


# STAT1 is a sex-specific tumor suppressor in colitis-associated colorectal cancer

Ilija Crnčec<sup>1</sup>, Madhura Modak<sup>2</sup>, Claire Gordziel<sup>3</sup>, Jasmin Svinka<sup>1</sup>, Irene Scharf<sup>1</sup>, Stefan Moritsch<sup>1</sup>, Paulina Pathria<sup>1</sup>, Michaela Schleder<sup>4,5</sup>, Lukas Kenner<sup>4,5,6</sup>, Gerald Timelthaler<sup>1</sup>, Mathias Müller<sup>7</sup>, Birgit Strobl<sup>7</sup>, Emilio Casanova<sup>4,8</sup>, Editha Bayer<sup>1</sup>, Thomas Mohr<sup>1</sup>, Johannes Stöckl<sup>2</sup>, Karlheinz Friedrich<sup>3,†</sup> and Robert Eferl<sup>1,†</sup> 

1 Institute of Cancer Research, Medical University Vienna & Comprehensive Cancer Center (CCC), Vienna, Austria

2 Institute of Immunology, Medical University Vienna, Austria

3 Institute of Biochemistry II, University Hospital Jena, Germany

4 Ludwig Boltzmann Institute for Cancer Research LBICR, Vienna, Austria

5 Department of Experimental Pathology and Laboratory Animal Pathology, Clinical Institute of Pathology, Medical University Vienna, Austria

6 Unit of Laboratory Animal Pathology, University of Veterinary Medicine Vienna, Austria

7 Institute of Animal Breeding and Genetics and Biomodels Austria, University of Veterinary Medicine Vienna, Austria

8 Department of Physiology, Center of Physiology and Pharmacology, Medical University Vienna, Austria

## Keywords

CD8<sup>+</sup> T cells; colitis; colorectal cancer; gender; sex; STAT1

## Correspondence

R. Eferl, Institute of Cancer Research, Medical University Vienna & Comprehensive Cancer Center (CCC), Borschkegasse 8a, 1090 Vienna, Austria  
Fax: +43 1 40160-957510  
Tel: +43 1 40160-57571  
E-mail: robert.eferl@meduniwien.ac.at

<sup>†</sup>These authors contributed equally to this work.

(Received 31 October 2017, revised 19 January 2018, accepted 21 January 2018, available online 20 February 2018)

doi:10.1002/1878-0261.12178

The interferon-inducible transcription factor STAT1 is a tumor suppressor in various malignancies. We investigated sex-specific STAT1 functions in colitis and colitis-associated colorectal cancer (CRC) using mice with specific STAT1 deletion in intestinal epithelial cells (STAT1<sup>ΔIEC</sup>). Male but not female STAT1<sup>ΔIEC</sup> mice were more resistant to DSS-induced colitis than sex-matched STAT1<sup>flox/flox</sup> controls and displayed reduced intraepithelial infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells. Moreover, DSS treatment failed to induce expression of T-cell-attracting chemokines in intestinal epithelial cells of male but not of female STAT1<sup>ΔIEC</sup> mice. Application of the AOM-DSS protocol for induction of colitis-associated CRC resulted in increased intestinal tumor load in male but not in female STAT1<sup>ΔIEC</sup> mice. A sex-specific stratification of human CRC patients corroborated the data obtained in mice and revealed that reduced tumor cell-intrinsic nuclear STAT1 protein expression is a poor prognostic factor in men but not in women. These data demonstrate that epithelial STAT1 is a male-specific tumor suppressor in CRC of mice and humans.

## 1. Introduction

The incidence of many cancers is significantly higher in men than in women with only a few exceptions.

This sex-specific susceptibility is due to occupational and behavioral factors, sex-related hormone signaling, and cancer-modulating genes on sex chromosomes (Clocchiatti *et al.*, 2016). Moreover, immune responses

## Abbreviations

AOM, azoxymethane; CMS, consensus molecular subtype; CRC, colorectal cancer; CXCL, chemokine (C-X-C motif) ligand; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IFN, interferon; IHC, immunohistochemistry; IL-6, interleukin 6; JAK, janus kinase; MACS, magnetic-activated cell sorting; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TCR, T-cell receptor.

and cancer immune surveillance are sex-dependent with women mounting stronger innate and adaptive immune responses than men do (Klein and Flanagan, 2016). CRC morbidity is similar in men and women but distinct sex-specific differences are apparent. Female CRC patients over 65 years show a lower 5-year survival rate than age-matched male patients because they develop mainly right-sided CRC in the proximal colon, which is more aggressive than left-sided CRC predominating in men. Due to the flat appearance, right-sided CRC is also more difficult to detect by colonoscopy than polypoid-like left-sided CRC. Despite these differences and an ongoing discussion on the clinical implementation of precision medicine, CRC guidelines for screening or therapy do not apply sex-specific recommendations. This might be due to the limited availability of sex-specific preclinical data as most animal studies avoided possible implications of estrogen signaling and used male mice for CRC induction (Kim *et al.*, 2015). Therefore, it is important to identify sex-related biological factors that affect CRC formation in men and women differently. These factors might be valuable therapy targets and markers for cancer prognosis after sex stratification of patients.

Colitis patients suffering from inflammatory bowel diseases (IBD) have an increased risk for development of CRC (Terzic *et al.*, 2010). Signal transducer and activator of transcription 1 (STAT1) is activated by interferon (IFN) signaling via tyrosine phosphorylation (Murray, 2007; Strobl *et al.*, 2011) and was found upregulated in the colon mucosa of IBD patients (Schreiber *et al.*, 2002). Murine studies have shown that STAT1 promotes colitis. DSS-induced colitis was reduced in STAT1 knockout mice (Bandyopadhyay *et al.*, 2008; Berry *et al.*, 2012) whereas mice with haploinsufficiency of SOCS1, a negative regulator of STAT1, showed more severe colitis (Horino *et al.*, 2008). However, a recent study has suggested that epithelial STAT1 protects from DSS-induced colitis in mice (Chiriac *et al.*, 2017).

STAT1 is commonly considered as a tumor suppressor (Meissl *et al.*, 2015) as observed in murine sarcomas (Shankaran *et al.*, 2001) and several mouse breast cancer models (Chan *et al.*, 2012; Klover *et al.*, 2010; Raven *et al.*, 2011; Schneckenleithner *et al.*, 2011). STAT1 inhibits proliferation and promotes apoptosis of tumor cells (Meissl *et al.*, 2015). Moreover, STAT1 promotes extrinsic, tumor-suppressive effects by enhancement of tumor immune surveillance through NK- and T cells (Meissl *et al.*, 2015). However, the role of STAT1 in CRC is controversial and data for colitis-associated CRC are limited. An oncogenic

function was suggested in SOCS1 knockout mice, which developed sporadic CRC with strong tumor cell-intrinsic STAT1 activation (Hanada *et al.*, 2006), but otherwise, Apc<sup>Min</sup>-induced intestinal tumorigenesis was not affected in STAT1 knockout mice (Liddle and Frank, 2008). STAT1 functions in CRC might be concealed by dual effects in tumor cells and stromal immune cells, which are both affected in STAT1 knockout mice. Conditional approaches are required to discriminate between these cellular compartments. We employed mice with specific deletion of STAT1 in intestinal epithelial cells to investigate tumor cell-intrinsic functions in colitis-associated CRC. We demonstrate a sex-specific and tumor-suppressive function of STAT1 in CRC of humans and mice.

## 2. Materials and methods

### 2.1. Mice and *in vivo* experiments

C57BL/6 STAT1<sup>ΔIEC</sup> (Villin<sup>Cre/+</sup> STAT1<sup>flox/flox</sup>) and control STAT1<sup>flox/flox</sup> mice (el Marjou *et al.*, 2004; Wallner *et al.*, 2012) were employed for tumor and colitis induction. Only littermates were used. They were cohoused until weaning age and then separated for experiments. For tumor induction, mice were treated with 12.5 mg·kg<sup>-1</sup> of AOM (Sigma, A5486) i.p. and subjected after a 5-day recovery period to two 5-day cycles of 2.5% DSS (MP Biomedicals, LLC 160110) and one 5-day cycle of 2% DSS (w/v) in drinking water with a 14-day interval of normal water between the cycles (Crnčec *et al.*, 2015). For induction of colitis, mice were given 2.5% DSS (w/v) in drinking water for 7 days followed by a 3-day recovery period. 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.

### 2.2. Histology and immunohistochemistry (IHC)

Intestines were flushed with PBS and 4% buffered formaldehyde and fixed as Swiss rolls in 4% formaldehyde (Crnčec *et al.*, 2015). Paraffin sections of 4 μm thickness were stained with H&E, alcian blue or IHC using standard procedures.

### 2.3. Isolation of intraepithelial and lamina propria lymphocytes

Intraepithelial and lamina propria lymphocytes were isolated as described (Weigmann *et al.*, 2007). In brief, colons of three mice per genotype and sex were flushed

with PBS and opened longitudinally. Large colon pieces were then shaken in a CMF/HEPES solution containing  $1 \times$  HBSS, FCS, 10 mM HEPES, and 25 mM sodium bicarbonate  $3 \times 15$  s to remove excess mucus, fecal material, and loose fat. Subsequently, they were washed in RPMI 1640 (Sigma-Aldrich #R8755) containing FCS. Large pieces were then sliced into 1- to 3-mm pieces and shaken in Hank's balanced salt solution containing 10% FCS, 5 mM EDTA, 15 mM HEPES and penicillin/streptomycin [ $100\text{U}\cdot\text{mL}^{-1}$ ] at  $37^\circ\text{C}$  for  $2 \times 15$  min. The supernatant of this preparation was used for isolation of intraepithelial lymphocytes. For lamina propria lymphocytes, the remaining pieces of colon were shaken in RPMI 1640 containing 5% FCS, 1 mM of  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$  and Collagenase [ $100\text{U}\cdot\text{mL}^{-1}$ ] at  $37^\circ\text{C}$  for  $2 \times 20$  min. The supernatant of both preparations was subjected to Percoll gradient centrifugation. The lymphocytes were collected at the interface between 44% and 67% layers for intraepithelial lymphocytes and 40% and 80% layers for lamina propria lymphocytes.

