OncoImmunology 4:4, e998529; April 2015; Published with license by Taylor & Francis Group, LLC

Myeloid *STAT3* promotes formation of colitis-associated colorectal cancer in mice

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Keywords: immune surveillance, immunoediting, tumor microenvironment, tumor stroma

Abbreviations: AOM, Azoxymethane, CRC, colorectal cancer; DSS, Dextransulfate; IHC, immunohistochemistry; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage

Myeloid cells lacking *STAT3* promote antitumor responses of NK and T cells but it is unknown if this crosstalk affects development of autochthonous tumors. We deleted *STAT3* in murine myeloid cells ($STAT3^{\Delta m}$) and examined the effect on the development of autochthonous colorectal cancers (CRCs). Formation of Azoxymethane/Dextransulfate (AOM/DSS)-induced CRCs was strongly suppressed in $STAT3^{\Delta m}$ mice. Gene expression profiling showed strong activation of T cells in the stroma of $STAT3^{\Delta m}$ CRCs. Moreover, $STAT3^{\Delta m}$ host mice were better able to control the growth of transplanted MC38 colorectal tumor cells which are known to be killed in a T cell-dependent manner. These data suggest that myeloid cells lacking *STAT3* control formation of CRCs mainly via cross activation of T cells. Interestingly, the few CRCs that formed in $STAT3^{\Delta m}$ mice displayed enhanced stromalization but appeared normal in size indicating that they have acquired ways to escape enhanced tumor surveillance. We found that CRCs in $STAT3^{\Delta m}$ mice consistently activate *STAT3* signaling which is implicated in immune evasion and might be a target to prevent tumor relapse.

Introduction

CRC represents the third most common form of cancer in humans. CRC develops frequently in the context of inflammatory bowel disease demonstrating a tumor-promoting role of inflammation.[1] Moreover, stroma immune cells support growth of CRC via production of cytokines that activate the oncogenic transcription factors NFκB and STAT3.[2] However, the stroma also contains immune cells with antitumor activities which force tumors to develop mechanisms of escape and immune suppression.[3] One paradigm is the ability of tumors to modulate polarization of TAMs. Th1 cytokines polarize macrophages toward the M1 phenotype which is responsible for innate immune attack of infectious pathogens but also neoplastic cells. Alternative activation by Th2 cytokines polarizes macrophages toward the M2 phenotype which is characterized by low expression of M1 markers (e.g., iNOS) but high expression of M2 markers (e.g., Arginase-1).[4] M2 macrophages have reduced antitumorigenic activities but promote angiogenesis and tumor progression.[4] Repolarization of M2 macrophages toward the M1 phenotype in tumors represents a promising option for antitumor therapies.[5] Another layer of complexity was recently discovered in breast tumors where an additional TAM population, distinguishably from M1 or M2 polarized macrophages, was identified.[6]

STAT3 phosphorylation at tyrosine residue 705 (pY-STAT3) via Janus kinases (JAKs) is induced by pro- and anti-

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Submitted: 10/23/2014; Revised: 12/10/2014; Accepted: 12/10/2014

http://dx.doi.org/10.1080/2162402X.2014.998529

inflammatory cytokines such as IL-6 and IL-10.[7] The oncogenic role of *STAT3* in CRC tumor cells has been demonstrated in several studies.[8-10] However, *STAT3* seems to have a suppressive role in advanced CRCs because Apc^{Min} mice lacking *STAT3* in tumor cells developed aggressive intestinal carcinomas that were not observed in Apc^{Min} control animals.[11-13] *STAT3* activation in tumor cells represents an important immune escape mechanism as cancer cells exploit *STAT3*-induced expression of VEGF and IL-10 to blunt maturation of stroma DCs. An immune suppressive action is further given by the *STAT3*-dependent repression of pro-inflammatory cytokines and chemokines including IFNγ, TNFα, IL-6, and CXCL10.[14,15]

Apart from tumor-cell specific functions, *STAT3* is considered to promote tumor formation when being activated in stroma cells of CRCs.[13,16,17] Tumor transplantation experiments into mice lacking *STAT3* in the hematopoietic system demonstrated that *STAT3* activation in immune cells prevents antitumorigenic activities of DCs, Th1 cells, NKs, and neutrophils.[18] This led to the concept of inhibiting stroma/hematopoietic *STAT3* as potential strategy for cancer immunotherapy.[19] Myeloid *STAT3* is considered of particular importance for the modulation of Th1-like antitumor responses.[20] Consequently, inhibition of *STAT3* in myeloid cells and B cells using CpG-STAT3 siRNA enhanced cytotoxic T cell (CTL)-mediated responses in a melanoma transplantation model.[21]

Here, we demonstrate that ablation of myeloid *STAT3* significantly interfered with development of autochthonous AOM/DSS-induced colorectal tumors in mice. This effect was mediated by an enhanced antitumor T cell response. Weighted gene co-expression network analysis (WGCNA) uncovered sustained T cell-mediated immune surveillance in escaped $STAT3^{\Delta m}$ tumors accompanied by enhanced *STAT3* signaling in tumors cells which might promote immune evasion.

