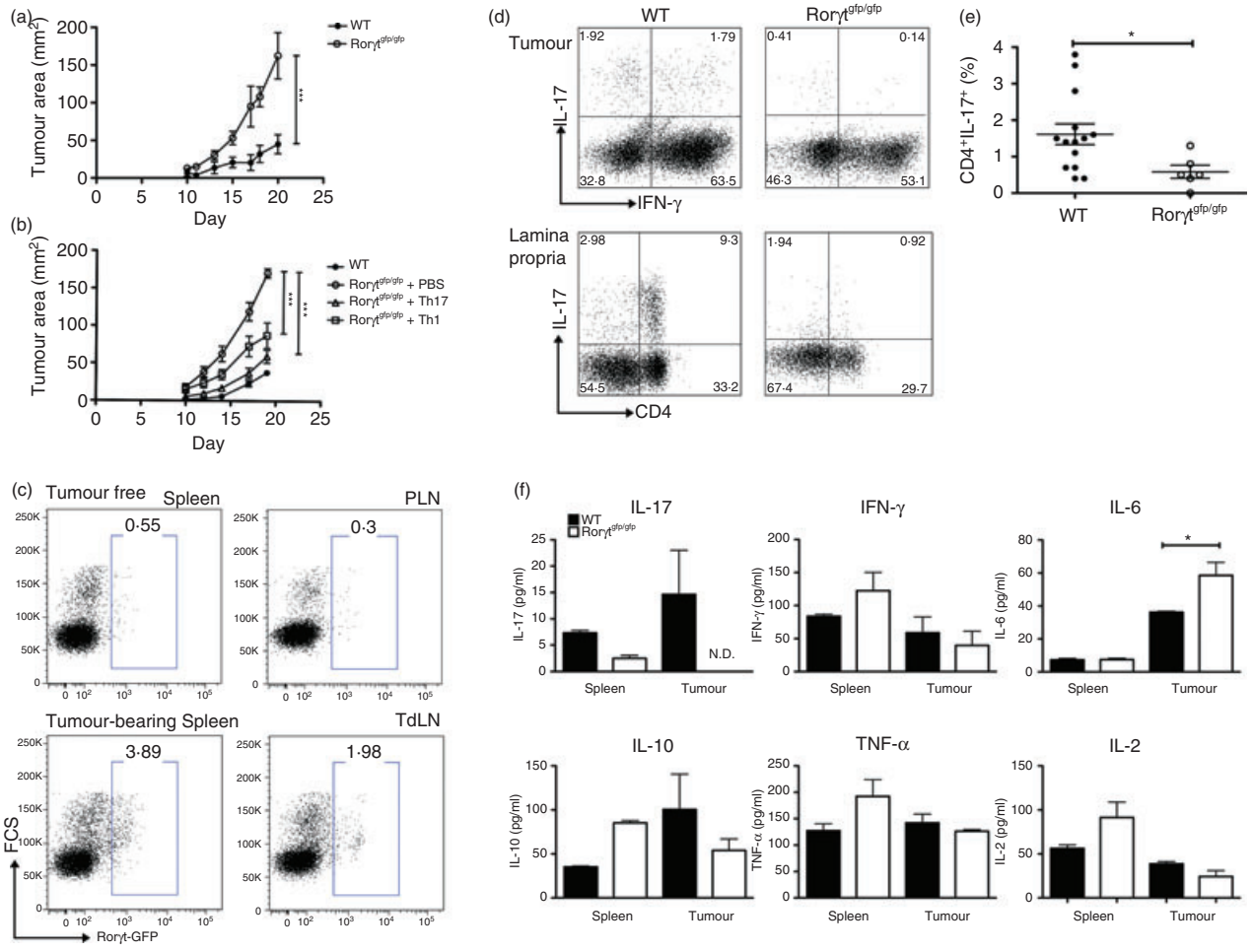

Figure 1. Reduced frequency of endogenous T helper type 17 (Th17) cells and enhanced tumour growth in Rorγt$^{gfp/gfp}$ mice. Tumour growth curves of wild-type (WT) and Rorγt$^{gfp/gfp}$ mice injected with B16.F10 cells (n = 5) (a). Tumour growth curves of wild-type (WT) and Rorγt$^{gfp/gfp}$ mice injected with B16.F10 cells that received PBS, 1 × 10^6 *in vitro*-generated Th17 or Th1 cells (n = 4) (b). Frequency of Th17 cells was monitored in tumour-free and tumour-bearing heterozygous Rorγt$^{gfp/+}$ mice by analysing Rorγt-GFP$^+$ cells in spleen, peripheral lymph node (PLN) or tumour-draining lymph nodes (TdLN) by FACS (c). Cells from tumour and lamina propria were isolated from tumour-bearing WT and Rorγt$^{gfp/gfp}$ mice, activated for 4 hr with PMA plus ionomycin in the presence of Brefeldin A and then stained to detect intracellular cytokines as described in the Materials and methods section. Dot plots show interleukin-17$^+$ (IL-17$^+$) cells in a CD3$^+$ CD4$^+$ gate (d). Percentage of tumour-infiltrating IL-17$^+$ cells in a CD3$^+$ CD4$^+$ gate from WT and Rorγt$^{gfp/gfp}$ mice (e). Splenocytes and tumour-infiltrating lymphocytes from WT and Rorγt$^{gfp/gfp}$ mice were stimulated for 4 hr with PMA plus ionomycin and cytokine concentrations in the supernatant were determined using the Th1/Th2/Th17 CBA kit. (f). Values expressed represent the mean ± SEM of at least three independent experiments *P < 0·05, **P < 0·01, ***P < 0·0001.

bone marrow (82·2 ± 9·9 mm^2) was less severe than in Rorγt$^{gfp/gfp}$ mice (162·3 ± 30·7 mm^2).

Next, we investigated the immunocompetence of Rorγt$^{gfp/gfp}$ mice by measuring the ability to mount a response against a foreign antigen. For this, WT and Rorγt$^{gfp/gfp}$ mice were transferred with CFSE-labelled OT-II CD4$^+$ T cells and immunized with OVA protein and LPS. Four days later we evaluated the proliferation of transferred cells in the spleen. OT-II CD4$^+$ T cells were activated to the same extent in WT and Rorγt$^{gfp/gfp}$ mice (Fig. 2b), indicating that immune responsiveness is not compromised in the latter. These data support that Rorγt$^{gfp/gfp}$ mice are a reliable model for studying the role of Th17 cells in tumour progression.

Th17 cells do not affect the recruitment or the immunosuppressive phenotype of myeloid-derived suppressor cells and regulatory T cells

Tumour evasion of the immune response is in part mediated through the activation and recruitment of immunosuppressive populations such MDSC and Treg cells to the tumour microenvironment. Inflammatory mediators produced by Th17 cells could participate in the mobiliza-