#### 2.4. Isolation of intestinal epithelial cells and MACS purification

Intestinal epithelial cells were isolated by shaking 1- to 3-mm colon pieces in Hank's balanced salt solution containing 10% FCS, 5 mM EDTA, 15 mM HEPES, and penicillin/streptomycin [ $100\text{U}\cdot\text{mL}^{-1}$ ] at  $37^\circ\text{C}$  for  $2 \times 15$  min. The IECs of the DSS-treated mice were subjected to MACS purification using BD IMag™ Streptavidin Particles Plus—DM system (BD Biosciences #557812) and biotinylated rat anti-mouse CD45 (clone 30-F11; BD Biosciences #553077) according to the manufacturer's instructions. Isolated IECs were tested by flow cytometry and showed 85–95% purity.

#### 2.5. Antibodies used for IHC stainings

IHC stainings were performed with antibodies for STAT1 (Santa Cruz #sc-592), phospho-STAT1 (Tyr701) (Cell Signaling #9167S), Ki67 (Novocastra #NCL-KI67-P), cleaved caspase-3 (Cell signaling #9661), STAT3 (Santa Cruz #sc-7179), phospho-STAT3 (Tyr705) (Cell signaling #9145), lysozyme (Dako #A0099), synaptophysin (Genetex GTX100865), endomucin (eBioscience #14-5851-82), granzyme B (Abcam #ab4059), p21 (Santa Cruz #sc-6246), c-Myc (Santa Cruz #sc-40), IL-6 (Abcam #ab6672) and detected with peroxidase-coupled secondary antibody (ID laboratories IDSTM003) using

AEC chromogen (ID laboratories #BP1108) or AEC staining kit (Sigma-Aldrich #AEC 101-1KT). BrdU incorporation was detected using the BrdU In-Situ Detection Kit (BD Biosciences #550803).

#### 2.6. Quantitation and grading of tumors

Pannoramic MIDI scanner (3DHistech Ltd.) was used to scan H&E-stained and IHC-stained Swiss rolls. Quantitation of tumor area and IHC stainings was performed using Definiens™ Tissue Studio histomorphometry software (Definiens AG, Munich, Germany). Tumor grading was performed by a board-certified pathologist.

#### 2.7. Colitis score

Scanned H&E Swiss rolls of colons were used to assess colitis in a blinded fashion by a board-certified pathologist according to the following criteria: inflammation score 0—rare or no inflammatory cells in lamina propria, 1—increased numbers of granulocytes in lamina propria, 2—confluence of inflammatory cells extending to submucosa, 3—transmural extension of inflammatory infiltrate; crypt damage 0—none, 1—loss of basal 1/3 of the crypt, 2—loss of basal 2/3 of the crypt, 3—entire crypt loss, 4—change in epithelial surface with erosion, 5—confluent erosion; ulceration 0—none, 1—1–2 ulcers focally, 2—3–4 ulcers focally, 3—confluent ulceration. The individual scores were added up to give a maximum of 11. This score was then multiplied by a multiplication factor on the basis of the area affected: 1—0–25% of the colon, 2—25–50% of the colon, 3—50–75% of the colon, 4—75–100% of the colon to give a final maximum score of 44.

#### 2.8. Flow cytometry

Flow cytometry analyses were performed using standard staining procedures. For membrane staining, cells were incubated with conjugated mAbs for 30 min at  $4^\circ\text{C}$ . For intracellular staining, cells were fixed with fixation buffer in the dark at  $4^\circ\text{C}$  for 20 min. Cells were then incubated with conjugated mAb in permeabilization buffer at  $4^\circ\text{C}$  for 1 h. Flow cytometry analyses were performed using LSRFortessa (Becton Dickinson, Heidelberg, Germany). Following reagents were used: Fixable Viability Dye eFluor506® (eBioscience #65-0866-14) and antibodies CD16/32 (BD Biosciences #553142; clone 2.4G2), CD45–BV421 (Biolegend 103133; clone 30-F11), CD45–APC–R700 (BD Biosciences #565478; clone 30-F11), CD8 $\alpha$  - APC (eBioscience #17-0081-83; clone 53-6.7), CD8 $\alpha$  - APC–R700 (BD Biosciences #564983;

clone 53-6.7), CD8 $\alpha$  - BV605 (Biolegend #100744; clone 53-6.7), CD4-PE-Cy.7 (eBioscience #25-0042-82; clone RM4-5), CD19 - PE-CF594 (BD Biosciences #562329; clone 1D3), TCR $\beta$  - APC-eFluor® 780 (eBioscience #47-5961-82; clone H57-597), TCR $\gamma\delta$  - PE (BD Biosciences #553178; clone GL3), granzyme B - PE (BD Biosciences #561142; clone GB11), isotype (BD Biosciences #556650; clone MOPC-21), CD326 - PE (BD Biosciences #563477; clone G8.8), fixation/permeabilization diluent (eBioscience #00-5223-56), permeabilization buffer (10x) (eBioscience #00-8333-56), fixation/permeabilization concentrate (eBioscience #00-5123-43).

## 2.9. Genotyping by polymerase chain reaction (PCR)

Genotyping of STAT1 was performed with primers 5'-TAGGCTCCCTCTTCCCTTC-3', 5'-ACACCATTG GCTTCACCTTC-3', and 5'-CCCCTGTCATCTGGA GTGAT-3'. The Cre transgene was detected with primers 5'-CGGTGCGATGCAACGAGTGATGAGG-3' and 5'CCAGAGACGGAAATCCATCGCTCG-3'.

## 2.10. RNA isolation and qRT-PCR

RNA was isolated using RNeasy Protect Mini Kit (Qiagen #74124) with an on-column DNase I digestion step with RNase-Free DNase Kit (Qiagen #79254). It was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad #170-8891). qRT-PCR was performed with an ABI 7500 cycler (Applied Biosystems, Foster City, CA, USA). Relative expression levels of transcripts were calculated using the comparative CT method and normalized for GAPDH. The following primers were used: STAT1, 5'-TGGTGAAATTGCAAG AGCTG-3' and 5'-TGTGTGCGTACCCAAGATGT-3'; IL-6, 5'-TGATGCACTTGCAAGAAAACA-3' and 5'-ACCAGAGGAAATTTTCAATAGGC-3'; CXCL-9, 5'-CGATCCACTACAAATCCCTCA-3' and 5'-TAGG CAGGTTTGATCTCCGT-3'; CXCL-10, 5'-CTCATC-CTGCTGGGTCTGAG-3' and 5'-CCTATGGCCCTC ATTCTCAC-3'; CXCL-11 5'-CTGCTGAGATGAAC AGGAAGG-3' and 5'-CGCCCTGTTTGAACAT AAG-3'.

## 2.11. Statistics

The normality of the data distribution was tested by Kolmogorov–Smirnov or D'Agostino–Pearson normality test. Significant differences in tumor load, multiplicity and size, colon length, colitis score, IHC stainings, qRT-PCR data, and CIBERSORT score

were calculated using unpaired *t*-test (for normal data) and Mann–Whitney test (for non-normal data). Multiple comparisons were calculated with one-way ANOVA test and Tukey's test, Bonferroni's post-test (for normal data) or Kruskal–Wallis test, and Dunn's post-test (for non-normal data). Significant differences in weight loss were calculated by performing an area under the curve (AUC) calculation and testing the AUC by independent *t*-test. Flow cytometry data from individual experiments were tested by a paired *t*-test. Significant differences in tumor grade frequency, frequency of the four CMS subgroups within patient samples, and IL-6 protein expression (assayed by IHC and quantitative histomorphometry with Definiens™ Tissue Studio software) were calculated by performing a  $\chi^2$  test. Association between expression of STAT1 and CXCL-9, CXCL-10, and CXCL-11 from human sample microarray data was calculated using Spearman's rank coefficient correlation. The strength of correlation was determined as follows:  $r = 0.00$ – $0.25$ —no correlation;  $r = 0.25$ – $0.50$ —weak positive correlation;  $r = 0.50$ – $0.75$ —positive correlation;  $r = 0.75$ – $1.00$ —strong positive correlation. Survival analyses were carried out using log-rank test. Significant differences between experimental groups were \* =  $P < .05$ , \*\* =  $P < .01$ , and \*\*\* =  $P < .001$ .

## 2.12. Analysis of human samples

Recently published survival data, derived from STAT1 and STAT3 IHC stainings of human CRC tissue microarrays (Gordziel *et al.*, 2013; Nivarthi *et al.*, 2016), were used for sex stratification and evaluation of the prognostic value of tumor cell-intrinsic nuclear STAT1 expression. Publicly available CRC microarray expression data (Guinney *et al.*, 2015) were used to examine the expression of STAT1, CXCL-9, CXCL-10, and CXCL-11 in patient samples. Stratification of the human sample microarray data into STAT1<sup>high</sup> and STAT1<sup>low</sup> groups was performed by fitting two Gaussian curves into the density distribution of STAT1 log<sub>2</sub> expression using the R package mixtools. Samples were ranked according to their STAT1 log<sub>2</sub> expression and ascribed posterior probabilities assigning them to either the STAT1<sup>high</sup> or the STAT1<sup>low</sup> group. The STAT1 log<sub>2</sub> expression of the first sample with the probability of belonging to the STAT1<sup>high</sup> group exceeding the probability of belonging to the STAT1<sup>low</sup> group was chosen as threshold and set at 8.52. Overall, 1479 human CRC samples were stratified according to sex and STAT1 expression forming STAT1<sup>high</sup> (534 samples) and STAT1<sup>low</sup> (945 samples)



groups. The CIBERSORT analysis was performed as described (Newman *et al.*, 2015).