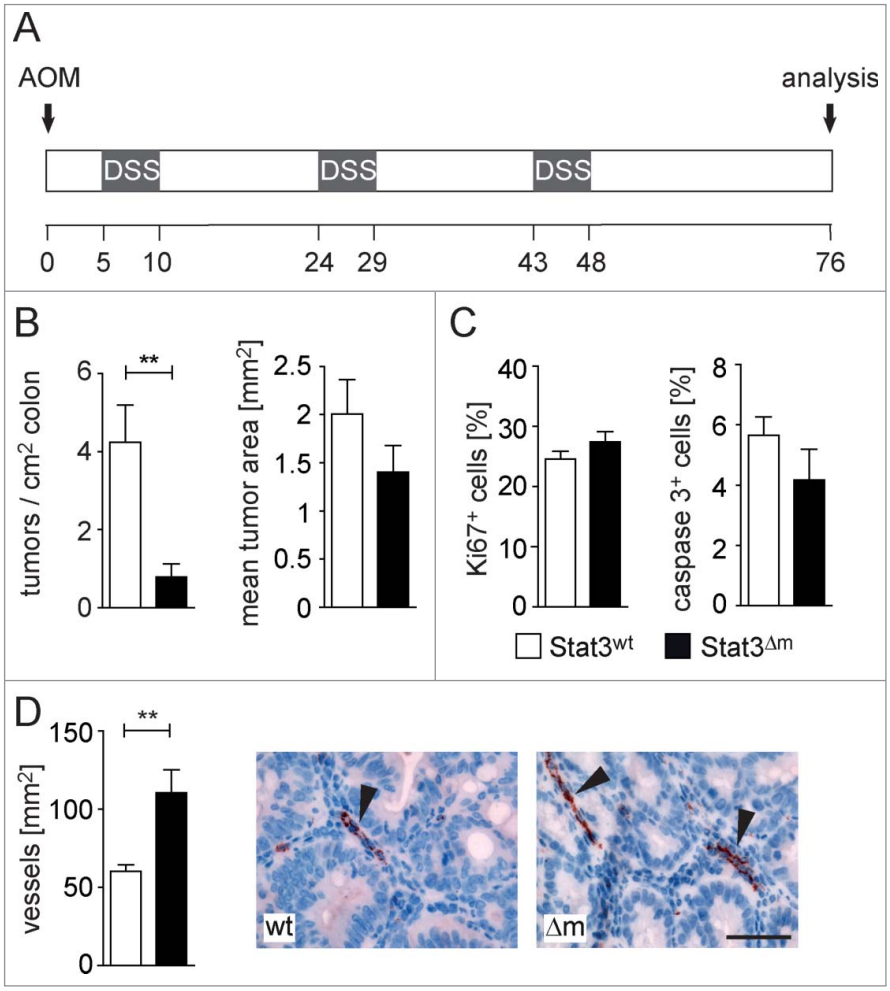


Figure 1. Myeloid *STAT3* promotes formation of CRCs. (**A**) Scheme of the AOM/DSS protocol for induction of CRCs. (**B**) Tumor multiplicity and mean tumor area of CRCs in male mice (n = 13 for $STAT3^{wt}$ and 16 for $STAT3^{\Delta m}$). Bars represent data +/- SEM. (**C**) Histomorphometric quantitation of IHC stainings for cell proliferation (Ki67) and apoptosis (cleaved caspase 3) in CRCs. Bars represent data +/− SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype. (**D**) Assessment of blood vessel densities in the stroma of CRCs. IHC stainings for vWF-positive blood vessels (arrowheads in images) were used for quantitation. Bars represent data +/− SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype. Scale bar indicates 50 μm.

Results

Myeloid *STAT3* promotes formation of colorectal tumors

We investigated the role of *STAT3* in the myeloid stroma cells of autochthonous colorectal tumors developing under inflammatory conditions using mice with conditional inactivation of *STAT3* in myeloid cells ($STAT3^{\Delta m}$ = $LysM^{Cre/Cre}$ $STAT3^{flox/flox}$) and corresponding controls ($STAT3^{wt}$ = $LysM^{Cre/Cre}$ $STAT3^{+/+}$). The LysMCre knock-in alleles were kept homozygous to account for potential Cre or LysM knock-in effects. Successful deletion of *STAT3* in myeloid cells was confirmed by Western blot of granulocyte-macrophage precursors, FACS-sorted peritoneal macrophages (**Fig. S1A**) and qPCR of FACS-sorted splenic macrophages (**Fig. S1B**). Histopathological analysis and IHC-staining revealed no major changes of cell differentiation or proliferation in colon and small intestine of $STAT3^{\Delta m}$ mice (**Fig. S2A**). Despite the observation that deletion of *STAT3* in myeloid cells provokes Th1 differentiation and development of colitis,[22,23] we could not detect severe colitis in $STAT3^{\Delta m}$ mice in the C57BL/6 genetic background (**Fig. S2B**). We only observed a consistently increased mean fluorescence intensity of the activating protein CD69 on $CD4^+$ T cells in the lamina propria of DSS-treated

STAT3$^{\Delta m}$ mice (**Fig. S3**) indicative for Th1 differentiation.[24]

To investigate the impact of myeloid *STAT3* in autochthonous tumors, we induced CRC using AOM/DSS (**Fig. 1A**). Tumor multiplicity was strongly reduced in STAT3$^{\Delta m}$ male mice but tumor size was not affected (**Fig. 1B**). Tumor analysis in female mice gave similar results (data not shown). Tumor parameters such as cell proliferation and cell survival were unaltered in STAT3$^{\Delta m}$ CRCs (**Fig. 1C**) whereas increased numbers of blood vessels were found in the tumor stroma (**Fig. 1D**).

Myeloid STAT3 regulates stromalization of CRCs

Tumor progression requires the reciprocal interaction between tumor and stroma cells. Remarkably, histopathologic and immunohistochemical examination revealed an increased stromalization of STAT3$^{\Delta m}$ CRCs (**Fig. 2A**). Histomorphometric quantitation of IHC-stained sections (**Fig. S4**) demonstrated that the relative percentages of most cell types were unaltered in the stroma of STAT3$^{\Delta m}$ CRCs (**Fig. 2B**). Interestingly, RORγt^{+} Th17 cells were almost absent (**Fig. 2B**). Among macrophages, we found a reduction of cells expressing the macrophage M2 polarization protein Arginase-1 paralleled by an increase in the number of M1 expressing iNOS-positive cells (**Fig. 2C**).

Increased stromalization is frequently associated with enhanced tumor progression and invasiveness. However, no significant alteration in the percentages of low grade tumors, high grade tumors and carcinomas was observed in STAT3$^{\Delta m}$ mice (**Fig. 2D**).