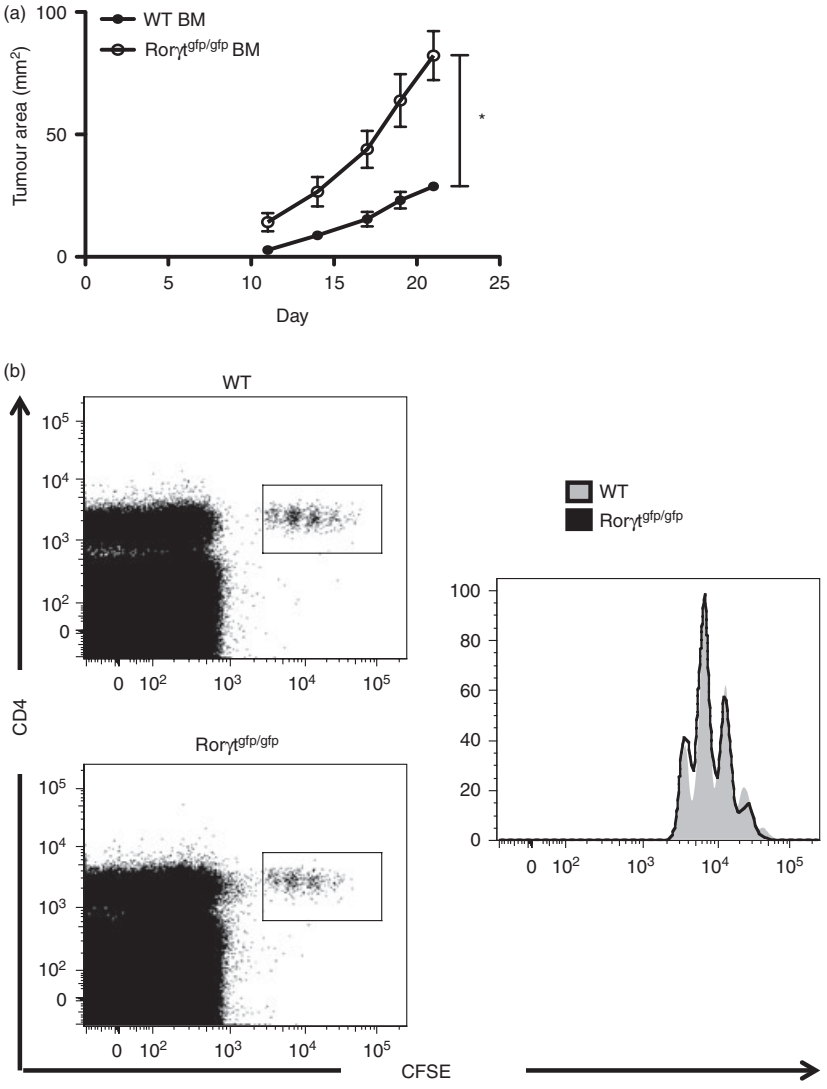

Figure 2. T helper type 17 (Th17) cell deficiency contributes to the enhanced tumour growth in Rorγt$^{gfp/gfp}$ mice. Tumour growth curves of transplanted wild-type (WT) mice that received bone marrow from WT or Rorγt$^{gfp/gfp}$ mice that were injected with B16.F10 cells ($n = 5$) (a). Wild-type mice were transferred with CFSE-stained OT-II CD4$^+$ T cells and 24 hr later, were challenged intraperitoneally with ovalbumin (OVA; 1 mg) + LPS (50 μg). Four days later, cell proliferation was analysed in splenocytes as CFSE dilution (b). (*$P < 0{\cdot}05$)

tion of immunosuppressive populations present in the tumour.[17] To study this possibility, WT and Rorγt$^{gfp/gfp}$ mice were injected with B16.F10 melanoma cells and analysed for the presence of MDSC and Treg cells in spleen and tumour. Our results show that in Rorγt$^{gfp/gfp}$ mice, the proportion of MDSC and Treg cells is not altered in the spleen or tumour compared with WT mice (Fig. 3a,b). To further characterize changes in these populations, we analysed the expression of CD49d, which distinguishes a subpopulation of MDSC that strongly suppresses T-cell proliferation.[26] CD49d^{high} MDSC were preferentially recruited into the tumour-draining lymph node and tumour, whereas an equal ratio of CD49d^{int} and CD49d^{high} MDSC was found in the spleen of tumour-bearing WT mice (see Supplementary material, Fig. S4A). The distribution of CD49d^{high} MDSC in Rorγt$^{gfp/gfp}$ mice was similar compared with that in WT mice (Fig. 3c) confirming that MDSC are not preferentially recruited and do not have a higher suppressive activity in Rorγt$^{gfp/gfp}$ mice. Th17 cells are able to recruit neutrophils to inflamed sites through the secretion of IL-17,[17] therefore we compared the presence of Gr-1$^+$ CD11b$^-$ neutrophils in spleen and tumour. A moderate decrease in the neutrophil population was observed in the spleen of Rorγt$^{gfp/gfp}$ mice compared with WT mice; however, this difference was not observed in the tumour (Fig. 3d), therefore the differences observed in tumour growth are not the result of changes in neutrophil recruitment in the tumour.

Next, we analysed the suppressive potential of Treg cells and we found that the expression of CD39 and CTLA-4 was up-regulated in tumour-infiltrating Treg cells compared with spleen Treg cells, whereas GARP and PD-L1 had similar expression levels in spleen and tumour Treg cells (see Supplementary material, Fig. S4B). As shown in Figure 3(e), both CD39 and CTLA-4 had similar expression levels in Treg cells from WT and Rorγt$^{gfp/gfp}$ mice.