### 3. Results

#### 3.1. Epithelial STAT1 is a sex-specific promoter of acute colitis

We employed mice with specific deletion of STAT1 in intestinal epithelial cells (STAT1<sup>ΔIEC</sup>) (el Marjou *et al.*, 2004; Wallner *et al.*, 2012) to investigate sex-specific functions in colitis and colitis-associated CRC. Deletion of STAT1 in intestinal epithelial cells (IECs) was confirmed by qRT-PCR for STAT1 mRNA and by STAT1 IHC staining of formalin-fixed and paraffin-embedded whole gut preparations (Swiss rolls) (Crnčec *et al.*, 2015) of male (Fig. S1A,B) and female (Fig. S1C,D) STAT1<sup>ΔIEC</sup> mice. Lamina propria immune cells of STAT1<sup>ΔIEC</sup> mice readily displayed STAT1 expression, which demonstrated specific ablation in IECs (Fig. S1A,C). The mucosal architecture in small intestine and colon was not affected by STAT1 ablation. Enterocytes, goblet cells, enteroendocrine cells, Paneth cells, and Ki67<sup>+</sup> cells in the intestinal crypts were present at normal numbers in STAT1<sup>ΔIEC</sup> mice (Fig. S2A–G).

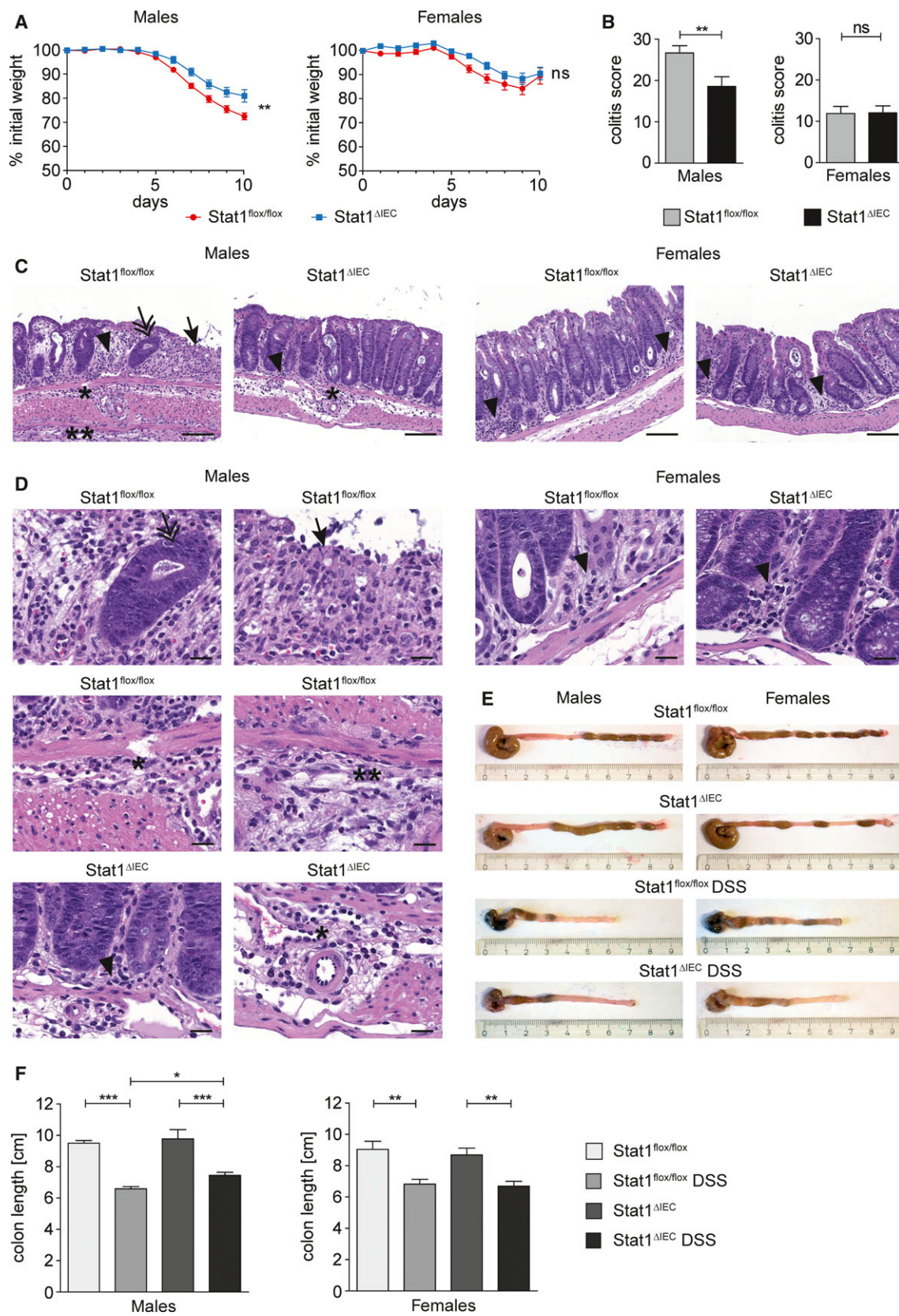
We performed short-term treatment of mice with DSS to investigate sex-specific STAT1 functions in acute colitis. Male STAT1<sup>ΔIEC</sup> mice were partially protected from loss in body weight (Fig. 1A), and the colitis score was attenuated (Fig. 1B–D). Shortening of the colon length, which is indicative of the severity of colitis (Okayasu *et al.*, 1990), was more pronounced in male STAT1<sup>flx/flx</sup> mice than in male STAT1<sup>ΔIEC</sup> mice (Fig. 1E,F). In contrast, female STAT1<sup>ΔIEC</sup> mice were not protected from acute colitis (Fig. 1A–F). These data demonstrate that epithelial STAT1 is a male-specific promoter of DSS-induced colitis.

Activated CD8<sup>+</sup> T cells express cytotoxic molecules such as perforin and granzyme B that can damage the intestinal epithelium and aggravate colitis (Monteleone *et al.*, 2012). Therefore, we characterized infiltration of lymphoid cells into the mucosa of DSS-treated mice by flow cytometry analysis. These analyses revealed reduced intraepithelial TCRαβ<sup>+</sup> and more specifically CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells in male but not in female STAT1<sup>ΔIEC</sup> mice (Fig. 2A,B; Fig. S3A). The percentage of CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells co-expressing the activation marker granzyme B was also reduced in male STAT1<sup>ΔIEC</sup> mice (Fig. 2C; Fig. S3B) although intracellular granzyme B expression levels were not affected (Fig. 2D). Numbers of intraepithelial CD4<sup>+</sup> TCRαβ<sup>+</sup> T cells and CD8<sup>+</sup> TCRγδ<sup>+</sup> T cells were unchanged in

male STAT1<sup>ΔIEC</sup> mice (Fig. S3C,D). T-cell populations in the lamina propria were also unchanged in DSS-treated male STAT1<sup>ΔIEC</sup> mice (Fig. S3E–I), but female STAT1<sup>ΔIEC</sup> mice showed increased numbers of CD4<sup>+</sup> TCRαβ<sup>+</sup> and CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells (Fig. S3E–G). However, intraepithelial TCRαβ<sup>+</sup> T-cell populations were generally more abundant than lamina propria TCRαβ<sup>+</sup> T cells (Fig. 2A; Fig. S3E). Moreover, the majority of CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells were located in the intestinal epithelium in DSS-treated mice (Fig. 2B; Fig. S3F). The data demonstrate that epithelial STAT1 is required for colitis-associated intraepithelial infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells in male but not in female mice.

#### 3.2. Epithelial STAT1 is a sex-specific inducer of T-cell-attracting chemokine expression in acute colitis

Our data indicate a sex-specific requirement of epithelial STAT1 for chemoattraction of CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells. IECs are a source for IL-6, which is a T-cell chemoattractant (Weissenbach *et al.*, 2004), in DSS-treated mice (Grivennikov *et al.*, 2009), and IFN-γ-regulated chemokines CXCL-9, CXCL-10, and CXCL-11 are secreted by epithelial cells and involved in inflammatory processes mediated by T cells (Marshall *et al.*, 2017). Reduced induction of CXCL-9 and CXCL-10 was recently demonstrated in bulk colon tissue of DSS-treated STAT1<sup>ΔIEC</sup> mice (Rauch *et al.*, 2015) but this study did not discriminate between expression in epithelial or inflammatory cells and sex. We hypothesized that STAT1 would regulate the production of these T-cell chemoattractants by IECs in a sex-specific manner. Therefore, we purified IECs from DSS-treated mice by MACS sorting and performed qRT-PCR analysis. DSS treatment induced mRNA expression of IL-6 in IECs of male and female STAT1<sup>flx/flx</sup> mice, but the induction was slightly attenuated in male STAT1<sup>ΔIEC</sup> mice (Fig. 3A). Expression of CXCL-9 mRNA was induced in male but not in female STAT1<sup>flx/flx</sup> mice although differences did not reach significance. Similar to IL-6, this induction was slightly attenuated in male STAT1<sup>ΔIEC</sup> mice (Fig. 3B). Expression of CXCL-10 mRNA was strongly induced in male but not in female STAT1<sup>flx/flx</sup> mice, albeit significantly attenuated in male STAT1<sup>ΔIEC</sup> mice (Fig. 3C). Expression of CXCL-11 mRNA was induced by DSS in STAT1<sup>flx/flx</sup> mice of both sexes but induction failed in IECs of male STAT1<sup>ΔIEC</sup> mice (Fig. 3D). These data revealed STAT1-dependent sex-specific effects of colitis on mRNA expression of T-cell-attracting chemokines CXCL-9