Myeloid *STAT3* is not required for NK cell-mediated immune surveillance of CRCs

Loss of *STAT3* in myeloid cells leads to an aberrant activation of NK and T cells.[18,25] Thus, we studied whether reduced colorectal tumorigenesis is caused by an improved immune surveillance exerted by NK or T cells. NK cell-dependent cytotoxicity was assessed *ex vivo* using spleen-derived NK cells and CFSE-stained YAC-1 target cells. No significant differences in killing efficacies of NK cells isolated from STAT3^{wt} or STAT3$^{\Delta m}$ mice were

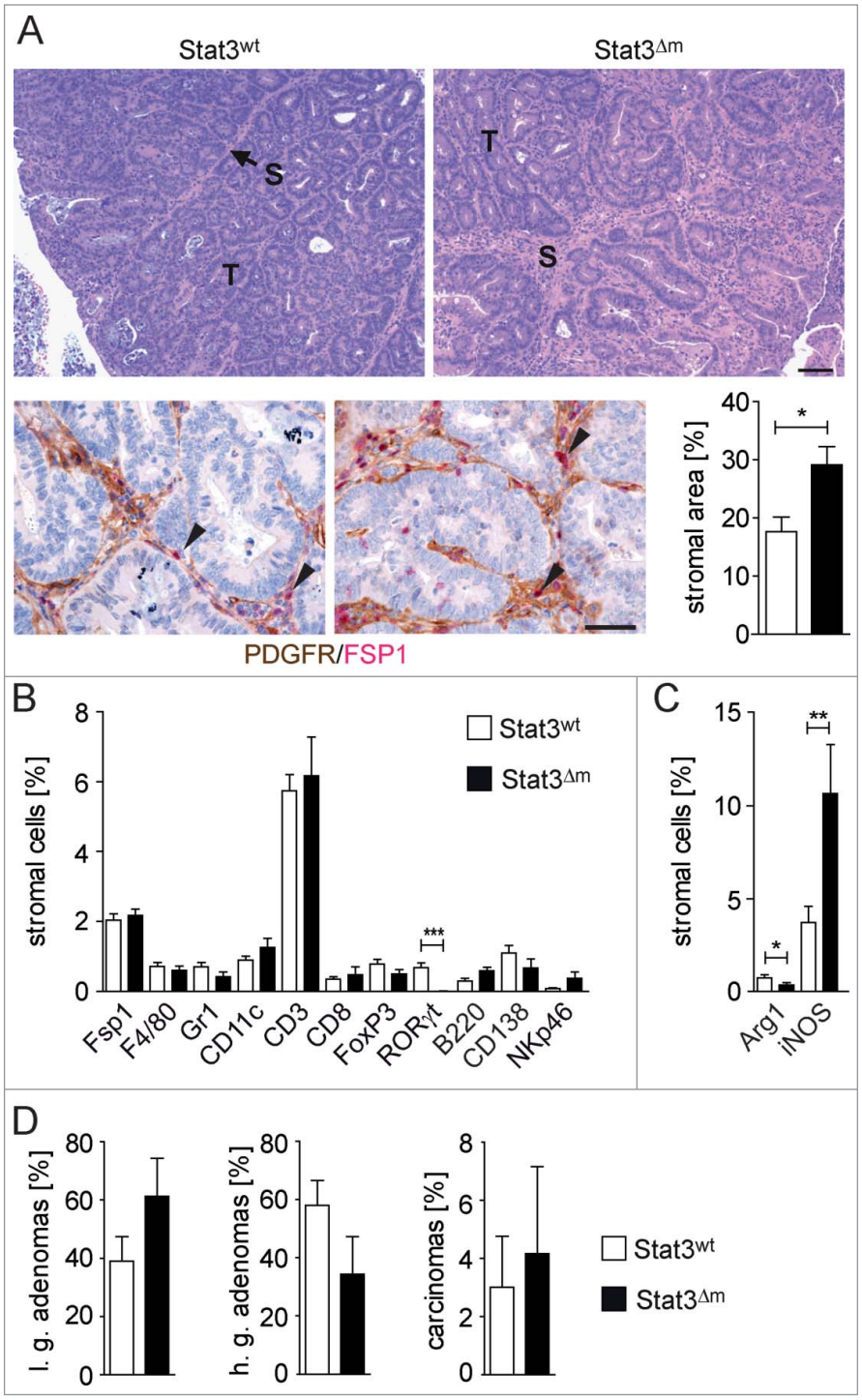


Figure 2. Enhanced stromalization of STAT3$^{\Delta m}$ tumors. (**A**) H&E staining (upper panel; T: tumor; S: stroma; scale bar indicates 100 μm) and double IHC for PDGFR/FSP1 (lower panel; scale bar indicates 50 μm) revealed increased stromal area in STAT3$^{\Delta m}$ tumors. FSP1^{+} fibroblasts are indicated by arrowheads. (**B**) IHC stainings with antibodies for indicated antigens were used for quantitative histomorphometry to define the composition of the tumor stroma. Bars represent data +/− SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype. (**C**) STAT3$^{\Delta m}$ tumors displayed reduced percentages of Arginase-1^{+} cells and increased percentages of iNOS^{+} cells. Bars represent data +/− SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype. (**D**) Histopathological grading of colon tumors revealed no significant change in STAT3$^{\Delta m}$ mice (l.g.: low grade; h.g.: high grade; $n = 97$ STAT3^{wt} and 26 STAT3$^{\Delta m}$ tumors).

