IL-10 directly activates and expands tumor resident CD8+ T cells without de novo infiltration from secondary lymphoid organs

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Running Title:
IL-10 activates tumor resident CD8+ T cells leading to tumor rejection

Precis:
In the absence of systemic immune activation or any contribution from lymphoid organs, IL-10 can uniquely activate and expand cytotoxic T cells within the tumor where it may offer an immunotherapeutic option, challenging prevailing views of IL-10 as a tumor supportive function.

Supported by Schering-Plough/Merck.
The authors declare no conflict of interest.
Abstract

The presence of activated intratumoral T cells correlates clinically with better prognosis in cancer patients. Although tumor vaccines can increase the number of tumor specific CD8+ T cells in systemic circulation, they frequently fail to increase the number of active and tumor reactive T cells within the tumor. Here we show that treatment with the pleiotropic cytokine IL-10 induces specific activation of tumor resident CD8+ T cells as well as their intratumoral expansion in several mouse tumor models. We found that inhibition of T cell trafficking from lymphoid organs did not impair IL-10-induced tumor rejection or the activation of tumor resident CD8+ T cells. Tumor resident CD8+ T cells expressed elevated levels of the IL-10 receptor and were directly activated by IL-10, resulting in prominent phosphorylation of STAT3 and STAT1. Although CD4+ T cells, regulatory T cells, NK cells, and dendritic cells have been reported as prominent targets of IL-10 in the tumor microenvironment, we found that expression of the IL-10R was required only on CD8+ T cells to facilitate IL-10-induced tumor rejection as well as in situ expansion and proliferation of tumor resident CD8 T cells. Together, our findings indicate that IL-10 activates CD8+ T cell-mediated tumor control and suggest that IL-10 may represent a potential tumor immunotherapy in human cancer patients.
Introduction

Interleukin-10 (IL-10) is a cytokine most recognized for its anti-inflammatory properties. IL-10 achieves this suppression of inflammatory responses by inhibiting the expression of MHC class II, co-stimulatory molecules and proinflammatory cytokines in APCs (1). This inhibition of APC function in turn limits the magnitude of T cell responses. In addition, IL-10 directly inhibits the activation and cytokine secretion of CD4+ T cells (2-4). The absence of IL-10 can lead to increased tumour clearance in response to intratumoral CpG injection (5), stronger T cell immunity induced by tumor cell vaccines (6) and it has been suggested that IL-10 contributes to the immune-suppressive environment of tumors.

Contrasting these findings, local release of IL-10 from transfected tumor cells (7-10) or therapeutic administration of IL-10 induces strong anti-tumor immune responses and leads to tumor rejection in a variety of mouse tumor models (11-13). Despite these findings, IL-10's direct target cell types and their location in vivo have not been identified. We and others have previously shown that the anti-tumor effect of IL-10 depends on CD8+ T cells (7, 9, 10, 12). In vitro, IL-10 induces the proliferation and cytotoxic activity of CD8+ T cells and is a chemoattractant for CD8+ T cells (14-17). However, whether IL-10 directly acts on CD8+ T cells during IL-10 mediated tumor rejection in vivo or if the activation of CD8+ T cells is a secondary event, mediated by other cells is not known.

Surprisingly, we show that IL-10 mediated activation of tumor resident CD8+ T cells alone is sufficient, to reject well established large tumors, without the requirement for other cells to respond to IL-10. Moreover, IL-10 induces the expansion of IL-10R
proficient CD8+ T cells trough proliferation.
Materials and Methods

Mice
Female C57BL/6 and BALB/c mice were obtained from The Jackson Laboratory.
C57BL/6 IL-10Rβ-/-, C57BL/6 OT-I, OT-I Rag1-/- CD45.1+/+ and OT-I Rag1-/-
IL10Rβ-/-CD45.1+/+ mice were maintained under specific pathogen-free conditions at
the animal facility of MRL/PA.
For the generation of mixed bone marrow chimeras lethally irradiated CD45.2+/+ hosts
received 5x10^6 bone marrow cells containing 50% from wildtype (WT) CD45.2+/+
donors and 50% from IL-10Rβ-/- CD45.1+/+ donors.
All animal procedures were approved by the IACUC of MRL/PA in accordance with
guidelines of AAALAC.

Tumor models
To generate the PDV6-OVA tumor cell line parental PDV6 cells (18) were transfected
with a plasmid expressing a CMV promoter driven cytoplasmic form of OVA (cOVA;
amino acids 20–145) (19).
Tumor implantations were done as previously described (12). Tumors were left untreated
for at least 10 days before injection with 5–10 μg of DNA using a hydrodynamic
technique.
PEG-IL-10 was injected at 0.1mg/kg twice a day.
CD8 T cell depletion was done as previously described (12).
FTY720 (Cayman Chemical) was dissolved in 20% 2-Hydroxypropyl-β-cyclodextrin
(Sigma) in PBS. A dose of 1mg/kg was administered by i.p. injection three times per
week. Tumor growth was monitored twice per week, recorded as $0.5 \times \text{length} \times (\text{width})^2$ and plotted as mean tumour volume ± SEM.