**Fig. 1.** Epithelial STAT1 is a sex-specific promoter of acute colitis. (A) Weight loss of DSS-treated male (nine STAT1<sup>fllox/fllox</sup>, nine STAT1<sup>ΔIEC</sup>) and female (eight STAT1<sup>fllox/fllox</sup>, nine STAT1<sup>ΔIEC</sup>) mice. (B) Colitis score of DSS-treated male (18 STAT1<sup>fllox/fllox</sup>, 18 STAT1<sup>ΔIEC</sup>) and female (10 STAT1<sup>fllox/fllox</sup>, 15 STAT1<sup>ΔIEC</sup>) mice. (C,D) H&E-stained images for evaluation of colitis in DSS-treated male and female STAT1<sup>fllox/fllox</sup> and STAT1<sup>ΔIEC</sup> mice. Scale bar indicates 100 μm (C) or 20 μm (D). Arrow: complete erosion of epithelial surface; arrowhead: immune infiltration into the mucosa; double arrow: epithelial regenerative atypia simulating dysplasia; x: immune infiltration into the submucosa; xx: immune infiltration into the subserosa. (E,F) Colon shortening in DSS-treated male (nine STAT1<sup>fllox/fllox</sup>, 12 STAT1<sup>ΔIEC</sup>) and female (eight STAT1<sup>fllox/fllox</sup>, seven STAT1<sup>ΔIEC</sup>) mice (≥ 5 control mice per sex and genotype). Bars represent data ± SEM. ns: not significant.

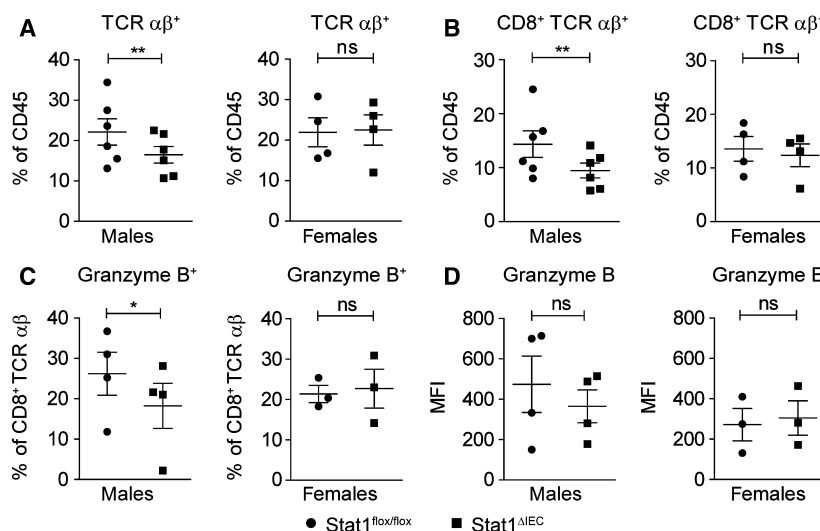
and 10 in IECs. Moreover, STAT1 was found to be required for mRNA induction of CXCL-11 in IECs of male but not of female mice. At the protein level, expression of IL-6 was reduced in DSS-treated male (Fig. 3E,G) but not female (Fig. 3F,G) STAT1<sup>ΔIEC</sup> mice. These data indicate a sex-specific requirement of epithelial STAT1 for production of distinct T-cell chemoattractants.

### 3.3. Epithelial STAT1 is a sex-specific tumor suppressor in colitis-associated CRC

DSS-induced colitis is the tumor-promoting condition in the azoxymethane/dextran sulfate sodium (AOM-DSS) model of colitis-associated CRC (Crnčec *et al.*, 2015), and we wondered if sex-specific STAT1 effects in colitis and CD8<sup>+</sup> T-cell infiltration impact tumor formation. Therefore, colorectal tumors were induced in STAT1<sup>ΔIEC</sup> mice with AOM-DSS (Crnčec *et al.*, 2015). Total tumor load was increased in male but not in female STAT1<sup>ΔIEC</sup> mice (Fig. 4A). Increased tumor load in STAT1<sup>ΔIEC</sup> males was due to increased tumor

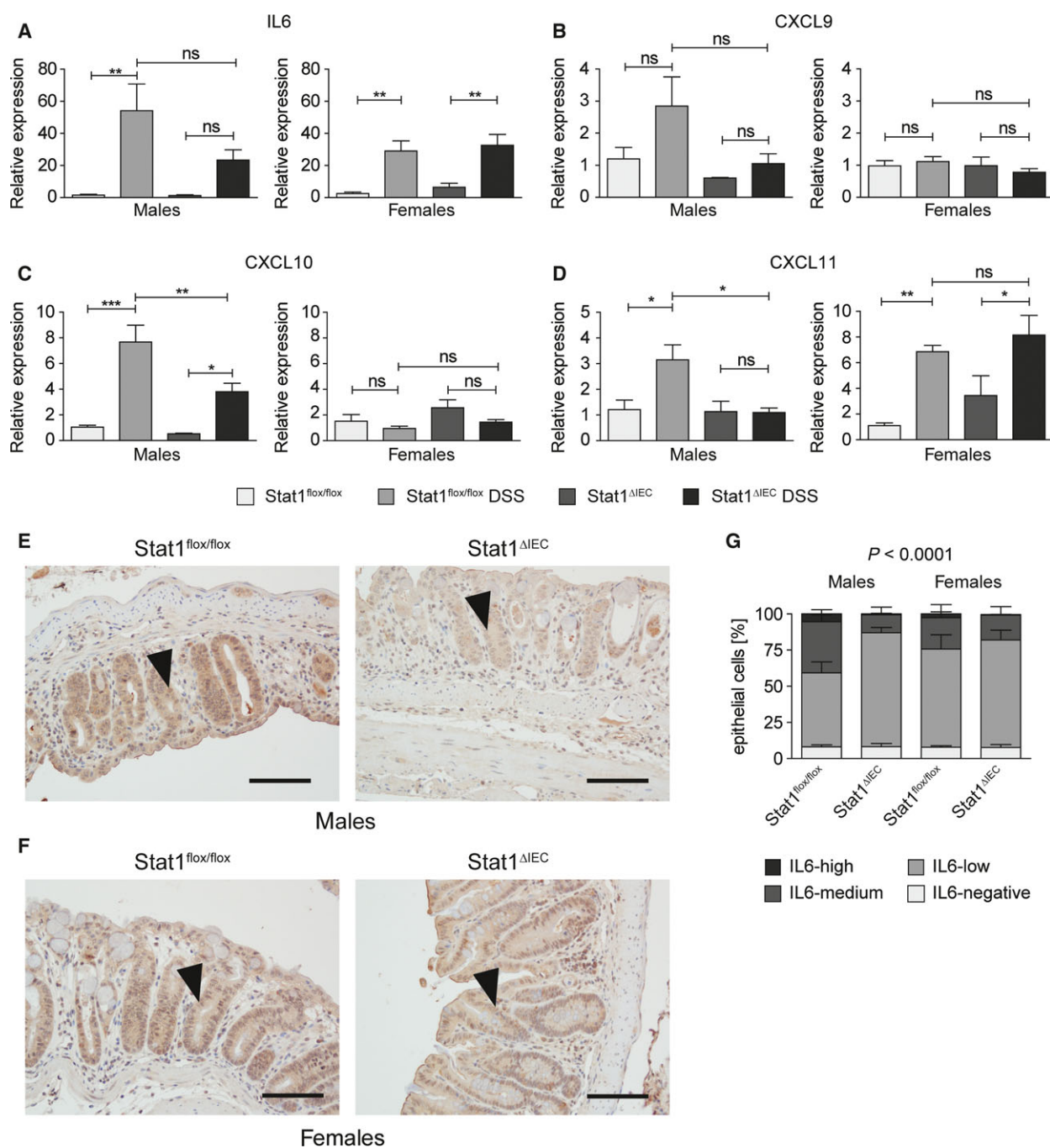
multiplicity (Fig. 4B), whereas the mean tumor size was not affected (Fig. 4C). Moreover, relative numbers of high-grade adenomas were increased in male STAT1<sup>ΔIEC</sup> mice (Fig. 4D; Fig. S4A). IHC stainings confirmed loss of STAT1 and pY-STAT1 (activated STAT1) in tumor cells of male and female STAT1<sup>ΔIEC</sup> mice (Fig. 4E, Fig. S4B,C), and no tumors that escaped STAT1 deletion were found. STAT1 activation was not prominent, and only a few tumor cells stained positive for pY-STAT1 in STAT1<sup>fllox/fllox</sup> mice (Fig. S4B,C). These data demonstrate a male-specific tumor-suppressive function of STAT1 in the formation and progression of colitis-associated CRC in mice.