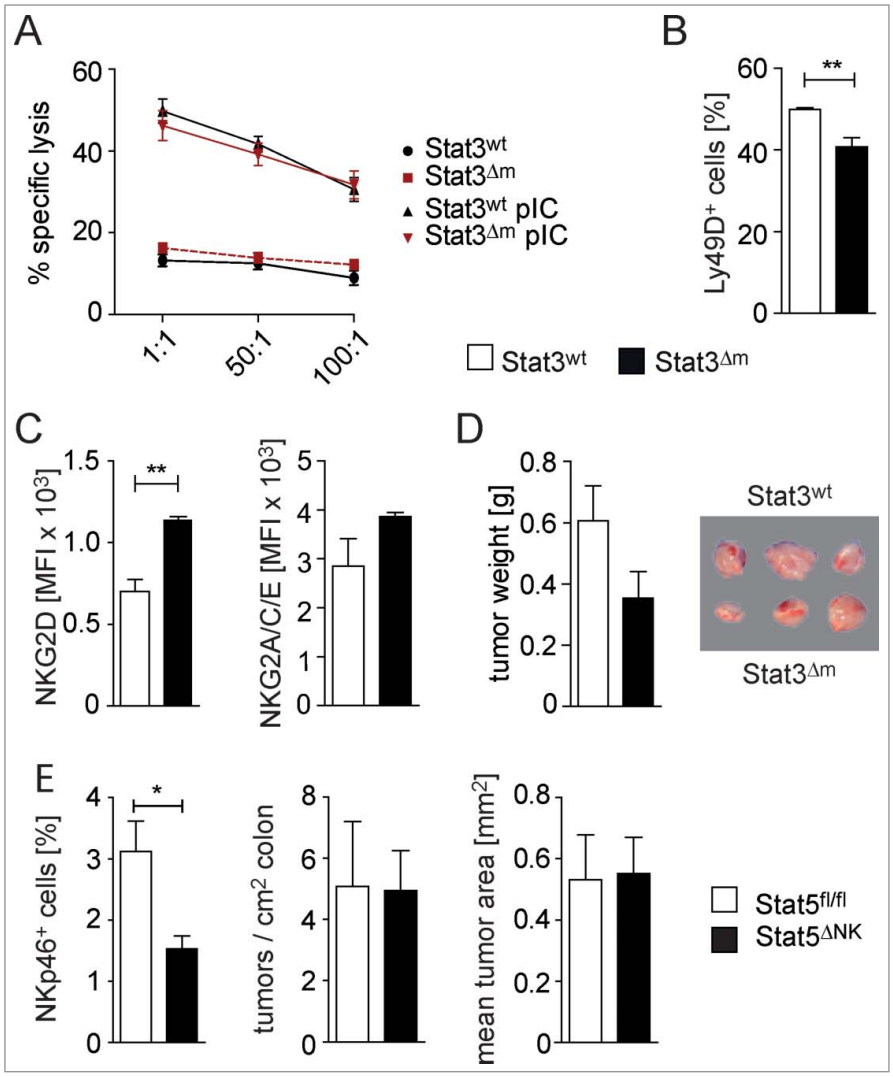


Figure 3. Tumor cytotoxicity of NK cells, isolated from spleens of STAT3^{wt} and STAT3$^{\Delta m}$ mice, and development of CRCs in mice with reduced NK cell numbers. (**A**) Flow cytometric analysis of specific lysis of CFSE-labeled YAC-1 target cells by splenocytes from STAT3^{wt} and STAT3$^{\Delta m}$ mice, injected with PBS or poly (I:C) ($n = 3$ per group). Different effector:target ratios are indicated. (**B**, **C**) Flow cytometric analysis of STAT3^{wt} and STAT3$^{\Delta m}$ splenocytes demonstrating reduced percentage of CD3$^-$ NKp46$^+$ gated Ly49D$^+$ NK cells but increased mean fluorescence intensity (MFI) of NKG2D on CD3$^-$ NKp46$^+$ gated NK cells in STAT3$^{\Delta m}$ mice ($n \geq 4$ mice per genotype). (**D**) Tumor weight and tumor images after transplantation of BCR/ABL^{p185} tumor cells into STAT3^{wt} and STAT3$^{\Delta m}$ host mice ($n = 15$ tumors in STAT3^{wt} and 11 tumors in STAT3$^{\Delta m}$ mice). (**E**) STAT5$^{\Delta NK}$ mice were employed as genetic model for NK ablation. The reduction of NK cell numbers in the intestine was confirmed by FACS analysis of mucosal cell preparations for CD45$^+$ CD3$^-$ CD19$^-$ NKp46$^+$ NK cells (left graph). Reduction of NK cell numbers did not affect tumor multiplicity and mean tumor area of AOM/DSS-induced CRCs ($n = 8$ STAT5$^{fl/fl}$ and 11 STAT5$^{\Delta NK}$ mice). Bars represent data +/− SEM.

detected (**Fig. 3A**). Similarly, numbers of NK cells in the spleen and the expression of NK cell maturation markers (CD27, KLRG-1) were unchanged (**Fig. S5A**) as were the percentages of NKG2D$^+$, NKG2A/C/E$^+$, DNAM-1$^+$, and Ly49C/I$^+$ NK cells (**Fig. S5B**). The only consistent changes were lower numbers of NK cells expressing the activating receptor Ly49D, which recognizes MHC-I alloantigens (**Fig. 3B**) and an enhanced mean fluorescence intensity of the activating receptor NKG2D in STAT3$^{\Delta m}$ mice (**Fig. 3C**). As NKG2D is an important receptor for the recognition of stress-induced ligands on tumors, we performed transplantation experiments with isogenic C57BL/6-derived BCR/ABL^{p185} leukemia cells that are sensitive to eradication by NK cells.[26] Although BCR/ABL^{p185}-derived tumors were slightly smaller in STAT3$^{\Delta m}$ host animals, indicative for an improved NK cell-mediated tumor surveillance, the differences did not reach statistical significance (**Fig. 3D**).

We next investigated the impact of NK cell-mediated immune surveillance on formation of autochthonous CRCs. For that purpose, we treated STAT5^{wt} and STAT5$^{\Delta NK}$ mice with AOM/DSS. STAT5$^{\Delta NK}$ mice represent a model for genetic ablation of NK cells.[27] However, no significant change in AOM/DSS-induced tumor formation was observed in STAT5$^{\Delta NK}$ mice despite of the substantial reduction of NKp46$^+$ cell numbers in the gut (**Fig. 3E**). These data suggest that NK cell-mediated killing activities do not significantly contribute to immune surveillance of AOM/DSS-induced CRCs.

Myeloid *STAT3* is required for T cell-mediated immune surveillance of CRCs

Apart from NK cells, CTLs and helper T cells are key determinants of tumor immune surveillance. Therefore, we performed *in vivo* cytotoxicity assays. m-TRP2$_{181\text{-}188}$-immunized STAT3$^{\Delta m}$ mice displayed a slight but significantly enhanced killing activity of m-TRP2$_{181\text{-}188}$-pulsed CFSE-labeled splenocytes *in vivo* (**Fig. 4A**) indicating enhanced CTL activity. To address whether the enhanced T cell-dependent cytotoxicity extends to CRCs, we transplanted isogenic colorectal MC38 adenocarcinoma cells. Remarkably, MC38 tumor growth, which is controlled by T cells,[28] was significantly impaired in STAT3$^{\Delta m}$ host animals (**Fig. 4B**). Reduced tumor growth was accompanied by higher numbers of CD4$^+$ T cells and slightly increased numbers of CD8$^+$ cytotoxic T lymphocytes in tumor cell preparations (**Fig. 4C**). The number of immature myeloid-derived suppressor cells (MDSCs) was also elevated in tumors of STAT3$^{\Delta m}$ host mice whereas TAMs were reduced (**Fig. 4C**). No changes of NK cell infiltration were observed (data not shown). Moreover, NKG2D ligand RaeI and MHC-I protein expression levels were similar on MC38 tumor cells that developed in STAT3^{wt} and STAT3$^{\Delta m}$ mice (data not shown).