**Tumor-infiltrating immune cell analysis and flow cytometry.**

Single-cell suspensions from tumors and lymph nodes were generated (12) and were directly used for flow cytometry analyses. To analyze STAT activation 5-7 tumors were pooled and T cells were purified by positive selection with anti-CD90 MACS multi-sort microbeads followed anti-CD45 MACS microbeads according to the manufacturer’s instructions (Miltenyi). Purified TILs were rested in 10% FCS, IMDM overnight followed by a 20 min stimulation with 100ng/ml mouse IL-10 (R&D).

Fluorescence-labeled antibodies against CD11b, CD8a, CD4, CD45, CD45.2, IL-10Ra, NK1.1, IFNγ, STAT1(pY701), STAT3(pY705), STAT4(pY693), STAT5(pY694) and BrdU were all purchased from BD Biosciences. Antibodies to CD45.1 and Foxp3 were purchased from e-Bioscience. For detection of intracellular IFNγ, cells were stained without further *in vitro* restimulation using the Cytofix/Cytoperm kit (BD Biosciences). Intracellular staining kits for BrdU (BD), Foxp3 and Phospho-STAT (Phosflow, BD) were used according to the manufacturer's instructions. Flow cytometry data were acquired on a FACSCantoII (BD) and analyzed with FlowJo software (Tree Star).

**IL-10 Minicircle**

The p2øC31.RSV.hAAT.bpA plasmid (encoding humanα1 anti trypsin) was provided by Dr. Zhi-Ying Chen (Stanford University, CA). For IL-10 minicircle the hAAT was replaced with mIL10 cDNA. Plasmids using the Ubiquitin promoter instead of the RSV
promoter were also generated. Injection of both minicircles resulted in comparable IL-10 serum levels.

Minicircle DNA production followed (20) with minor modifications.

**Adoptive T cell transfer**

Splenocyte suspensions of WT (CD45.1+) and IL-10Rb-/- (CD45.1+) OT-I mice were adoptively transferred into congenic hosts (CD45.2+) bearing PDV6-OVA tumors via tail vein injection. The frequency of Tg CD8+ T cells was determined by FACS before transfer and equal numbers of WT and IL-10Rb-/- CD8+ OT-I T cells were transferred.

**In vitro activation of CD8+ OT-I T cells**

Splenocyte suspensions of C57BL/6 OT-I mice were activated with 1 μg/ml SIINFEKL peptide (Bio-Synthesis) in 24 well plates.

**Gene expression analysis and statistical methods**

All mRNA analysis was performed using quantitative RT-PCR as previously described (12). Statistical analysis was performed using Prism software.
Results

**IL-10 treatment induces activation of CD8+ T cells and tumor rejection**

We have recently shown that pegylated IL-10 (PEG-IL-10) controls tumor growth and induces tumor rejection in a wide variety of tumor models (Suppl. Fig.1A) (12). mRNA analysis of tumors from control and IL-10 treated mice revealed that IL-10 induces a strong increase in the expression of the cytotoxic effector molecules Granzyme B and Perforin and of the effector cytokine IFNγ. In contrast, IL-10 induced only minor changes of these effector molecules in the tumor draining lymph node (TdLN) (Suppl Fig.1B). Isolation of inflammatory cells from tumors showed that PEG-IL-10 induced an increased presence of intratumoral CD8+ T cells (Suppl Fig.1C). Antibody mediated ablation of CD8+ T cells abrogated tumor rejection (Suppl Fig.1D). However, most tumor models required PEG-IL-10 to be administered twice a day, making the usage of PEG-IL-10 for further mechanistic studies tedious and financially prohibitive. Therefore we used the hydrodynamic delivery of an IL-10 encoding minicircle plasmid (21) to further study the molecular mechanisms underlying the potent anti-tumor efficacy of IL-10. The location of the delivery and expression of such intravenously injected plasmids is predominantly in the liver, resulting in high prevalence of secreted proteins in the serum. Mice with established PDV6 tumors injected with the IL-10 minicircle, displayed very high IL-10 serum concentrations immediately after the injection (**Fig.1A**), that subsequently stabilized in the 10 – 100 ng/ml range. IL-10 was undetectable after control minicircle injection (data not shown). The IL-10 levels were sufficient to promote rejection of established PDV6 tumors (**Fig.1B**). In contrast, IL-10 treatment was ineffective in IL-10Rb-/- mice (Suppl Fig.2A,B), indicating that IL-10 acts on the host but not the tumor cells and in RAG-/- mice (Suppl. Fig.2C), indicating the importance of
the adaptive immune system in the IL-10 response. Likewise, established CT26 tumors were rejected in IL-10 treated mice, but not in control mice (Suppl. Fig 3A grey lines). These data confirm the previously reported potent anti-tumor efficacy of IL-10 and demonstrate the usefulness of the IL-10 minicircle for further mechanistic studies.