To get mechanistic insight, we analyzed sex-specific STAT1 effects on tumor parameters, STAT3 activation, and expression of STAT1 target genes implicated in tumorigenesis. Numbers of proliferating cells were not significantly altered in both sexes (Fig. 4F), but apoptotic cells were reduced in tumors of male STAT1<sup>ΔIEC</sup> mice (Fig. 4G). Despite the described function of STAT1 in tumor angiogenesis (Meissl *et al.*, 2015), no effect of STAT1 ablation on vessel density was



**Fig. 2.** Epithelial STAT1 is a sex-specific promoter of intraepithelial CD8<sup>+</sup> T-cell infiltration in acute colitis. (A–C) Flow cytometry data for intraepithelial infiltration of TCRαβ<sup>+</sup> T cells (A), CD8<sup>+</sup> TCRαβ<sup>+</sup> T cells (B), and CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells (C) into the mucosa of male and female STAT1<sup>fllox/fllox</sup> and STAT1<sup>ΔIEC</sup> mice during DSS-induced acute colitis. Each data point represents a biological replicate with cells pooled from three mice. (D) Mean fluorescence intensity for granzyme B expression in intraepithelial CD8<sup>+</sup> T cells during DSS-induced acute colitis. Each data point represents a biological replicate with cells pooled from three mice. ns: not significant.



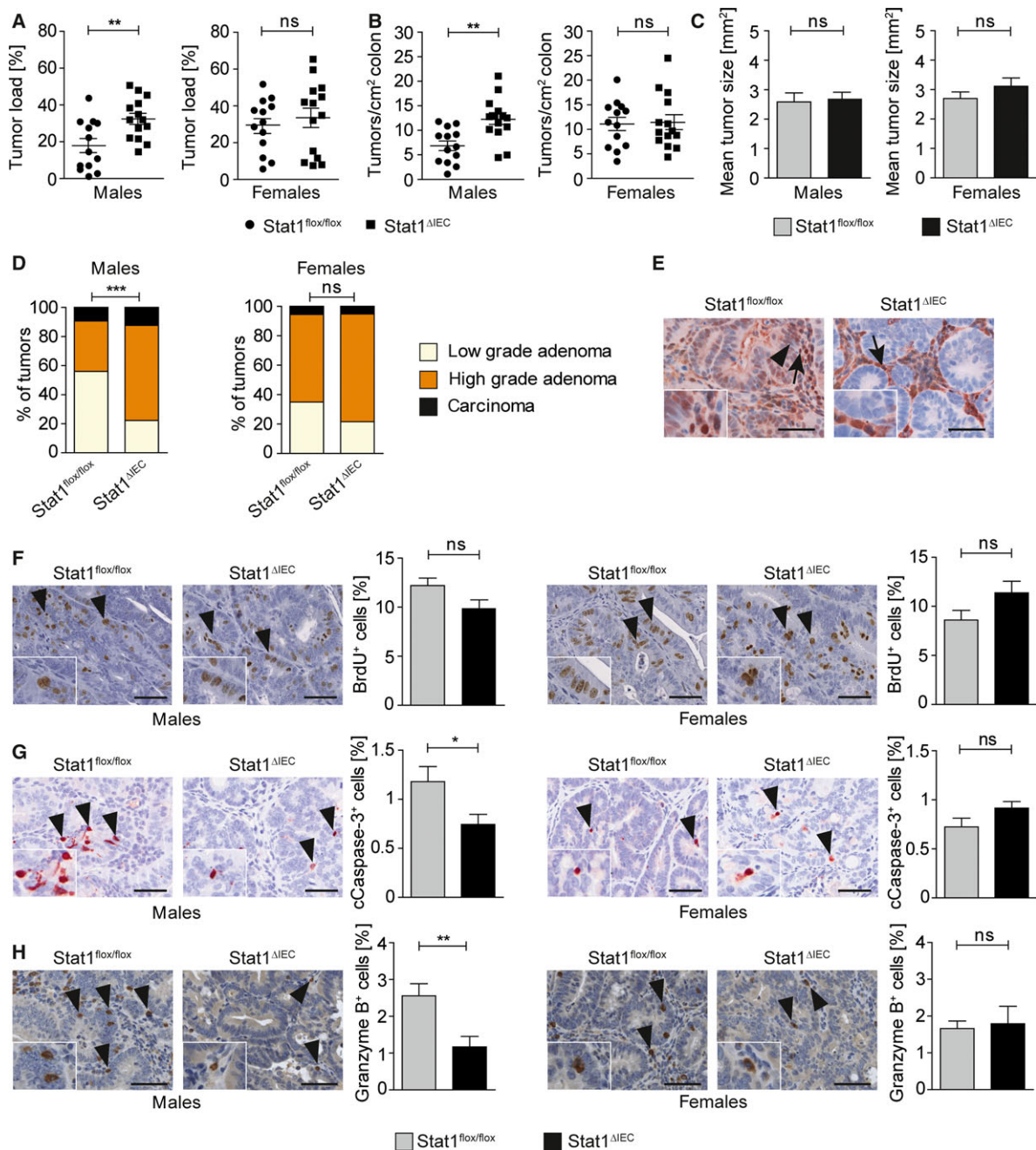


**Fig. 3.** Epithelial STAT1 is a sex-specific promoter of chemokine expression in acute colitis. (A–D) qRT-PCR analysis for mRNA expression of IL-6 (A), CXCL-9 (B), CXCL-10 (C), and CXCL-11 (D) in isolated IECs of untreated versus DSS-treated male and female *STAT1*<sup>flox/flox</sup> and *STAT1*<sup>ΔIEC</sup> mice (4–6 biological replicates per treatment, sex, and genotype). Bars represent data  $\pm$  SEM. ns: not significant. (E, F) Representative IHC stainings for IL-6 (images; positive epithelial cells are indicated by arrowheads; scale bars indicate 50  $\mu$ m) in DSS-treated male (E) and female (F) *STAT1*<sup>flox/flox</sup> and *STAT1*<sup>ΔIEC</sup> mice. (G) Quantification of positive epithelial cells with different staining intensities (bar diagrams; automated quantitative histomorphometry of four animals per genotype and sex) in DSS-treated male and female *STAT1*<sup>flox/flox</sup> and *STAT1*<sup>ΔIEC</sup> mice. Bars represent data  $\pm$  SEM.

observed (Fig. S5A). The closely related transcription factor STAT3 acts as an oncogene and conditional deletion in intestinal epithelial cells of mice interfered

with AOM-DSS-induced CRC formation (Bollrath et al., 2009). Activation of STAT3 is frequently enhanced when STAT1 is deleted (Regis et al., 2008).





**Fig. 4.** Epithelial STAT1 is a sex-specific suppressor of colitis-associated CRC. (A) Tumor load (% tumor area per total colon area) in male and female STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice. (B) Tumor multiplicity (number of tumors per cm<sup>2</sup> colon) in male and female STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice. (C) Mean tumor size in male and female STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice (automated quantitative histomorphometry of  $\geq 75$  tumors per genotype in  $\geq 13$  animals per genotype). Bars represent data  $\pm$  SEM. (D) Histopathological grading of colon tumors in male and female STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice ( $\geq 75$  tumors per genotype in  $\geq 13$  animals per genotype). (E) IHC for STAT1 in tumors of STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice (insets show high magnifications; positive epithelial cells are indicated by arrowheads; positive stroma cells are indicated by arrows; scale bar indicates 50  $\mu$ m). (F,G) BrdU IHC stainings for cell proliferation (F) and cleaved caspase-3 IHC stainings for apoptosis (G) in colon tumors of male and female STAT1<sup>flox/flox</sup> and STAT1<sup>ΔIEC</sup> mice (images; scale bar indicates 50  $\mu$ m; positive cells are indicated by arrowheads and shown in detail by insets). Positive cells were quantified by automated quantitative histomorphometry ( $\geq 8$  tumors per genotype in  $\geq 3$  animals per genotype). Bars represent data  $\pm$  SEM. (H) IHC staining for granzyme B (images; scale bar indicates 100  $\mu$ m; positive cells are indicated by arrowheads) and histomorphometric quantitation of granzyme B<sup>+</sup> cells in colon tumors ( $\geq 9$  tumors per genotype in  $\geq 3$  animals per genotype). Bars represent data  $\pm$  SEM. ns: not significant.

Although numbers of STAT3-expressing tumor cells were unchanged (Fig. S5B), we found increased STAT3 activation in STAT1<sup>ΔIEC</sup> tumors (Fig. S5C). This molecular effect was present in both sexes (Fig. S5C) suggesting that STAT3 does not contribute to STAT1-dependent sex-specific differences in tumorigenesis. Other STAT1 target genes such as p21 and c-Myc were not differentially expressed in STAT1<sup>fllox/fllox</sup> and STAT1<sup>ΔIEC</sup> tumors (Fig. S5D,E). Moreover, IHC staining revealed reduced numbers of granzyme B<sup>+</sup> cells in tumors of male but not of female STAT1<sup>ΔIEC</sup> mice (Fig. 4H). This sex-specific effect might blunt cytotoxic activity and lead to increased tumor load in male STAT1<sup>ΔIEC</sup> mice.