These data demonstrate enhanced T cell-mediated killing of MC38 colorectal tumors cells in STAT3$^{\Delta m}$ host mice.

We next investigated if enhanced T cell activity is also evident in the stroma of autochthonous AOM/DSS-induced STAT3$^{\Delta m}$ CRCs. Tumor and stroma tissue were separated by microdissection and microarray analysis was performed with isolated RNA. Genes indicative for T cell activation were upregulated in the stroma of STAT3$^{\Delta m}$ CRCs (**Fig. 4D**). In human CRCs, a gene expression profile called Immunologic Constant of Rejection (ICR) signature[29] is indicative for T cell activation and better prognosis of patients. Several genes of the human ICR signature such as stat1 were also found upregulated in murine STAT3$^{\Delta m}$ CRCs (**Fig. 4E**). STAT1 is a key factor in immune surveillance[30] and it has been shown that deletion of *STAT3* shifts gp130-type cytokine signaling from a *STAT3* to a *STAT1* response.[31] Consistently, increased numbers of stroma cells with STAT1 protein expression and STAT1 activation were detected in STAT3$^{\Delta m}$ CRCs (**Fig. 4F**). Taken together, these data support the concept that CRC growth in STAT3$^{\Delta m}$ mice is limited by T cell-mediated immune surveillance.

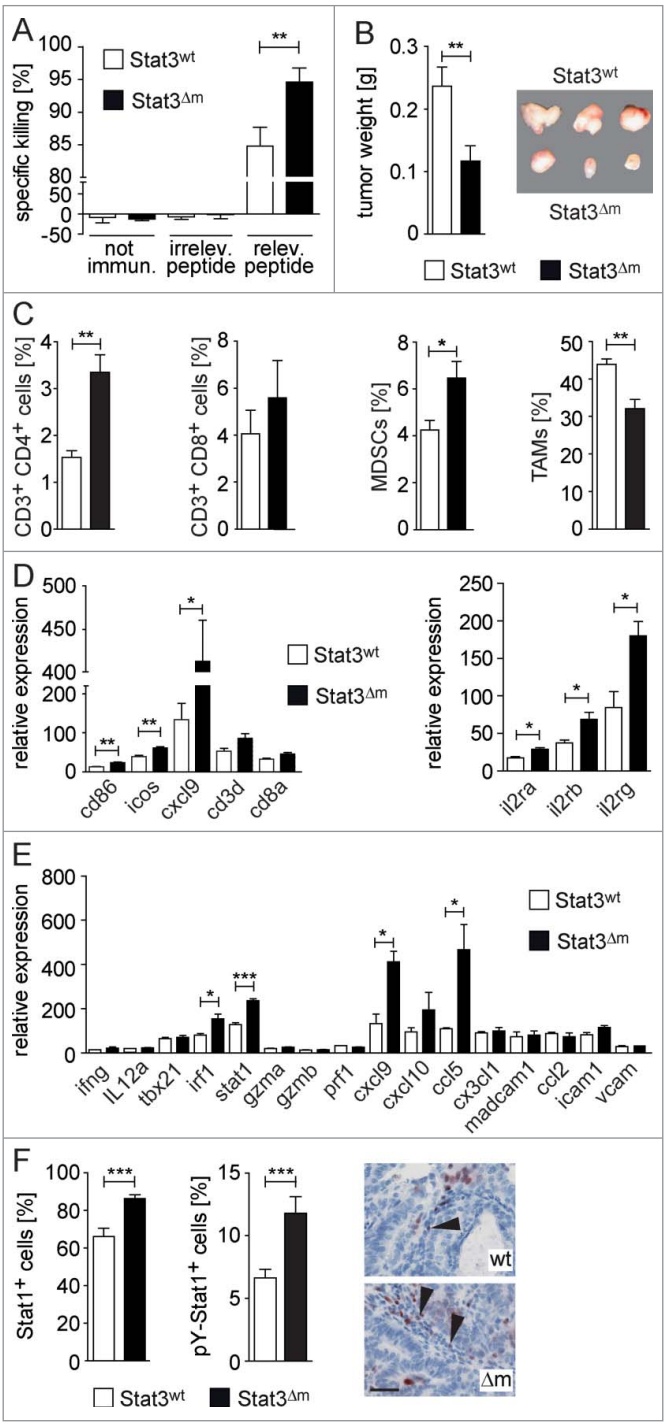


Figure 4. Characterization of cytotoxic T cell activity in STAT3$^{\Delta m}$ mice. (**A**) *In vivo* cytotoxicity assay in STAT3^{wt} and STAT3$^{\Delta m}$ mice immunized with m-TRP2$_{181\text{-}188}$. CFSE^{low} target cells were used as internal control. CFSE^{high} target cells were loaded with an irrelevant peptide or m-TRP2$_{181-188}$ (relevant peptide). Specific killing was calculated after FACS analysis of CFSE-labeled target cells ($n = 4$ mice per group). (**B**) Tumor weight of transplanted MC38 tumor cells into STAT3^{wt} and STAT3$^{\Delta m}$ mice ($n = 12$ tumors in STAT3^{wt} and 14 tumors in STAT3$^{\Delta m}$ mice). (**C**) FACS analysis of immune cell preparations revealed increased numbers of CD4$^+$ T cells, MDSCs (Gr1^{hi} CD11b$^+$) and reduced numbers of TAMs (Gr1^{lo} CD11b$^+$ CD11c^{lo}) in MC38 tumors of STAT3$^{\Delta m}$ hosts. Bars represent data +/– SEM. (**D**) Affymetrix RNA expression data of laser-capture microdissected stroma tissue from STAT3^{wt} and STAT3$^{\Delta m}$ CRCs for genes indicative for T cell activation (left graph) and IL-2 receptors (right graph). (**E**) Affymetrix RNA expression data of ICR genes measured in the tumor stroma. (**F**) STAT1 protein expression and activation in the stroma of STAT3$^{\Delta m}$ CRCs. Images show IHC-stained tissue sections of stroma cells positive for phosphorylated (activated) pY-STAT1 (arrowheads). Scale bar indicates 50 μm. Corresponding IHC stainings were used for histomorphometric quantitation of STAT1- and pY-STAT1-positive cells in the tumor stroma (bar diagrams). Bars represent data +/– SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype.