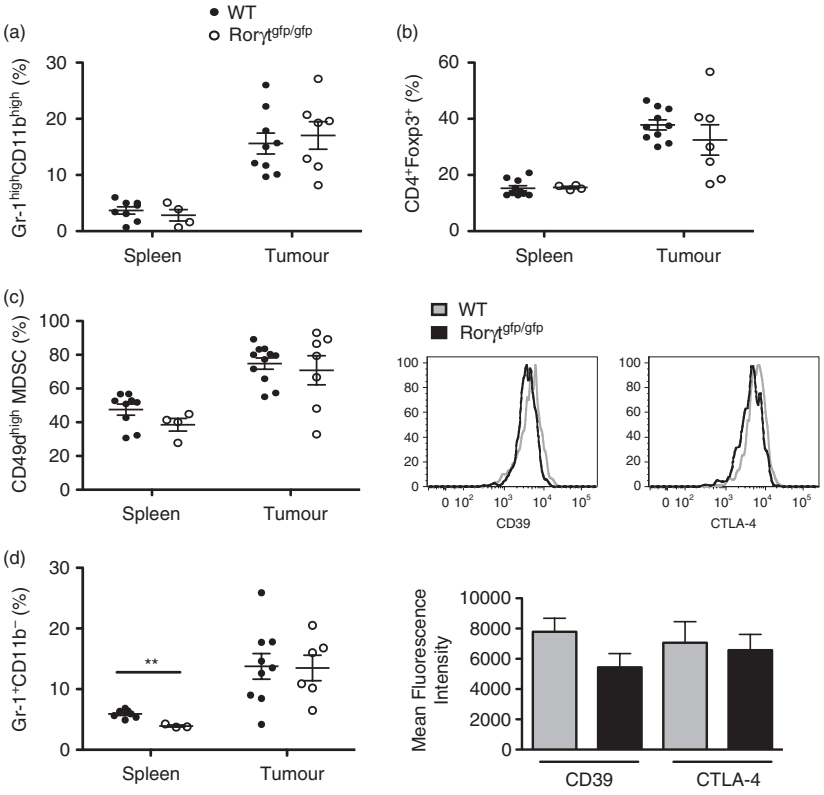

Figure 3. Myeloid-derived suppressor cells (MDSC) and regulatory T (Treg) cells from Rorγt$^{gfp/gfp}$ mice do not present an altered recruitment to the tumour or phenotype. Splenocytes and tumour-infiltrating cells from wild-type (WT) and Rorγt$^{gfp/gfp}$ mice were isolated and analysed by FACS. Frequency of Gr-1^{high}CD11b^{high} MDSC in a PI$^-$ gate (a). Foxp3$^+$ cells in a CD4$^+$ gate (b). Percentage of CD49d^{high} cells in a Gr-1^{high} CD11b^{high} gate (c). Frequency of Gr-1^{high} CD11b$^-$ neutrophils in a PI$^-$ gate (d). CD39 and CTLA-4 mean fluorescence intensity in a CD4$^+$ Foxp3$^+$ gate in cells isolated from WT and Rorγt$^{gfp/gfp}$ tumours (e). Bars show mean ± SEM.

Our results indicate that mice with decreased endogenous Th17 cells are more vulnerable against tumour development but they present no differences in the infiltration and immunosuppressive function of MDSC or Treg cells in the tumours.

Effect of Th17 cells in the recruitment of effector populations to the tumour

To understand the mechanisms underlying the enhanced tumour growth observed in Rorγt$^{gfp/gfp}$ mice, we compared the presence of effector populations such as natural killer cells, CD8$^+$ T cells and CD4$^+$ T cells in the tumour of Rorγt$^{gfp/gfp}$ and WT mice. Although we observed no differences in the percentage of natural killer cells in WT versus Rorγt$^{gfp/gfp}$ mice (Fig. 4a), we observed a significant increase in the percentage of tumour-infiltrating IFN-γ-producing CD8$^+$ T cells in Rorγt$^{gfp/gfp}$ mice compared with WT mice (Fig. 4b,c).

We also observed a higher proportion of Th1 cells in the CD4$^+$ population in the spleen of Rorγt$^{gfp/gfp}$ mice compared with WT mice (Fig. 4e); however, we observed no difference in the levels of secreted IFN-γ either in spleen or tumour between WT and Rorγt$^{gfp/gfp}$ mice (Fig. 1c). Importantly, our data also showed a significant reduction of total CD4$^+$ T cells in the tumour while maintaining similar levels in the spleen, compared with WT mice (Fig. 4d), suggesting that in Rorγt$^{gfp/gfp}$ mice there is an overall decrease in Th1 cells in the tumour. These results suggest that enhanced tumour growth observed in Rorγt$^{gfp/gfp}$ mice could be the outcome, at least in part, of a decrease of Th1 effector cells in the tumour, despite the observed increase in CD8$^+$ T cells.

To confirm that tumour progression is mediated by the decline in the percentage of effector rather than by the accumulation of immunosuppressive populations within the tumour, we studied the percentage of MDSC, Treg cells and Th1 cells in tumours of different size from WT mice. We observed a reduction in Th1 cells but no evident accumulation of MDSC or Treg cells in advanced tumours (Fig. 5). This suggests that the degree of tumour severity is not correlated with the expansion of immunosuppressive populations but rather with a decline in effector cells.

Discussion

Although Th17 cells have been found in higher frequency in patients with cancer and are particularly enriched in human and mouse tumours, the function of Th17 cells in tumour immunity remains controversial, because they have been described as presenting both tumour-promoting and anti-tumour properties. The main pro-tumour role of Th17 cells is based on their production of IL-17 and its pro-angiogenic effect over surrounding vascular endothelial cells and fibroblasts. It has been demonstrated