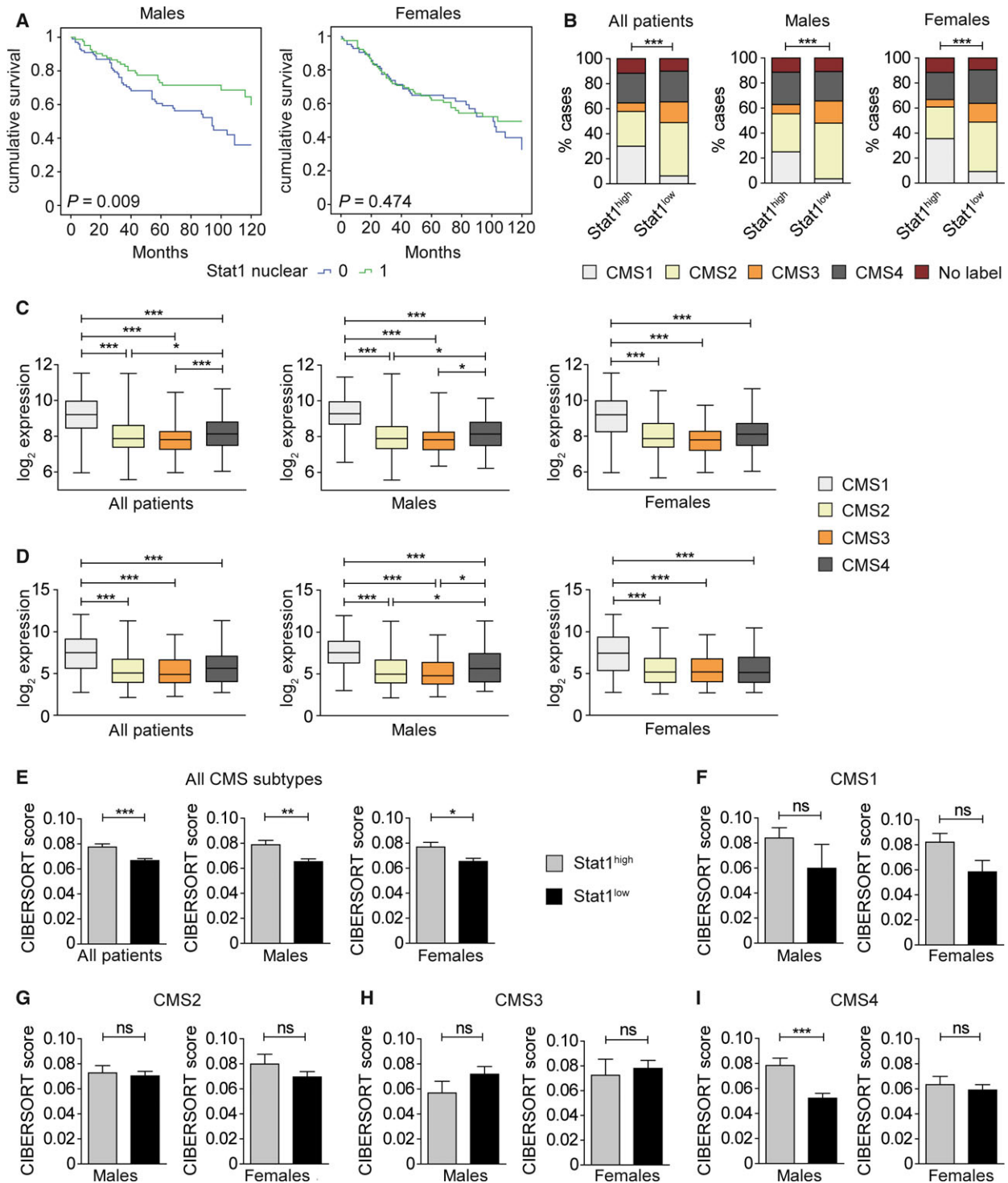
### 3.4. Tumor cell-intrinsic nuclear STAT1 is a sex-specific prognostic factor in human CRC

We recently performed STAT1 and STAT3 IHC stainings of human CRC tissue microarrays (Gordziel *et al.*, 2013; Nivarthi *et al.*, 2016). A distribution analysis of nuclear versus cytoplasmic STAT1 in cancer cells of these tissue microarrays revealed that most colorectal tumors were negative for STAT1 in both compartments or positive for STAT1 in the nucleus with or without cytoplasmic STAT1 expression (Fig. S6A). A similar distribution was obtained after sex stratification of CRC in men and women (Fig. S6B). Survival analyses demonstrated that tumor cell-intrinsic nuclear STAT1 protein expression is a beneficial prognostic factor in CRC (Gordziel *et al.*, 2013). However, sex stratification of the survival data revealed that this prognostic value is male-specific (Fig. 5A). Tumor cell-intrinsic cytoplasmic STAT1 was not identified as prognostic factor after sex stratification but there was a trend toward favorable prognosis in male patients (Fig. S6C). We also demonstrated that cytoplasmic but not nuclear STAT3 represents a prognostic factor in human CRC (Gordziel *et al.*, 2013). Stratification of these data revealed no sex bias for the prognostic value of cytoplasmic STAT3 (Fig. S6D), although there was a trend toward favorable prognosis in male patients. Moreover, neither nuclear STAT3 nor concomitant nuclear STAT1 and nuclear STAT3 represented a sex-specific prognostic factor (Fig. S6E,F). A sex-specific correlation with clinicopathological parameters showed that tumor grading or tumor staging was not prognostic in male and female CRC patients (Fig. S7A,B). However, vein invasion of tumor cells and lymph node metastasis was a significant factor for bad prognosis in both sexes (Fig. S7C,D). Metastasis is the cause for mortality in about 90% of cancer patients (Spano *et al.*, 2012). Therefore, we

investigated if the good prognosis of male patients with prominent STAT1 expression in cancer cells is due to reduced vein invasion or lymph node metastasis. Interestingly, we could neither identify a significant correlation between tumor cell-intrinsic nuclear STAT1 expression and vein invasion (Table S1) nor lymph node metastasis (Table S2) in both sexes. This suggests that tumor cell-intrinsic STAT1 is implicated in the invasion–metastasis cascade of male CRC patients at a stage beyond vein invasion. In summary, our data demonstrate that expression of tumor cell-intrinsic nuclear STAT1 protein is a male-specific prognostic factor in human CRC. Moreover, sex stratification can significantly refine the prognostic value of STAT1 in CRC patients.

### 3.5. Sex-specific correlation between STAT1 expression and abundance of CD8<sup>+</sup> T cells in the CMS4 subgroup of human CRC

We next stratified publicly available CRC gene expression data for the recently described consensus molecular subtypes (CMS) of CRC (Guinney *et al.*, 2015) into STAT1<sup>high</sup>- and STAT1<sup>low</sup>-expressing subgroups (Fig. S8) to assess correlations between STAT1, IL-6, CXCL-9, CXCL-10, CXCL-11 cytokine/chemokine expression, CD8<sup>+</sup> T-cell infiltration, and sex. The percentage of CMS1 tumors was higher within the STAT1<sup>high</sup> subgroup compared to the STAT1<sup>low</sup> subgroup irrespective of sex, which is most likely due to the strong immune cell infiltration of this subtype (Guinney *et al.*, 2015). In contrast, the percentages of CMS2 and CMS3 were lower in the STAT1<sup>high</sup> subgroup and the CMS4 subtype was equally distributed between STAT1<sup>high</sup> and STAT1<sup>low</sup> subgroups (Fig. 5B). Expression of STAT1 did not correlate with IL-6 (Fig. S9B–D) but correlated positively with T-cell-attracting chemokines CXCL-9, CXCL-10, and CXCL-11 in all four CMS subtypes irrespective of the sex (Fig. S10B–D, S11B–D, S12A–C). STAT1, IL-6, CXCL-9, CXCL-10, and CXCL-11 were most prominently expressed in CMS1 and CMS4 subtypes (Fig. 5C–D; Fig. S9A, S10A, S11A). The CMS4 subtype, which is characterized by invasiveness and bad prognosis (Guinney *et al.*, 2015), showed a sex-specific increase in STAT1 and CXCL-11 expressions. Both genes were expressed at significantly higher levels in CMS4 than in CMS2 or CMS3 subtypes of male but not of female patients (Fig. 5C,D). In contrast, IL-6, CXCL-9, and CXCL-10 were significantly increased in CMS4 tumors of both sexes (Fig. S9A, S10A, S11A). Importantly, immune phenotyping by CIBERSORT (Newman *et al.*, 2015) demonstrated that STAT1



**Fig. 5.** Tumor cell-intrinsic nuclear STAT1 is a sex-specific prognostic marker for human CRC. (A) Survival curves of male and female CRC patients with or without tumor cell-intrinsic nuclear STAT1 expression. The analysis is based on published survival data (Gordziel *et al.*, 2013) that were used for sex stratification. (B) STAT1 expression- and sex-based stratification of CMS1-4 subtypes of CRC (Guinney *et al.*, 2015). (C,D) STAT1 (C) and CXCL11 (D) log<sub>2</sub> expression within CMS1-4 subtypes of CRC (Guinney *et al.*, 2015) in all patients and after sex stratification. (E) CIBERSORT analysis for CD8<sup>+</sup> T-cell infiltration in STAT1<sup>high</sup> and STAT1<sup>low</sup> CMS1—four subtypes of CRC without (all patients) and with sex stratification. (F-I) CIBERSORT analysis for CD8<sup>+</sup> T-cell infiltration in sex-stratified STAT1<sup>high</sup> and STAT1<sup>low</sup> CMS1 (F), CMS2 (G), CMS3 (H), and CMS4 (I) subtypes of CRC. Note that high STAT1 expression is indicative of CD8<sup>+</sup> T-cell infiltration in CMS4 CRC of male but not of female patients.



expression correlated with CD8<sup>+</sup> T-cell infiltration in CRC (Fig. 5E). Stratification of these data according to the CMS subtype (Fig. 5F–I) revealed a male-specific positive correlation between STAT1 expression and CD8<sup>+</sup> T-cell infiltration in CMS4 (Fig. 5I). These data suggest that STAT1 regulates CD8<sup>+</sup> T-cell infiltration in CMS4 tumors of male but not of female patients.