Next, we investigated IFNγ induction in tumor resident CD8+ T cells by IL-10 using flow cytometry. Cells obtained from CT26 tumors or TdLN of control or IL-10 treated mice were stained for intracellular IFNγ without any further in vitro restimulation. IFNγ was not detectable in CD4+ and CD8+ T cells isolated from TdLN of control or IL-10 treated mice. A small percentage of CD4+ tumor infiltrating T cells (TILs) produced IFNγ but this population did not change in response to IL-10 treatment. In contrast, the frequency of CD8+ TILs producing IFNγ increased on average three-fold upon IL-10 treatment (Fig.1C, D, (12)). These data show that IL-10 treatment leads to an increase in the frequency of IFNγ producing CD8+ T cells in the tumor.

**IL-10 mediated tumor rejection does not require de-novo T cell infiltration**

IFNγ induces the chemokines MIG (CXCL9) and IP-10 (CXCL10), two potent chemoattractants for IFNγ producing T cells (22). The mRNA of MIG and IP10 was induced in the tumor but not in secondary lymphoid organs (Fig.2A). Both chemokines were also potently induced in the serum of IL-10 treated, tumor bearing animals (Fig.2B). Therefore, we asked if chemokine mediated T cell migration could be responsible for the anti-tumor efficacy of IL-10. Mice deficient for the MIG and IP10 receptor (CXCR3-/-) bearing large PDV6 tumors were treated with IL-10. IL-10 treatment induced rejection of tumors in CXCR3 -/- mice with an identical kinetic as in WT mice (Fig.2C), indicating that CXCR3 is not essential or even rate limiting for IL-10 mediated tumor rejection.
Since chemokine receptor usage typically shows some redundancy, T cell migration was blocked using a more general approach. To this end we used FTY720, an analog of sphingosine-1-phosphate that blocks the egress of lymphocytes from lymphoid organs (23). First we verified that FTY720 inhibits the migration of newly activated CD8+ T cells from the lymph node to the tumor. CFSE-labeled OT-I CD8+ T cells, expressing a TCR specific for the Ovalbumin-derived peptide SIINFEKL bound to H-2Kb, were adoptively transferred into mice with established PDV6-OVA tumors. One day later FTY720 treatment was started to analyze the proliferation and accumulation of OT-I cells in the LN and the tumor (Fig.3A). OT-I cells in the LN of control and FTY720 treated mice had proliferated to an equal extent three days after transfer and the frequency of OT-I cells was identical. Only very few OT-I cells were detected in tumors three days after transfer (data not shown). Seven days after the transfer a high frequency of OT-I cells was found in the tumors of control mice, but not of FTY720 treated mice, indicating complete inhibition of CD8+ T cell migration from the lymph node to the tumor (Fig.3B).

We next determined the effect of FTY720 treatment on IL-10 mediated tumor rejection. Mice with established PDV6 tumors were injected three times a week with FTY720 starting three days before minicircle injection. The efficacy of FTY720 was confirmed by analysis of the T cell numbers in the blood (Fig.3C). The frequency of CD8+ T cells in control mice that had not received FTY720 was on average 10% of total PBMC. The frequency of CD8 T cells in the blood of IL-10 treated mice was initially comparable, but was reduced after prolonged exposure to IL-10. In FTY720 treated mice, the frequency of CD8+ T cells in the blood of was reduced to around 1% irrespective of IL-10 treatment.
showing that FTY720 inhibits T cell egress from lymphoid organs even under IL-10 treatment.

However, the blockade of T cell migration by FTY720 did not alter the anti-tumor efficacy (Fig.3D). IL-10 induced rejection of established PDV6 tumors followed a similar kinetic in both buffer and FTY720 treated mice. Identical results were obtained in CT-26 tumor bearing Balb/C mice (Suppl Fig 3A). Despite inhibition of de-novo T cell infiltration, IL-10 treatment increased the activity of tumor resident CD8+ T cells (Suppl. Fig 3 C,D).

These results show that IL-10 does not require the involvement of LN CD8+ T cells but rather directly activates tumor resident CD8+ T cells.

**Activated CD8+ T cells express elevated levels of IL-10Ra**

To determine the mechanism underlying this differential behavior of LN and tumor derived CD8+ T cells we analyzed the IL-10 receptor expression on these cells (Fig.4A). Indeed, tumor-infiltrating CD8+ T cells expressed significantly higher levels of the IL-10 receptor than CD8+ T cells from the TdLN. CD4+ TILs also showed a higher expression of the IL-10 receptor compared to their LN counterparts; however the levels were lower than on CD8+ TILs, especially for effector CD4+Foxp3- TILs. NK cells and CD11b+ cells from the tumor expressed similar or slightly reduced IL-10 receptor levels when compared to the corresponding lymph node population.