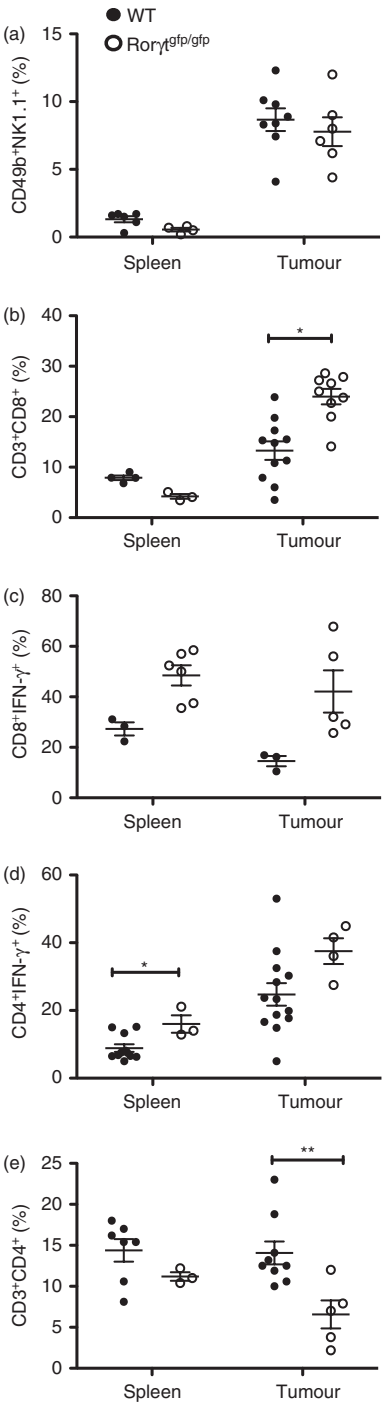

Figure 4. Altered recruitment of $CD4^+$ and $CD8^+$ T-cell effector populations in $Ror\gamma t^{gfp/gfp}$ mice. Cells from spleen and tumour of wild-type (WT) and $Ror\gamma t^{gfp/gfp}$ mice were isolated and cell populations were analysed by FACS. Percentage of $CD49b^+$ $NK1.1^+$ natural killer cells in a PI^- gate (a).Total and interferon-γ^+ (IFN-γ^+) $CD8^+$ T cells (b and c). Total and IFN-γ^+ $CD4^+$ T cells (d and e). Bars show mean ± SEM. $^{**}P < 0{\cdot}001$.

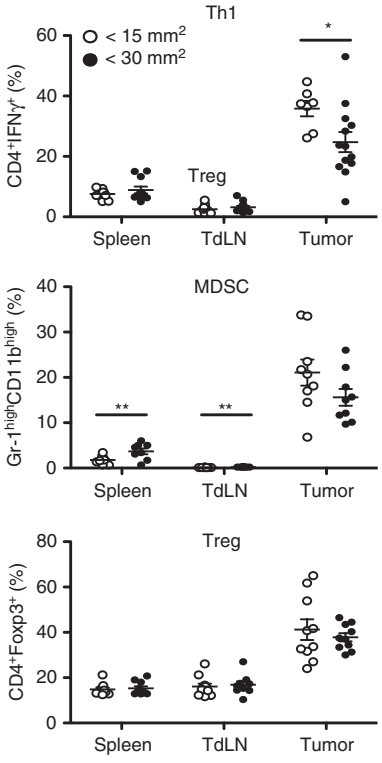

Figure 5. Infiltration of effector T helper type 1 (Th1) cells takes place during initial tumour development and declines with tumour growth. Wild-type mice injected with B16.F10 melanoma cells were divided in two groups that were euthanized at two different points of tumour development, before tumour size had reached 15 mm^2 and when the tumour had grown over 30 mm^2. Th1, myeloid-derived suppressor cell (MDSC) and regulatory T (Treg) cell frequencies in spleen, tumour-draining lymph node (TdLN) and tumour were analysed by FACS. Data are presented as mean ± SEM.$^*P < 0{\cdot}05$.

that by acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators,[27,28] including vascular endothelial growth factor, which promotes tumour angiogenesis.[29] Additional reports have analysed the role of Th17 function by deleting IL-17 or the IL-17 receptor in mice, while overlooking other cytokines and factors secreted by Th17 cells.[5–8] The group of Yu has reported that IL-17-deficient mice present a reduction in tumour growth and progression. They propose that IL-17 could contribute to tumour growth by stimulation of tumour cell proliferation through the induction of IL-6 in the tumour environment and activation of STAT3 in tumour cells.[9] In the same line of evidence, using IL-17R-deficient mice, another group has reported that IL-17 promotes the tumour growth through the recruitment of MDSC to the tumour microenvironment.[22]

Although IL-17 is the signature cytokine produced by Th17 cells, the production of this cytokine is not the only function of Th17 cells, so the biological activity of Th17 cells should not be equated to biological activity of IL-17. In our model, RORγt-deficient mice lack Th17 cells and all the biological functions related to these cells. This includes not only IL-17 production but other cytokines and chemokines produced by Th17 cells capable of

altering the tumour microenvironment, such as CCL20, IFN-γ and granulocyte–macrophage colony-stimulating factor, the latter being recently proposed as a novel Th17s 'weapon'.[30–32] This important difference in the models used could explain why in the absence of Th17 cells we observe an increase in tumour burden whereas other studies using IL-17 and IL-17R-deficient mice have reported that IL-17 promotes tumour growth.