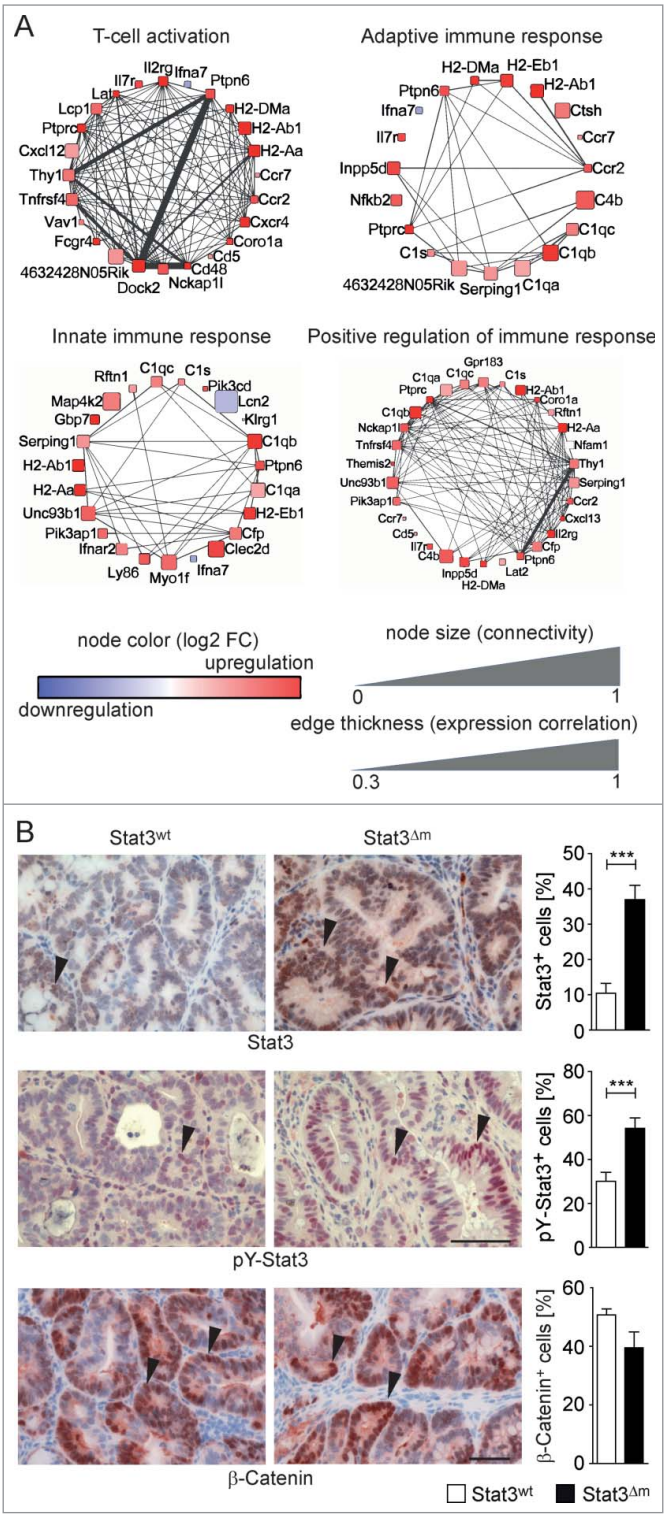
CRCs of STAT3$^{\Delta m}$ mice escape T cell-mediated killing

In order to gain further insight into immune-mediated killing and putative tumor cell-autonomous escape mechanisms of CRCs, microarray data from tumor and stroma tissues were analyzed by WGCNA. Modules of high correlation for genes implicated in immune responses and cytokine production were identified with microarray data from stroma tissue (**Fig. S6**). In line with the increased density of blood vessels observed in the stroma of STAT3$^{\Delta m}$ tumors (**Fig. 1D**) we also identified a module containing genes implicated in angiogenesis (**Fig. S6**). This module was further stratified into positive and negative regulators of angiogenesis which revealed a bias toward increased expression of positive regulators and decreased expression of negative regulators in the stroma of STAT3$^{\Delta m}$ tumors (**Fig. S7A**). Network plots for GO terms in immune modules revealed a clear result with drastic upregulation of corresponding genes in the stroma of STAT3$^{\Delta m}$ tumors (**Fig. 5A**; **Fig. S7B**). Upregulation of cytokine/chemokine receptors, MHC-II molecules and complement factors was identified in network plots (**Fig. 5A**; **Fig. S7B**). Implicated genes suggested a strong enhancement of immune responses related to T cell activation. In summary, these data provide strong evidence for a pronounced T cell dependent immune surveillance occurring in AOM/DSS-induced tumors in the absence of myeloid *STAT3*.

Cell-autonomous changes of putative immunoediting pathways in STAT3$^{\Delta m}$ CRCs

Despite maintained T cell activation in the stroma, tumors of STAT3$^{\Delta m}$ mice did not show a significant reduction in size (**Fig. 1B**). This indicates that established CRCs in STAT3$^{\Delta m}$ mice must have developed strategies to escape the enhanced tumor surveillance. We thus investigated the microarray expression data of the microdissected tumor cell tissue separately for processes involved in immunoediting. RNA expression of immunosuppressive tumor-derived factors (**Fig. S8A**) and NKG2D stress-induced ligands (**Fig. S8B**) were unchanged. Unaltered protein expression of the NKG2D ligand RaeI was confirmed by IHC (data not shown). As STAT3$^{\Delta m}$ CRCs displayed increased expressing of complement factors in the stroma (**Fig. 5A**; "adaptive immune response") we investigated expression of mCRPs, which protect tumor cells from complement attack. No differences were observed (**Fig. S8C**).