We next addressed the question why CD8+ TILs express more IL-10 receptor than their lymph node counterparts. While CD8+ T cells in the lymph node mostly consist of naïve T cells, tumor-infiltrating T cells must have been previously activated to allow for
migration to the tumor. Therefore, the control of IL-10 receptor expression on CD8+ T cells in response to antigen specific stimulation was assessed. Splenocytes from OT-I TCR transgenic mice were stimulated with SIINFEKL peptide \textit{in vitro} and IL-10 receptor surface expression on CD8+ T cells was analyzed. Indeed, activated CD8+ OT-I T cells expressed substantially increased levels of the IL-10 receptor when compared to naïve OT-I cells (Fig.4B). To confirm that activated T cells also increase the expression of IL-10 receptor \textit{in vivo} we transferred CD45.1+ CD8+ OT-I T cells into congenic CD45.2+/+ C57BL/6 mice with established PDV6-OVA tumors. As shown before (Fig.4A), the host CD8+ T cells in the TdLN had undetectable IL-10Ra surface levels. In contrast, OT-I cells in the lymph node expressed elevated levels of IL-10Ra on their surface (Fig.4C). The IL-10 receptor expression was maintained on OT-I cells isolated from the tumor. Host CD8+ T cells from the tumor also showed strong IL-10Ra expression in agreement with previous data (Fig.4C and 4A). Together, these data show that antigen mediated activation of CD8+ T cells leads to increased expression of the IL-10 receptor.

\textbf{IL-10R expression on CD8+ T cells is necessary and sufficient for CD8+ T cell activation by IL-10}

The high level of IL-10 receptor expression on tumor-infiltrating CD8+ T cells, regulatory CD4+ T cells and myeloid cells (Fig.4A) led us to wonder which population might contribute to the observed activation and expansion of CD8+ T cells. To answer this question we employed an adoptive transfer of WT and IL-10Rb-/- CD8+ T cells. WT and IL-10 receptor deficient OT-I CD8+ T cells were co-transferred into congenic WT C57BL/6 mice with established PDV6-OVA tumors (Fig.5A, B). One week after the
T cell transfer mice either received the IL-10 or control minicircle. Five days later we determined the frequency of activated CD8+ T cells by flow cytometry for intracellular IFNγ, since IFNγ expression is essential for IL-10 mediated PDV6 tumor rejection (12). As shown above for CD8+ T cells infiltrating CT26 tumors (Fig.1C), the frequency of IFNγ producing host CD8+ T cells infiltrating PDV6-OVA tumors was increased on average three-fold after IL-10 treatment (Fig.5C). In the tumors of control treated mice a similar percentage of WT and IL-10 receptor deficient OT-I T cells expressed IFNγ (Fig.5D). After IL-10 treatment the frequency of IFNγ producing WT cells in the tumor increased approximately three-fold while the frequency of IFNγ producing IL-10Rb-/- OT-I cells stayed constant (Fig.5D). The frequency of IFNγ producing host CD8+ T cells, WT and IL-10 receptor deficient OT-I T cells in the tumor draining lymph node was low, but identical, irrespective of the presence or absence of IL-10 (data not shown).

These results show that the expression of the IL-10 receptor on tumor resident CD8+ T cells is necessary for the induction of IFNγ after IL-10 treatment. We next asked, if other cells in the micro-environment were required to receive the IL-10 signal or if IL-10R expression on CD8+ T cells alone might be sufficient for their activation. We used IL-10Rb-/- recipients for the adoptive co-transfer of WT and IL10Rb-/- OT-I CD8+ T cells. In this scenario only the WT OT-I cells expressed the IL-10 receptor. In contrast to WT hosts, the frequency of IFNγ producing host CD8+ T cells infiltrating PDV6-OVA tumors was not increased in IL-10Rb-/- hosts after IL-10 treatment (Fig.5E). However, the frequency of IFNγ producing tumor resident WT OT-I cells increased again by approximately three-fold after IL-10 treatment (Fig.5F).
These results show that the expression of the IL-10 receptor is only required on tumor resident CD8+ T cells, to mediate the accumulation of activated, IFN\(\gamma\) expressing intratumoral CD8+ T cells in response to IL-10.