In this work, a new approach was taken to study the function of endogenous Th17 cells in tumour development. Here we analyse how the endogenous development of Th17 cells influences tumour growth in a model of intradermal melanoma. In agreement with previous evidence from other groups,[8,10] our results show that Th17 cells confer protection against tumour development. Moreover, we did not find evidence to associate Th17-mediated inflammation with recruitment of immunosuppressive populations in the tumour. Instead, we show that Th17 cells promote antitumour responses by enhancing the recruitment of $CD4^+$ T cells to the tumour.

Given that Th17 cells represent a minor percentage of immune cells in the tumour, it is notable that a reduction in this population can produce such profound effects. Despite expressing phenotypic markers of terminally differentiated effector cells, it has been demonstrated that human and mouse primary Th17 cells also present stem cell-like properties. For instance, it has been described that Th17 cells have a high proliferation potential, self renewal, multipotency and resistance to apoptosis compared with Th1 cells.[33,34] Interestingly, Th17 cells are significantly more effective than Th1 cells at mediating tumour rejection; however, this strong effect is dependent on the acquisition of Th1-like features, including IFN-γ production.[10,34] This strongly suggests that Th17 cells are polyfunctional and may represent a critical component for a long-term anti-tumour response as a persistent source of Th1-like effector cells.

In our model, adoptively transferred Th17 cells presented features of multipotency because upon transfer into Rorγt$^{gfp/gfp}$ mice, a percentage of these cells are converted into IFN-γ-producing cells within the tumour. This observation confirms that *in vitro*-generated Th17 cells present stem cell-like features and may require conversion to Th1 cells to exert their anti-tumour function in our model as well.

Immunosuppression within the tumour is the major obstacle for the antitumour response and represents a critical barrier for the development of successful cancer immunotherapy. Two central components that contribute to immunosuppression in the tumour are MDSC and Treg cells. We observed that MDSC and Treg cells are dramatically expanded in tumour compared with spleen and tumour-draining lymph nodes (Fig. 5). It was recently reported that IL-17 is required for the infiltration and tumour-promoting activity of MDSC in a subcutaneous lymphoma model.[22] In contrast, we provide evidence that a decrease in endogenous Th17 cells does not affect the percentages of MDSC and Treg cell populations in intradermal melanoma (Fig. 3), indicating that the enhanced tumour growth rate observed in Rorγt$^{gfp/gfp}$ mice is not associated with an increased percentage of these populations in the tumour. MDSC-mediated suppressive mechanisms have been extensively described but until recently, no surface markers had been associated with the suppressive properties of MDSC. Recently, CD49d was reported to be expressed exclusively on monocytic MDSC, which are more efficient at suppressing T-cell proliferation compared with $CD49d^-$ MDSC.[26] Analysis of the expression of CD49d in WT mice showed that $CD49d^{high}$ MDSC are predominantly accumulated in the tumour-draining lymph node and tumour (see Supplementary material, Fig. S4A). However, we observed no change in the proportion of the $CD49^{high}$ subpopulation in the spleen or within tumours of Rorγt$^{gfp/gfp}$ mice compared with WT mice, suggesting that the suppressive potential of MDSC present in the tumour of Rorγt$^{gfp/gfp}$ mice is similar compared with WT mice. In contrast with MDSC, several surface proteins have been described to mediate immunosuppressive mechanisms by Treg. CD39 and CTLA-4 suppress T-cell activation,[35] PD-L1 expression induces T-cell death,[36] while GARP participates in the release of surface-bound transforming growth factor-β[37]. Our analysis showed an increase in the expression levels of CD39 and CTLA-4 in Treg cells isolated from the tumour compared with spleen Treg cells (see Supplementary material, Fig. S4B), suggesting that these mechanisms play an important role in the regulation of T-cell priming and expansion in the tumour. However, we found no difference in the expression of CD39 or CTLA-4 in Treg cells from Rorγt$^{gfp/gfp}$ mice, indicating that the degree of immunosuppression mediated by Treg cells in these mice is comparable with that seen in WT mice. Altogether, we show that a decrease of Th17 cells does not influence the recruitment or phenotype of immunosuppressive populations in melanoma tumours.