We next used the Molecular Signature Database of the Broad Institute to identify differentially regulated signaling pathways in tumor cells. Genes upregulated in STAT3$^{\Delta m}$ tumor cells (>2-fold) overlapped with genes upregulated in colonic tissue after treatment with IL-22 (a potent inducer of STAT3)[32] indicating enhanced STAT3 signaling. Since STAT3 activation is a prominent mechanism involved in immune escape of tumor cells,[33] we stained STAT3$^{\Delta m}$ tumors for STAT3 and activated nuclear pY-STAT3. Consistent with the overlap analysis, elevated levels of *STAT3* and activated pY-STAT3 were evident in STAT3$^{\Delta m}$ tumor cells (**Fig. 5B**). The overlap analysis also suggested increased expression of c-Myc, a *STAT3* target gene,[34] in STAT3$^{\Delta m}$ tumor cells which was confirmed by IHC (data not shown). In contrast, no obvious change in nuclear ß-Catenin, the major driver of CRC formation, was observed (**Fig. 5B**). These data suggest that STAT3$^{\Delta m}$ CRCs activate STAT3 signaling in tumor cells for immune escape.



Discussion

Ablation of hematopoietic *STAT3* results in antitumor immune responses in murine models of tumor cell transplantation.[14] These models have certain disadvantages since tumor cell lines have been cultured for a long period of time and may not ideally reflect tumor development *in vivo*. Our knowledge on the role of hematopoietic STAT3 in autochthonous tumors is limited. It is also unclear if and how tumors will adapt to a different microenvironment upon deletion of *STAT3* to circumvent and escape the improved immune surveillance. Thus, we deleted *STAT3* in myeloid cells in a murine model of autochthonous CRCs. The use of an autochthonous model allowed us to study

Figure 5. Weighted Gene Co-expression Network Analysis (WGCNA) of Affymetrix RNA expression data obtained with laser-capture microdissected stroma of STAT3^{wt} and STAT3$^{\Delta m}$ CRCs. Modules of highly correlated genes were identified for immune response genes. (**A**) Selected network plots for GO terms in immune modules. Blue node color represents downregulation and red node color represents upregulation of gene expression in the stroma of STAT3$^{\Delta m}$ tumors. The node size is associated with the gene's co-expression in the entire data set. The edge (line) thickness is linked to the gene's connectivity (co-expression within the module). (**B**) Enhanced STAT3 expression and activation in STAT3$^{\Delta m}$ CRCs. IHC stainings for total STAT3, phosphorylated pY-STAT3 and ß-Catenin proteins were used for histomorphometric quantitation of positive tumor cells (arrowheads in images). Bars represent data +/− SEM of $n \geq 9$ tumors in ≥ 3 animals per genotype. Scale bar indicates 50 μm.

cellular and molecular consequences of myeloid *STAT3* ablation on the tumor stroma as well as on the tumor cells themselves. We found that ablation of myeloid *STAT3* interfered with formation of inflammation-associated CRCs. Only few tumors evolved that seemed having adapted to escape the strong immunogenic microenvironment.

Inflammation is a strong promoter for CRC development but inflammatory infiltrates also contain immune cells with antitumor activities.[1] Loss of STAT3 in hematopoietic cells provokes inflammation in the gut albeit it remained unclear whether this condition promotes or prevents formation of colitis-associated CRCs.[23,35,36] Interestingly, we have not observed severe spontaneous colitis in $STAT3^{\Delta m}$ mice. $STAT3^{\Delta m}$ and $STAT3^{wt}$ mice also responded to a comparable extent to DSS treatment regarding the activation status of intestinal inflammatory cells and ulceration score. Several reasons may underlie this discrepancy. It may be caused by the different C57BL/6 genetic background or alternatively may be related to a different intestinal microflora as $STAT3^{\Delta m}$ associated enterocolitis reacts to ablation of TLR4 signaling.[22]

The increased angiogenesis observed in $STAT3^{\Delta m}$ CRCs remains controversial because it has been demonstrated that *STAT3* activation in MDSCs and TAMs of transplanted B16 melanomas favors tumor angiogenesis.[37] However, the altered stromalization and chemokine/cytokine expression might have pleiotropic effects on angiogenesis pathways in $STAT3^{\Delta m}$ CRCs. The observed bias toward increased expression of positive regulators and decreased expression of negative regulators in the stroma of $STAT3^{\Delta m}$ tumors might result in a net increase of angiogenesis that cannot be attributed to a single factor. Interestingly, $ROR\gamma t^+$ Th17 cells were almost completely absent in the stroma of $STAT3^{\Delta m}$ tumors. The role of this cell type in cancer is controversial and promoting as well as suppressive effects on angiogenesis have been reported.[38] It has been demonstrated that inflammatory cytokines from local macrophages and dendritic cells can promote a Th17-Th1 "phenotypic drift" that leads to gradual acquisition of Th1-associated gene expression by Th17 cells. This is marked by loss of Th17 cell molecules including $ROR\gamma t$.[39] It is possible that the *STAT3*-deficient myeloid cells promote a Th17-Th1 "phenotypic drift" thereby depleting the $ROR\gamma t^+$ Th17 cell population in $STAT3^{\Delta m}$ tumors.

Upon challenge with transplanted tumor cells, the MC38 studies suggest that enhanced immune surveillance in $STAT3^{\Delta m}$ mice largely relies on myeloid cross activation of T cells. Most importantly, the stroma gene expression signatures in $STAT3^{\Delta m}$ tumors are clearly indicative for T cell activation whereas NK cell activities seem to play a minor role. In line with this hypothesis, CRC formation was not promoted in $STAT5^{\Delta NK}$ mice which display reduced NK cell numbers and NK cell-dependent tumor surveillance[27] including a significant reduction of $Nkp46^+$ NK cells in the gut. The presence of $CD3^+$, $CD8^+$, and $CD45RO^+$ stroma T cell infiltration and their intratumoral location in CRCs (Immunoscore) represents a better prognostic marker than conventional TNM staging.[40] The additional determination of a core gene expression signature called ICR further refines the accuracy of prognosis.[29] T cells were the most abundant stroma cell type in AOM/DSS-induced CRCs. Importantly, $STAT3^{\Delta m}$ tumors displayed upregulation of ICR genes irf1, stat1, cxcl9, and ccl5 indicating that they represent predictive markers in mice and humans.