**IL-10R\(b\) expression is required on endogenous CD8+ T cells for activation and IFN\(\gamma\) induction by IL-10**

T cell receptor transgenes may have a comparatively high affinity to their cognate antigen and the antigen is more prominently expressed than endogenous tumor antigens. To investigate a natural affinity range of antigen TCR pairings, mixed bone marrow chimeras were established with WT (CD45.2+/+) and IL-10 receptor deficient (CD45.1+/+) bone marrow. Three month after the transfer, MC38 tumors were implanted. Mice with established tumors were injected with the IL-10 minicircle and intracellular IFN\(\gamma\) was measured by flow cytometry (Fig.6A,B). In tumors from control mice, around 4% of both WT and IL-10R\(b\)-/- CD8+ T cells expressed IFN\(\gamma\) (Fig.6C). In contrast, 10-20% of WT CD8+ T cells in tumors of mice treated with the IL-10 minicircle expressed IFN\(\gamma\), while the frequency of IFN\(\gamma\) producing IL-10R\(b\)-/- CD8+ T cells was not increased.

Taken together, these results show that IL-10 directly activates tumor resident CD8+ T cells to express IFN\(\gamma\).

**IL-10 treatment leads to the preferred accumulation of WT CD8+ T cells in the tumor**

T cells and in particular CD8+ T cells are usually rare in tumors but their increased presence confers favorable prognosis to tumor patients (24). We used mixed bone
marrow chimeras to investigate if the IL-10R status would change CD8+ T cells presence in tumors in dependence of treatment. In control mice, both WT and IL-10R-/- CD8+ T cells were present in lymph nodes and tumors at equal numbers (Fig.6D). The ratio of WT to IL-10R-/- CD8+ T cells in the TdLN remained similar upon IL-10 treatment, even if IL-10R-/- cells appeared to be at a slight disadvantage in later stages of IL-10 exposure (Fig.6E). Surprisingly however, IL-10 treatment not only lead to a strong increase in IFNγ producing WT tumor resident CD8+ T cells (Fig.5B) but also to a preferred accumulation of WT tumor resident CD8+ T cells over IL-10Rb-/- cells. In the mixed bone marrow chimeras the frequency of WT CD8+ T cells in the tumors of IL-10 treated mice was increased from 60% to 90% of all CD8+ T cells with prolonged presence of IL-10. WT CD4+ T cells also accumulated over IL-10Rb-/- CD4+ cells in the tumor but only after prolonged elevation of IL-10 and the differences were less pronounced than for CD8+ T cells (Fig.6H, I).

**IL-10R induces accumulation of antigen specific tumor resident CD8+ T cells**

To confirm the preferred accumulation of WT CD8+ T cells in IL-10 treated tumors we analysed the behavior of WT and IL10Rb-/- OT-I CD8+ T cells after co-transfer into mice carrying large ovalbumin expressing tumors. In control treated mice, both T cell genotypes were present at an equal proportion in the TdLN and the tumor (Fig.7 A-C). In IL-10 treated animals, tumor resident IL-10R proficient cells had again an advantage over IL-10R-/- OT-I CD8+ T cells, while the difference was not statistically significant in the lymph node (Fig.7 A-C).
A similar effect was also observed in IL-10Rb-/- hosts indicating the requirement of the IL-10 signaling in only the CD8+ T cells (Fig 7D). The increased accumulation of tumor resident CD8+ T cells in response to IL-10 is therefore dependent on direct activation of the IL-10R on the CD8+ T cell, rather than an effect on macrophages, dendritic cells or CD4+ helper cells.

It was unclear how the accumulation of tumor resident WT over IL-10Rb-/- CD8+ T cells is regulated. To start elucidating the underlying mechanism the proliferation of tumor resident OT-I CD8+ T cells after adoptive transfer in tumor bearing hosts was measured (Fig.7E). To this end mice were injected with BrdU 16 hours before flow cytometry was performed. In the TdLN and tumor of control treated mice the frequency of BrdU+ WT and IL-10Rb-/- OT-I was identical (data not shown). In contrast, a higher frequency of WT than IL-10Rb-/- OT-I cells had incorporated BrdU in IL-10 treated mice. This difference in proliferation between WT and IL-10Rb-/- OT-I cells was more pronounced in the tumor than in the TdLN (Fig.7E,F).

This result suggests that tumor resident CD8+ T cells proliferate in vivo in response to IL-10 treatment and that this effect is primarily induced directly in CD8+ T cells.

**IL-10 activates a unique combination of STATs in tumor resident CD8+ T cells**

To explain why CD8+ T cells from lymph nodes react different to IL-10 than tumor resident CD8 T cells we asked if the higher expression of the IL-10R on tumor resident CD8+ T cells would result in a differential signaling downstream of the receptor. To this end, we performed flow cytometry for phosphorylated STATs (pSTAT) in T cells. Both in CD8+ T cells isolated from the TdLN and the tumor IL-10 induced pSTAT3 (Fig.7G).
However, the levels of pSTAT3 were almost 10 fold higher in tumor derived CD8+ T cells than in TdLN derived CD8+ T cells. Tumor resident CD8+ T cells also had higher levels of pSTAT3 than CD4+ TILs in response to IL-10 while LN CD8+ and CD4+ T cells displayed similar amounts of pSTAT3. Interestingly, IL-10 signaling also led to the activation of STAT1 but only in tumor derived CD8+ T cells (Fig.7G). IL-10 did not activate STAT1 in CD8+ T cells obtained from the TdLN nor in CD4+ T cells isolated from the LN or tumor. This data suggest that the high expression of the IL-10 receptor on tumor resident CD8+ T cells leads to a unique activation pattern of STAT3 and STAT1 in these cells.
Discussion