By comparing immunosuppressive populations (MDSC and Treg cells) and effector Th1 cells during initial and advanced stages of tumour development in WT mice, we observed that immunosuppressive and effector cells infiltrate the tumour from early development. Whereas the percentage of MDSC and Treg cells in the tumour is sustained, there is a significant drop in the percentage of Th1 cells (Fig. 5). This suggested that the tumour severity is not correlated with the expansion of immunosuppressive populations but rather with a decline in effector cells. The percentage of Th1 cells was moderately increased in the spleen and tumour of Rorγt$^{gfp/gfp}$ mice compared with WT mice (Fig. 4e); however, tumours of Rorγt$^{gfp/gfp}$ mice were significantly less infiltrated by total $CD4^+$ T cells compared with WT mice. This finding is consistent with

decreased infiltration of $CD4^+$ T cells in tumour-burdened lungs of IL-17 knockout mice[8] and increased $CD4^+$ T-cell infiltration in a model that enhances Th17 differentiation in the tumour microenvironment.[11]

Our results support the concept that Th17 cells promote signalling in the tumour environment that specifically favours the trafficking of effector $CD4^+$ T cells. In human ovarian cancer ascites, the levels of IL-17 are positively correlated with that of CXCL9 and CXCL10, two chemokines that are associated with a Th1 response.[2] Additionally, the group of Dutton demonstrated that OVA-specific Tc17 cells, IL-17-producing $CD8^+$ T cells, adoptively transferred into tumour-bearing mice control tumour growth in a model of melanoma. They observed that these Tc17 cells are a source for various cytokines and chemokines that elicit further chemokines involved in the attraction of inflammatory cells to the tumour, including $CXCR3^+$ effector cells and neutrophils.[38] Additional evidence of the recruitment of Th1 effector cells by Th17 cells came from a study in mice showing the protective role of Th17 cells in response to vaccination during *Mycobacterium tuberculosis* challenge. The authors proposed that vaccination induces IL-17-producing $CD4^+$ T cells that populate the lung and, after challenge, trigger the production of chemokines such as CXCL9, CXCL10 and CXCL11 that recruit IFN-γ-producing $CD4^+$ T cells, which ultimately restrict bacterial growth.[39] It remains to be studied whether in our model, Th17 cells in the tumour can influence chemokine expression in melanoma tumour and stromal cells as a mechanism to coordinate the recruitment of $CXCR3^+$ effector Th1 cells to the tumour thus favouring anti-tumour responses.

It has been proposed that a shift in the ratio of the Th17/Treg numbers leads to reduced tumour growth and improved survival in a model of pancreatic cancer.[11] Although Rorγt$^{gfp/gfp}$ mice have a higher proportion of Th1 cells, it is possible that the absolute number of cells in the tumour is reduced compared with that in WT mice, because of a significant decrease in total $CD4^+$ T cells. It is possible that changes in the ratio of effector Th1 and Th17 cells versus immunosuppressive populations also have a contributory effect on tumour progression.

A surprising difference observed in the tumour environment of Rorγt$^{gfp/gfp}$ mice was a higher frequency of $CD8^+$ T cells. If such an increase were accompanied by a stronger tumour-specific cytotoxic response, the expected outcome would be a reduction in tumour size. As this is not the case, the enhanced tumour growth that is seen in these mice suggests that a better assessment of cytotoxic functions of $CD8^+$ T cells is needed to fully comprehend how impaired Th17 development affects the anti-tumour response. It is important to note that tumour rejection following adoptive transfer of tumour-specific $CD8^+$ T cells can be greatly enhanced when co-transferred with Th17 cells,[33] therefore it is possible that disruption of the collaborative response between Th17 and $CD8^+$ T cells also contributes to enhanced tumour growth in Rorγt$^{gfp/gfp}$ mice.

In conclusion, our data show that Th17 cells do not affect the recruitment of immunosuppressive populations such as Treg cells or MDSC to the tumour, but favour the recruitment of effector Th1 cells. This evidence suggests that Th17 cells orchestrate the recruitment of Th1 effector cells to the tumour, and therefore strongly argues in favour of a protective role of Th17 cells during anti-tumour responses.

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Disclosures

The authors declare no conflict of interest.

References

1 Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, Chang A, Zou W. Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol* 2007; **178**:6730–3.

2 Kryczek I, Banerjee M, Cheng P *et al.* Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 2009; **114**:1141–9.

3 Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang RF. Generation and regulation of human $CD4^+$ IL-17-producing T cells in ovarian cancer. *Proc Natl Acad Sci USA* 2008; **105**:15505–10.

4 Su X, Ye J, Hsueh EC, Zhang Y, Hoft DF, Peng G. Tumor microenvironments direct the recruitment and expansion of human Th17 cells. *J Immunol* 2010; **184**:1630–41.