The concept of immunoediting suggests that tumors – incompletely eradicated by the immune system – remain dormant and eventually escape immune attack (i.e. they become edited).[33] CRCs in $STAT3^{\Delta m}$ mice face sustained stroma T cell activities that might force them to develop additional immune evasion mechanisms. The importance of *STAT3* activation in tumor cells for immune evasion is well documented[41,42] and has been demonstrated for various cancers including glioblastoma[43,44] and squamous cell carcinoma.[45] *STAT3* is predominantly activated by IL-6 and IL-11 cytokines in CRCs.[46] This cytokine-mediated *STAT3* activation is usually transient but cancer cells have developed mechanisms for persistent activation of *STAT3* that depends on sphingosine signaling.[47,48] CRCs in $STAT3^{\Delta m}$ mice might represent a subset of survivors with particular high pY-STAT3 levels in tumor cells that selectively escaped sustained T cell activities. pY-STAT3 autoregulates its own expression[49] leading to a positive feed forward loop that might explain the high levels of total STAT3 protein in $STAT3^{\Delta m}$ tumor cells.

STAT3 inhibitors are in clinical trials for various types of cancers[50] and several immunotherapy strategies for CRC are currently tested.[51] Our data suggest that application of *STAT3* inhibitors interferes with immune evasion of CRC tumor cells which would improve the efficacy of immunotherapy strategies and prevent relapse.

Methods

Mice and *in vivo* experiments

C57BL/6 $STAT3^{\Delta m}$ (=$LysM^{Cre/Cre}$ $STAT3^{flox/flox}$) and $STAT3^{wt}$ control (=$LysM^{Cre/Cre}$ $STAT3^{+/+}$) mice[52,53] were employed for tumor induction with AOM/DSS. For tumor transplantation studies, 1×10^5 of BCR/ABL^{p185} or 1×10^6 of MC38 cells were injected subcutaneously. Tumors were weighed and analyzed by flow cytometry 11 d (BCR/ABL^{p185}) and 14 d (MC38) after transplantation. *In vivo* cytotoxicity of CTL cells was evaluated as previously described (supplemental methods).[54] $F4/80^+$ peritoneal macrophages were FACS sorted 4 h after i.p. injection of 4% thioglycerol (Sigma). All mouse experiments were performed in accordance with Austrian and European laws and with the general regulations specified by the Good Science Practices guidelines of the Medical University of Vienna.

In vitro cytotoxicity assay of NK cells

Mice were injected i.p. with PBS or 100 μg poly (I:C) to activate NK cells and spleen cells were isolated after 18 h. Splenocytes were incubated with 5×10^4 CFSE-stained (2.5 μM) YAC-1 target cells at effector-to-target ratios of 100:1, 50:1 and 25:1 in triplicates in 96-well plates. To assess the extent of spontaneously occurring apoptosis, tumor cells were incubated in the absence of NK cells. After 6 h 7-AAD Viability Solution was added and cytotoxicity was quantified by flow cytometry.

Cytotoxicity was normalized to quantified NK numbers of splenic single cell suspensions.

Histology and immunohistochemistry (IHC)

Intestines were flushed with PBS and 4% buffered formaldehyde and fixed as Swiss roles in 4% formaldehyde. 4 μm paraffin sections were stained with H&E, alcian blue or IHC using standard procedures (supplemental methods).

Flow cytometric analysis

For staining of macrophages, splenic immune cells and lamina propria lymphocytes, cells were preincubated with PBS containing 0.5% BSA and FCγIII/IIR antibody to block nonspecific binding. The Cell Trace CFSE Cell Proliferation Kit (Invitrogen) and the 7-AAD Viability Solution (eBioscience) was used according to the manufacturer's instruction. Cells were stained with corresponding antibodies (supplemental methods) and analyzed using a FACSCanto™ II flow cytometer (BD Biosciences) or a FACS Fortessa (Beckton Dickinson) and data were calculated with FACSDiva software (BD Biosciences).

Microarray analysis

RNA from microdissected paraffin material was labeled and hybridized to mouse whole genome GeneChip Mouse Gene 2.0ST arrays (Affymetrix) according to manufacturer's instructions. Microarray data were analyzed using Limma package of R with the parameters "mouse," "tissue type" (tumor and stroma) and "genotype" using a paired approach with "mouse" as pairing variable.[55] p values were adjusted according to Benjamini-Hochberg.[56] An adjusted p value < 0.05 was considered as significant. Data are available at GEO via accession number GSE60871.

Weighted Gene Co-expression Network Analysis (WGCNA)

The Pearson correlations were calculated for all pairs of genes in the samples. The resulting correlation matrix was transformed into a matrix of connection strengths by raising the absolute value of the correlation coefficients to the power ß. A ß of 6 was used to satisfy the scale free topology criterion.[57] The top 25% of genes displaying the highest variability over the entire dataset were selected for module detection. From these genes, modules were determined using the "blockweise" module detection function of the WGCNA package with default values except for the minimal module size and the merge cut height parameters (set to 20 and 0.05, respectively). Biological functions of the single modules were determined using the GO term enrichment analysis function of WGCNA. Only "biological function" ontology was included into the analysis.[58] p values of enriched terms were adjusted as above according to Benjamini-Hochberg. An adjusted p value < 0.05 was considered as significant.

Statistics

Significant differences in tumor parameters, IHC stainings and RT-PCR data were calculated with GraphPad Prism 5 software using unpaired t test and Mann–Whitney test. Multiple comparisons were calculated with One-way Anova and Bonferroni post-test. Significant differences between experimental groups were: $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.005$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Ludwig Boltzmann Gesellschaft (LBG), the Austrian Science Fund (FWF) grant SFB F28 to RE, BS, VS, and MM, the FWF Doktoratskolleg-plus grant "Inflammation and Immunity" to RE, BS, VS, and MM, the FWF grants P25925-B20 and P26908-B20 to RE, the Comprehensive Cancer Center (CCC) Vienna Research Grant to RE, and the Italian Cancer Research Association (AIRC) grant IG13009 to VP.

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