IL-10 is generally considered an immune suppressive cytokine and is often cited as one of the molecules responsible for the immune-suppressive environment in tumors. In contrast to this widely held belief, and confirming earlier studies (11-13), we show here that IL-10 induces potent anti-tumor responses. This anti-tumor activity required the presence of CD8+ T cells and IL-10 increased the cytotoxic activity of these cells. We show here, for the first time, that in vivo treatment with IL-10 directly induces specific activation and expansion of tumor resident CD8+ T cells. In human tumors, the number and the activity of intratumoral CD8+ T cells correlates with an improved prognosis for cancer patients (25). High frequency of tumor-specific T cells in the blood, as can be achieved with cancer vaccines, does not necessarily correlate with improved prognosis (26, 27). Restricted migration of T cells into the tumor can explain why vaccine induced increases in tumor-specific T cells have not translated into effective clinical outcomes (28). Interestingly, IL-10 treatment seems to circumvent this problem as T cell trafficking from lymphoid organs is not required for T cell activation in established tumors.

Inhibition of T cell trafficking with FY720 does not prevent IL-10 induced tumor rejection or the activation of tumor resident CD8+ T cells. Our data therefore suggest that IL-10 treatment does not require de-novo priming of tumor-reactive CD8+ T cells in the draining lymph node, but that reactivation of tumor-resident CD8+ T cells can be sufficient to reject tumors.

It is important to note that tertiary lymphoid structures as described in human colon and lung cancers were not found in any of the tumors analyzed in this study and a previous study (12), CD8+ T cells were rather equally distributed throughout the tumor tissue.
The potent anti-tumor efficacy of IL-10 is in stark contrast to its better known immune suppressive capacity in infection and autoimmune models as well as its described contribution to the immune suppressive environment of the tumor.

The stage and location of the immune response as well as the IL-10 target cell driving the immune response at this stage seem to most affect the immunoregulatory function of IL-10. The inhibitory function of IL-10 on T cell responses is most prominent during the priming phase of the immune response. Here, IL-10 mainly inhibits the function of DCs and macrophages, limiting the initial activation of T cells. Moreover, IL-10 can also directly affect CD4+ T cells, inhibiting their activation, proliferation and cytokine production. In contrast, IL-10 stimulates CD8+ T cells in vitro (14-17). However, in a recent study addressing the impact of IL-10 on CD8+ T cells in vivo, it was shown that IL-10 can inhibit pathogen-specific CD8+ T cells directly (29). In this study WT and IL-10Rb deficient OT-I CD8+ T cells were cotransferred into mice 24h after infection with Ovalbumin expressing *Listeria monocytogenes*, when infection induced IL-10 serum levels are highest, and naïve WT CD8+ T cells in the spleen were limited in their expansion. Therefore, IL-10 inhibits the priming and early activation of the transferred CD8+ T cells in secondary lymphoid organs. In our adoptive transfer experiments IL-10 treatment started after the OT-I transfer, when cells had extensively proliferated and migrated into the tumour. Here, in contrast to naïve CD8+ T cells, IL-10 does not inhibit, but potently stimulates activated, tumor-resident CD8+ T cells.

Underlying this different outcome of IL-10 treatment on CD8+ T cell activity might be the levels of IL-10R expression in naïve versus activated cells. Tumor-resident CD8+ T cells express higher levels of IL-10R, leading to a different signalling downstream of the
receptor than in their naïve lymph node counterparts. While lymph-node derived T cells show intermediate levels of pSTAT3, tumor derived CD8+ T cells show high levels of activated pSTAT3 and pSTAT1 in response to IL-10. In many cell types STAT3 and STAT1 have opposing roles in the control of proliferation and the regulation of immune response. STAT3 can promote proliferation and survival. In contrast, STAT1 signalling promotes immune responses, but can induce pro-apoptotic and anti-proliferative pathways (30). The role of these transcription factors in the IL-10 induced activation of tumor-resident CD8+ T cells has not been addressed. Interestingly, IL-21 and IL-27, two cytokines that show anti-tumor efficacy (31, 32), likewise induce preferentially pSTAT1 and pSTAT3 (33, 34) indicating that this combined activation of STATs in CD8+ T cells might be especially beneficial for the induction of productive anti-tumor immune responses.