5 Benchetrit F. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* 2002; **99**:2114–21.

6 Numasaki M, Fukushi J, Ono M *et al.* Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003; **101**:2620–7.

7 Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. *Blood* 2009; **114**:357–9.

8 Martin-Orozco N, Muranski P, Chung Y *et al.* T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity* 2009; **31**:787–98.

9 Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. *J Exp Med* 2009; **206**:1457–64.

10 Muranski P, Boni A, Antony PA *et al.* Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 2008; **112**:362–73.

11 Gnerlich JL, Mitchem JB, Weir JS *et al.* Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer. *J Immunol* 2010; **185**:4063–71.

12 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**:883–99.

13 Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**:162–74.

14 Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol* 2006; **176**:284–90.

15 Cheng P, Corzo CA, Luetteke N *et al.* Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J Exp Med* 2008; **205**:2235–49.

16 Ghannam S, Pene J, Torcy-Moquet G, Jorgensen C, Yssel H. Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. *J Immunol* 2010; **185**:302–12.
17 Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 2008; **28**:454–67.
18 Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E_2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* 2007; **67**:4507–13.
19 Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J Immunol* 2008; **181**:4666–75.
20 Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* 2009; **27**:485–517.
21 Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. Gr-1^+ $CD115^+$ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 2006; **66**:1123–31.
22 He D, Li H, Yusuf N, Elmets CA, Li J, Mountz JD, Xu H. IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. *J Immunol* 2010; **184**:2281–8.
23 Yang Z, Zhang B, Li D, Lv M, Huang C, Shen GX, Huang B. Mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment via IL-17 pathway in murine hepatocarcinoma model. *PLoS ONE* 2010; **5**:e8922.
24 Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17^+ T helper cells. *Cell* 2006; **126**:1121–33.
25 Eberl G, Marmon S, Sunshine MJ, Rennert PD, Choi Y, Littman DR. An essential function for the nuclear receptor RORγ(t) in the generation of fetal lymphoid tissue inducer cells. *Nat Immunol* 2004; **5**:64–73.
26 Haile LA, Gamrekelashvili J, Manns MP, Korangy F, Greten TF. CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice. *J Immunol* 2010; **185**:203–10.
27 Numasaki M, Lotze MT, Sasaki H. Interleukin-17 augments tumor necrosis factor-α-induced elaboration of proangiogenic factors from fibroblasts. *Immunol Lett* 2004; **93**:39–43.
28 Takahashi H, Numasaki M, Lotze MT, Sasaki H. Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. *Immunol Lett* 2005; **98**:189–93.
29 Honorati MC, Neri S, Cattini L, Facchini A. Interleukin-17, a regulator of angiogenic factor release by synovial fibroblasts. *Osteoarthritis Cartilage* 2006; **14**:345–52.
30 Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, Suter T, Becher B. RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 2011; **12**:560–7.
31 El-Behi M, Ciric B, Dai H *et al.* The encephalitogenicity of T_H17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol* 2011; **12**:568–75.
32 McGeachy MJ. GM-CSF: the secret weapon in the T_H17 arsenal. *Nat Immunol* 2011; **12**:521–2.
33 Kryczek I, Zhao E, Liu Y *et al.* Human TH17 cells are long-lived effector memory cells. *Sci Transl Med* 2011; **3**:104ra0.
34 Muranski P, Borman ZA, Kerkar SP *et al.* Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity* 2011; **35**:972–85.
35 Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol* 2008; **8**:523–32.
36 Zhou Q, Munger ME, Highfill SL *et al.* Program death-1 signaling and regulatory T cells collaborate to resist the function of adoptively transferred cytotoxic T lymphocytes in advanced acute myeloid leukemia. *Blood* 2010; **116**:2484–93.
37 Battaglia M, Roncarolo MG. The Tregs' world according to GARP. *Eur J Immunol* 2009; **39**:3296–300.
38 Garcia-Hernandez Mde L, Hamada H, Reome JB, Misra SK, Tighe MP, Dutton RW. Adoptive transfer of tumor-specific Tc17 effector T cells controls the growth of B16 melanoma in mice. *J Immunol* 2010; **184**:4215–27.
39 Khader SA, Bell GK, Pearl JE *et al.* IL-23 and IL-17 in the establishment of protective pulmonary $CD4^+$ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol* 2007; **8**:369–77.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rorγ$t^{gfp/gfp}$ mice present enhanced tumour growth.

Figure S2. T helper type 17 cells produce interferon-γ upon adoptive transfer to wild-type or Rorγ$t^{gfp/gfp}$ mice.

Figure S3. Impaired T helper type 17 polarization in Rorγ$t^{gfp/gfp}$ mice.

Figure S4. Myeloid-derived suppressor cell and regulatory T cell surface markers related to a suppressive phenotype are up-regulated in the tumour microenvironment.