In summary, we have shown here that therapeutic IL-10 treatment leads to the direct activation of tumor-resident CD8+ T cells and has potent anti-tumor efficacy in several mouse tumor models. This data, together with the finding that higher doses of IL-10 can increase the production of IFNγ and Granzymes in peripheral blood of humans (35, 36), argue that IL-10 should be tested as tumor immunotherapy in human cancer patients.
References

Figure Legends

Figure 1. IL-10 controls tumour growth.

A and B, C57BL/6 mice with established PDV6 tumours were injected with 10 μg control or IL-10 minicircle. A, Serum IL-10 levels in individual mice after minicircle injection. B, Tumour growth after minicircle injection (Arrow). (At least two separate experiments, at least 5 mice per group with similar results). ***, p < 0.001

C,D, Intracellular IFNγ in tumour and TDLN derived T cells without any further *in vitro* restimulation 6 days after minicircle injection of CT26 bearing mice. C, Representative FACS plots gated for CD4+ or CD8+ T cells. Numbers indicate percentage of IFNγ-positive cells. D, summary graph. (representative of more than three experiments with 2-3 mice per group).

Figure 2. IL-10 efficacy is independent of increased MIG and IP10 expression

A, B Treatment of PDV6 bearing C57BL/6 mice with PEG-IL-10.

A, mRNA expression of MIG and IP10 in tumours and tumour-draining lymph nodes of PDV6-bearing mice treated with PEG-IL-10 for 5 days. B, Serum MIG and IP10 levels in individual mice 9 days after start of IL-10 treatment. (two independent experiments with similar results). C, Tumor growth after IL-10 minicircle treatment (5μg) in PDV6 tumor bearing C57BL/6 WT and CXCR3−/− mice. (two independent experiments with at least 5 mice per group, identical results).

Figure 3. Anti-tumour function of IL-10 is intact in mice with blocked T cell migration. A, OT-I adoptive transfer and FTY720 function in tumor bearing mice.
Transfer of splenocytes from CD45.1+ OT-I mice containing 3 x 10^6 CD8+ T cells into PDV6-OVA bearing C57BL/6 mice followed by FTY720 treatment after 1 day (3mg/kg, every other day). A, Representative dot plot and B, summary graph showing the frequency of OT-I CD8+ T cells among CD8+ T cells in control and FTY720 treated mice in TdLN on day 3 and tumour on day 7 after T cell transfer. C, IL-10 and FTY720 treatment to evaluate IL-10 efficacy in the absence of T cell migration. PDV6-bearing mice were treated with 1 mg FTY720 per kg body weight three times a week starting 3 days before minicircle injection. C, Frequency of CD4+ and CD8+ T cells in the blood (after 28 days of FTY720 or control treatment). D, Tumour growth. Arrow depicts time of minicircle injection. (Representative for two separate experiments with 7-10 mice / group with similar results) ***, P < 0.001

**Figure 4. IL-10R is up-regulated on activated CD8+ T cells**

A, Flow cytometry analysis of IL-10Ra expression on indicated cell types. B, Splenocytes from OT-I TCR Tg mice were stimulated with 1 μg/ml SIINFEKL peptide. At the indicated time points, TCR Tg CD8+ T cells were analysed for the surface expression of IL-10Ra. C, Splenocytes from CD45.1+ OT-I mice containing 1.9 x 10^6 CD8+ T cells were adoptively transferred into PDV6-OVA bearing C57BL/6 mice. 9 days later the IL-10Ra surface expression of host and OT-I CD8+ T cells from the tumour or tumour-draining lymph node was analysed. Data are from one of at least two independent experiments consisting of 2-3 mice per group with similar results.
Figure 5. IL-10R expression on CD8+ T cells is necessary and sufficient for increased frequency of IFNγ producing CD8+ T cells in tumours of IL-10 treated mice.

A, Schematic outlining the experimental approach. Splenocytes containing 1.9 x 10^6 CD8+ T cells from CD45.1+/+ IL-10Rb-/- and CD45.1+/+ WT OT-I mice were cotransferred at a 1:1 ratio into PDV6-OVA bearing CD45.1-/- C57BL/6 mice. 8 days later mice were injected with 5 µg control or IL-10 minicircle. 5 days after minicircle injection tumour and lymph node cells were stained for intracellular IFNγ without any further in vitro restimulation. B, Representative dot plot gated on CD8+ T cells showing the identification of the host and adoptively transferred OT-I CD8+ T cells.

C, Representative dot plots (left) and summary graph (right) for intracellular IFNγ staining in host CD8+ TILs of WT recipients. D, Representative dot plots (left) and summary graph (right) for intracellular IFNγ staining in WT and IL-10Rb-/- OT-I CD8+ TILs in WT hosts. The lines connect the results for WT and IL10Rb-/- OT-I cells from the same mouse. E, Representative dot plots (left) and summary graph (right) for intracellular IFNγ staining in host CD8+ TILs of IL-10Rb-/- recipients. F, Representative dot plots (left) and summary graph (right) for intracellular IFNγ staining in WT and IL-10Rb-/- OT-I CD8+ TILs in IL-10Rb-/- hosts.

Data shown are representative of three or more experiments with 2-5 mice per group. Numbers in dot plots indicate percentage of IFNγ-positive cells.

Figure 6. IL-10 acts directly on CD8+ T cells and leads to the preferred accumulation of WT CD8+ T cells in the tumour.
A, Schematic outlining the experimental approach. Lethally irradiated C57BL/6 mice received 5 x 10^6 bone marrow cells from CD45.1+/+ IL-10Rb/-/ and CD45.1-/- WT mice at a 1:1 ratio. 3 months later mice were implanted intradermally with 1 x 10^6 MC38 cells. After tumours were established mice received 5 μg control or IL-10 minicircle. 3, 10 and 16 days after minicircle injection tumour and lymph node cells were stained for intracellular IFNγ without any further in vitro restimulation.

B, Representative dot plots and C, summary graph for intracellular IFNγ staining in CD8+ TILs. Data shown are for tumour-infiltrating CD8+ T cells and are pooled from all time points. Results are representative of two experiments with 2-4 mice per group and time point. Numbers in dot plots indicate percentage of IFNγ-positive cells.

D, Representative dot plots and summary graph for the frequency of WT and IL-10Rb/-/ CD8+ T cells in E, the tumour-draining lymph node of control treated mixed BM chimera, F, the tumor of control treated mice as well as G, in the tumor of IL-10 treated animals. H, Representative dot plots and I, summary graph for the frequency of WT and IL-10Rb/-/ CD4+ T cells in the tumour of IL-10 treated of mixed BM chimera.

**Figure 7. WT CD8+ T cells proliferate more than IL-10Rb/-/ CD8+ T cells in IL-10 treated mice.**

Experimental approach as shown in Figure 5A.

A, Dot plot gated on CD8+ T cells showing the identification of the host and adoptively transferred OT-I CD8+ T cells. B, Representative dot plots showing the frequency of WT and IL-10Rb/-/ OT-I CD8+ T cells in the tumour and tumour-draining lymph node and C, D summary graphs showing the frequency of WT and IL-10Rb/-/ OT-I cells as
percentage of total transferred cells in WT (C) or IL-10Rb-/− mice (D). E,F Mice were injected with 1 mg/ml BrdU i.p. 16 h prior to FACS analysis. E, Representative dot plots showing BrdU incorporation in WT and IL-10Rb-/− OT-I CD8+ T cells in the tumour and tumour-draining lymph node of IL-10 treated mice and F, summary graph representing the ratio of BrdU+ WT to IL-10Rb-/− OT-I cells in control and IL-10 treated animals. G, T cells from tumours and tumour-draining lymph nodes of CT26-bearing mice were isolated, rested overnight and stimulated with 100 ng/ml IL-10 for 20 min. Cells were fixed immediately and stained for the indicated phosphorylated STAT proteins. Data are from one of two independent experiments with similar results.
Emmerich Figure 1
Emmerich Figure 2
**Emmerich Figure 3**

A: LN day 3 and tumor day 7 analysis

- **ctrl**: 7.3% CD45.2+OT-I cells
- **CD45.2**: 8.8% CD45.2+OT-I cells
- **FTY720**: 0.2% CD45.2+OT-I cells

B: OT-I (% of CD8+)

C: Blood analysis

- **-FTY720**: CD4: 33%, CD8: 1.2%
- **+FTY720**: CD4: 17%, CD8: 1.3%

D: Tumor growth

- **-FTY720**: Control: 1200 mm³, IL-10: 600 mm³
- **+FTY720**: Control: 900 mm³, IL-10: 300 mm³
Emmerich Figure 4

A

<table>
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<th>CD8+</th>
<th>CD4+Foxp3-</th>
<th>CD4+Foxp3+</th>
<th>NK1.1+</th>
<th>CD11b+</th>
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<tbody>
<tr>
<td>TdLN</td>
<td>Tumor</td>
<td>IL-10Ra</td>
<td>isotype</td>
<td></td>
</tr>
</tbody>
</table>

B

OT-I *in vitro*

- day 0
- day 1
- day 2
- day 3
- day 4

C

OT-I *in vivo*

<table>
<thead>
<tr>
<th>Host CD8+ (CD45.1-)</th>
<th>OT-I (CD45.1+)</th>
</tr>
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<tbody>
<tr>
<td>TdLN</td>
<td>Tumor</td>
</tr>
</tbody>
</table>

IL-10Ra

Emmerich Figure 4
Emmerich Figure 5
Emmerich Figure 6
Emmerich Figure 7
IL-10 directly activates and expands tumor resident CD8+ T cells without de novo infiltration from secondary lymphoid organs

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Cancer Res  Published OnlineFirst May 11, 2012.