#### 4. Discussion

The identity of sex-specific molecular factors in cancer formation and progression remains scarce (Clocchiatti *et al.*, 2016). We investigated sex-specific STAT1 functions in colitis and colitis-associated CRC using mice with specific STAT1 deletion in intestinal epithelial cells (STAT1<sup>ΔIEC</sup>). Male but not female STAT1<sup>ΔIEC</sup> mice were more resistant to DSS-induced colitis than sex-matched controls and displayed reduced intraepithelial infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells. The AOM-DSS protocol for induction of colitis-associated CRC resulted in increased intestinal tumor load in male but not in female STAT1<sup>ΔIEC</sup> mice. A sex-specific stratification of human CRC patients corroborated the data obtained in mice and revealed that reduced tumor cell-intrinsic nuclear STAT1 protein expression is a poor prognostic factor in men but not in women. Our data demonstrate that epithelial STAT1 is a sex-specific tumor suppressor in CRC of mice and humans. To our knowledge, epithelial STAT1 is the first factor that acts as a male-specific tumor suppressor in CRC.

DSS-induced colitis was attenuated in male STAT1<sup>ΔIEC</sup> mice. This observation contrasts a recent study that showed aggravated colitis in DSS-treated STAT1<sup>ΔIEC</sup> mice (Chiriatic *et al.*, 2017). We suspect that different microbiota conditions in the animal facilities account for this discrepancy. Concomitant with attenuated colitis, intraepithelial infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells was reduced in male STAT1<sup>ΔIEC</sup> mice. CXCL-9, CXCL-10, and CXCL-11 chemokines attract CXCR-3-expressing CD8<sup>+</sup> T cells and promote Th1 responses during inflammatory conditions (Groom and Luster, 2011). These chemokines are regulated by IFN-γ and produced by epithelial cells (Marshall *et al.*, 2017). We observed a DSS-mediated induction of IL-6, CXCL-9, CXCL-10, and CXCL-11 mRNA expression levels in IECs of male control mice. This induction was blunted in male STAT1<sup>ΔIEC</sup> mice, which might account for the reduced infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells. We confirmed blunted IL-6 induction in male STAT1<sup>ΔIEC</sup> mice at the protein level because mRNA data were ambiguous and did not reach significance. Only IL-6 and CXCL-11 mRNA were induced by DSS in epithelial cells of female

control mice, and induction was maintained in sex-matched STAT1<sup>ΔIEC</sup> mice, which was confirmed for IL-6 at the protein level. These data suggest a male-specific requirement for epithelial STAT1 in IL-6, CXCL-9, CXCL-10, and CXCL-11 expression during DSS-induced colitis. The CXCL-11 gene contains a frameshift mutation in the C57BL/6 mouse strain (Sierro *et al.*, 2007), but the promoter is still regulated by IFN-γ (Benson and Ernst, 2009). Therefore, blunted CXCL-11 expression cannot explain reduced infiltration of CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells in STAT1<sup>ΔIEC</sup> mice (which are C57BL/6). However, CXCL-10 has overlapping functions with CXCL-11 (Groom and Luster, 2011) and impaired induction might contribute to reduced infiltration.

Colitis is the tumor-promoting condition in the AOM-DSS model (Crnčec *et al.*, 2015). It is therefore counterintuitive that the tumor load was increased in AOM-DSS-treated male STAT1<sup>ΔIEC</sup> mice although severity of DSS-induced colitis was reduced. However, inflammation has a dual role in tumorigenesis and it depends on the immune cell composition whether tumor formation is promoted or inhibited (Monteleone *et al.*, 2012). CD8<sup>+</sup> T cells can aggravate tissue damage, which may sustain a tumor-promoting chronic inflammation (Monteleone *et al.*, 2012), but they also release different cytotoxic molecules such as IFN-γ which kill dysplastic cells (Restifo *et al.*, 2012). Accordingly, antibody-mediated depletion of CD8<sup>+</sup> T cells led to increased tumor load in AOM-DSS-treated mice (Pastille *et al.*, 2014). In addition, AOM-DSS-treated mice with ectopic Smad7 expression in T cells showed more severe colitis but reduced tumor load, which was associated with increased tumor immune surveillance by CD8<sup>+</sup> T cells (Rizzo *et al.*, 2011). This suggests that tumor suppression by CD8<sup>+</sup> T cells prevails over tumor-promoting effects of colitis in the AOM-DSS model. Therefore, reduced numbers of intraepithelial CD8<sup>+</sup> TCRαβ<sup>+</sup> granzyme B<sup>+</sup> T cells during DSS-induced colitis might have created an immune-privileged microenvironment in male STAT1<sup>ΔIEC</sup> mice that led to a higher tumor load despite of reduced colitis.

Reduced intraepithelial granzyme B<sup>+</sup>-cell infiltration during DSS-induced colitis was preserved in AOM-DSS-induced tumors of male STAT1<sup>ΔIEC</sup> mice, which contained lower numbers of granzyme B<sup>+</sup> cells than sex-matched control tumors. This occurred without implication of intratumoral chemokine expression because RNA-seq analysis did not reveal reduced mRNA levels for CXCL-9, CXCL-10, and CXCL-11 in bulk tumor tissue of male STAT1<sup>ΔIEC</sup> mice (data not shown). Low numbers of intratumoral granzyme-expressing CD8<sup>+</sup> T cells are associated with bad



prognosis of human CRC (Angell and Galon, 2013; Galon *et al.*, 2006, 2013). Consistently, reduced infiltration of intratumoral granzyme-expressing cells correlated with an increased tumor load and a higher percentage of high-grade tumors in male STAT1<sup>ΔIEC</sup> mice. Moreover, apoptosis was reduced which might be due to impaired granzyme B<sup>+</sup>-cell-mediated killing.

We recently employed IHC staining of tissue microarrays and identified nuclear STAT1 expression in cancer cells as a beneficial prognostic factor for CRC patients (Gordziel *et al.*, 2013). Prompted by our sex-specific observations in mice, we stratified these data according to sex. This strategy significantly improved the prognostic value of STAT1 in the male cohort but abolished it in the female cohort indicating that tumor cell-intrinsic nuclear STAT1 suppresses CRC progression in male but not in female patients. Moreover, we identified a male-specific positive correlation between STAT1 expression and CD8<sup>+</sup> T-cell infiltration in the CMS4 subtype of human CRC (Guinney *et al.*, 2015) using CIBERSORT analysis of bulk gene expression data (Newman *et al.*, 2015). It has to be shown if tumor cell-intrinsic STAT1 is responsible for this sex-specific effect because bulk gene expression data do not discriminate between tumor and stromal cells. CMS4 tumors displayed also sex-specific differences in STAT1/CXCL-11 expression, but these differences were likely too subtle to explain enhanced male-specific CD8<sup>+</sup> T-cell infiltration.

In summary, we identified epithelial STAT1 as a molecular factor that affects CRC formation in a sex-specific manner. Consequently, the prognostic value of tumor cell-intrinsic STAT1 improved significantly after sex stratification of CRC patients. Our data also recommend sex stratification prior to evaluation of therapeutic efficacy of JAK-STAT inhibitors in IBD patients (Sandborn *et al.*, 2012, 2014) to account for sex-specific STAT1 effects on inflammation.

## Acknowledgements

We thank Nicole Boucheron for support with the flow cytometry analysis. This work was supported by the Austrian Science Fund (FWF) Doktoratskolleg-plus grant 'Inflammation and Immunity' (W1212, to RE and MM), the FWF grants P25925-B20, P26908-B20, P29222-B28 (to RE), and the FWF SFB F6101 and F6106 (to MM).

## Author contributions

IC, RE, and KF involved in conceptualization; IC and MMo involved in methodology; IC, MMo, CG, JS, IS,

SM, PP, and GT investigated the study; RE wrote the original draft; IC, MMo, JS, LK, MM, BS, and RE wrote, reviewed and edited the manuscript; MM and RE acquired the funding; MS, LK, EC, MM, BS, EB, TM, and JS provided the resources; RE supervised the study.

## References

- Angell H and Galon J (2013) From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Curr Opin Immunol* **25**, 261–267.
- Bandyopadhyay SK, de la Motte CA, Kessler SP, Hascall VC, Hill DR and Strong SA (2008) Hyaluronan-mediated leukocyte adhesion and dextran sulfate sodium-induced colitis are attenuated in the absence of signal transducer and activator of transcription 1. *Am J Pathol* **173**, 1361–1368.
- Benson SA and Ernst JD (2009) TLR2-dependent inhibition of macrophage responses to IFN-gamma is mediated by distinct, gene-specific mechanisms. *PLoS One* **4**, e6329.
- Berry D, Schwab C, Milinovich G, Reichert J, Ben Mahfoudh K, Decker T, Engel M, Hai B, Hainzl E, Heider S *et al.* (2012) Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. *ISME J* **6**, 2091–2106.
- Bollrath J, Pesse TJ, von Burstin VA, Putoczki T, Bennecke M, Bateman T, Nebelsiek T, Lundgren-May T, Canli O, Schwitalla S *et al.* (2009) gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. *Cancer Cell* **15**, 91–102.
- Chan SR, Vermi W, Luo J, Lucini L, Rickert C, Fowler AM, Lonardi S, Arthur C, Young LJ, Levy DE *et al.* (2012) STAT1-deficient mice spontaneously develop estrogen receptor alpha-positive luminal mammary carcinomas. *Breast Cancer Res* **14**, R16.
- Chiriac MT, Buchen B, Wandersee A, Hundorfean G, Gunther C, Bourjau Y, Doyle SE, Frey B, Ekici AB, Buttner C *et al.* (2017) Activation of epithelial STAT1 by interleukin 28 controls mucosal healing in mice with colitis and is increased in mucosa of patients with inflammatory bowel disease. *Gastroenterology* **153**, 123–138.
- Clocchiatti A, Cora E, Zhang Y and Dotto GP (2016) Sexual dimorphism in cancer. *Nat Rev Cancer* **16**, 330–339.
- Crnčec I, Pathria P, Svinka J and Eferl R (2015) Induction of colorectal cancer in mice and histomorphometric evaluation of tumors. *Methods in Mol Biol* **1267**, 145–164.
- Galon J, Angell HK, Bedognetti D and Marincola FM (2013) The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. *Immunity* **39**, 11–26.

- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P *et al.* (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960–1964.
- Gordziel C, Bratsch J, Moriggl R, Knosel T and Friedrich K (2013) Both STAT1 and STAT3 are favourable prognostic determinants in colorectal carcinoma. *Br J Cancer* **109**, 138–146.
- Grivennikov S, Karin E, Terzic J, Mucida D, Yu GY, Vallabhapurapu S, Scheller J, Rose-John S, Cheroutre H, Eckmann L *et al.* (2009) IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. *Cancer Cell* **15**, 103–113.
- Groom JR and Luster AD (2011) CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* **89**, 207–215.
- Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P *et al.* (2015) The consensus molecular subtypes of colorectal cancer. *Nat Med* **21**, 1350–1356.
- Hanada T, Kobayashi T, Chinen T, Saeki K, Takaki H, Koga K, Minoda Y, Sanada T, Yoshioka T, Mimata H *et al.* (2006) IFN $\gamma$ -dependent, spontaneous development of colorectal carcinomas in SOCS1-deficient mice. *J Exp Med* **203**, 1391–1397.
- Horino J, Fujimoto M, Terabe F, Serada S, Takahashi T, Soma Y, Tanaka K, Chinen T, Yoshimura A, Nomura S *et al.* (2008) Suppressor of cytokine signaling-1 ameliorates dextran sulfate sodium-induced colitis in mice. *Int Immunol* **20**, 753–762.
- Kim SE, Paik HY, Yoon H, Lee JE, Kim N and Sung MK (2015) Sex- and gender-specific disparities in colorectal cancer risk. *World J Gastroenterol* **21**, 5167–5175.
- Klein SL and Flanagan KL (2016) Sex differences in immune responses. *Nat Rev Immunol* **16**, 626–638.
- Klover PJ, Muller WJ, Robinson GW, Pfeiffer RM, Yamaji D and Hennighausen L (2010) Loss of STAT1 from mouse mammary epithelium results in an increased Neu-induced tumor burden. *Neoplasia* **12**, 899–905.
- Liddle FJ and Frank DA (2008) STAT1 expression is not required for polyp formation in Min mice. *Mol Carcinog* **47**, 75–79.
- el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D and Robine S (2004) Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* **39**, 186–193.
- Marshall A, Celentano A, Cirillo N, McCullough M and Porter S (2017) Tissue-specific regulation of CXCL9/10/11 chemokines in keratinocytes: implications for oral inflammatory disease. *PLoS ONE* **12**, e0172821.
- Meissl K, Macho-Maschler S, Muller M and Strobl B (2015) The good and the bad faces of STAT1 in solid tumours. *Cytokine* **89**, 12–20.
- Monteleone G, Pallone F and Stolfi C (2012) The dual role of inflammation in colon carcinogenesis. *Int J Mol Sci* **13**, 11071–11084.
- Murray PJ (2007) The JAK-STAT signaling pathway: input and output integration. *J Immunol* **178**, 2623–2629.
- Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M and Alizadeh AA (2015) Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* **12**, 453–457.
- Nivarthi H, Gordziel C, Themanns M, Kramer N, Eberl M, Rabe B, Schleder M, Rose-John S, Knosel T, Kenner L *et al.* (2016) The ratio of STAT1 to STAT3 expression is a determinant of colorectal cancer growth. *Oncotarget* **7**, 51096–51106.
- Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y and Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98**, 694–702.
- Pastille E, Bardini K, Fleissner D, Adamczyk A, Frede A, Wadwa M, von Smolinski D, Kasper S, Sparwasser T, Gruber AD *et al.* (2014) Transient ablation of regulatory T cells improves antitumor immunity in colitis-associated colon cancer. *Cancer Res* **74**, 4258–4269.
- Rauch I, Rosebrock F, Hainzl E, Heider S, Majoros A, Wienerroither S, Strobl B, Stockinger S, Kenner L, Muller M *et al.* (2015) Noncanonical effects of IRF9 in intestinal inflammation: more than type I and type III interferons. *Mol Cell Biol* **35**, 2332–2343.
- Raven JF, Williams V, Wang S, Tremblay ML, Muller WJ, Durbin JE and Koromilas AE (2011) Stat1 is a suppressor of ErbB2/Neu-mediated cellular transformation and mouse mammary gland tumor formation. *Cell Cycle* **10**, 794–804.
- Regis G, Pensa S, Boselli D, Novelli F and Poli V (2008) Ups and downs: the STAT1:STAT3 seesaw of Interferon and gp130 receptor signalling. *Semin Cell Dev Biol* **19**, 351–359.
- Restifo NP, Dudley ME and Rosenberg SA (2012) Adoptive immunotherapy for cancer: harnessing the T cell response. *Nat Rev Immunol* **12**, 269–281.
- Rizzo A, Waldner MJ, Stolfi C, Sarra M, Fina D, Becker C, Neurath MF, Macdonald TT, Pallone F, Monteleone G *et al.* (2011) Smad7 expression in T cells prevents colitis-associated cancer. *Cancer Res* **71**, 7423–7432.
- Sandborn WJ, Ghosh S, Panes J, Vranic I, Su C, Rousell S, Niezychowski W; Study A3921063 Investigators (2012) Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med* **367**, 616–624.

- Sandborn WJ, Ghosh S, Panes J, Vranic I, Wang W, Niezychowski W; Study A3921063 Investigators (2014) A phase 2 study of tofacitinib, an oral Janus kinase inhibitor, in patients with Crohn's disease. *Clin Gastroenterol Hepatol* **12**, 1485–1493. e1482.
- Schneckenleithner C, Bago-Horvath Z, Dolznig H, Neugebauer N, Kollmann K, Kolbe T, Decker T, Kerjaschki D, Wagner KU, Muller M *et al.* (2011) Putting the brakes on mammary tumorigenesis: loss of STAT1 predisposes to intraepithelial neoplasias. *Oncotarget* **2**, 1043–1054.
- Schreiber S, Rosenstiel P, Hampe J, Nikolaus S, Groessner B, Schottelius A, Kuhbacher T, Hamling J, Folsch UR and Seegert D (2002) Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease. *Gut* **51**, 379–385.
- Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ and Schreiber RD (2001) IFN $\gamma$  and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107–1111.
- Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M *et al.* (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci USA* **104**, 14759–14764.
- Spano D, Heck C, De Antonellis P, Christofori G and Zollo M (2012) Molecular networks that regulate cancer metastasis. *Semin Cancer Biol* **22**, 234–249.
- Strobl B, Stoiber D, Sexl V and Mueller M (2011) Tyrosine kinase 2 (TYK2) in cytokine signalling and host immunity. *Front Biosci (Landmark Ed)* **16**, 3214–3232.
- Terzic J, Grivennikov S, Karin E and Karin M (2010) Inflammation and colon cancer. *Gastroenterology* **138**, 2101–2114. e2105
- Wallner B, Leitner NR, Vielnascher RM, Kernbauer E, Kolbe T, Karaghiosoff M, Rulicke T, Decker T and Muller M (2012) Generation of mice with a conditional Stat1 null allele. *Transgenic Res* **21**, 217–224.
- Weigmann B, Tubbe I, Seidel D, Nicolaev A, Becker C and Neurath MF (2007) Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nature Protoc* **2**, 2307–2311.
- Weissenbach M, Claßen T, Weber C, Spitzer D, Wirth D, Vestweber D, Heinrich PC and Schaper F (2004) Interleukin-6 is a direct mediator of T cell migration. *Eur J Immunol* **34**, 2895–2906.

## Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Specific deletion of STAT1 in intestinal epithelial cells of STAT1 $\Delta$ IEC mice.

**Fig. S2.** STAT1 is not required for development of the intestinal architecture.

**Fig. S3.** Gating strategy for flow cytometry analyses and lamina propria immune cells in STAT1 $^{flox/flox}$  and STAT1 $\Delta$ IEC mice during DSS-induced colitis.

**Fig. S4.** Activation status of STAT1 in AOM-DSS-induced tumors.

**Fig. S5.** Blood vessel formation and STAT1 target gene expression in AOM-DSS-induced colorectal tumors.

**Fig. S6.** Tumor cell-intrinsic cytoplasmic STAT1 or nuclear STAT3 is not a sex-specific prognostic marker for human CRC.

**Fig. S7.** Vein invasion and lymph node metastases are negative prognostic markers for human CRC in male and female patients.

**Fig. S8.** Strategy for stratification of human CMS1-4 subtypes of CRC into STAT1 $^{high}$ - and STAT1 $^{low}$ -expressing subgroups.

**Fig. S9.** IL-6 expression does not correlate with STAT1 expression in human CRC.

**Fig. S10.** CXCL-9 expression correlates positively with STAT1 expression in human CRC.

**Fig. S11.** CXCL-10 expression correlates positively with STAT1 expression in human CRC.

**Fig. S12.** CXCL-11 expression correlates positively with STAT1 expression in human CRC.

**Table S1.** No correlation of tumor cell-intrinsic nuclear STAT1 expression and vein invasion.

**Table S2.** No correlation of tumor cell-intrinsic nuclear STAT1 expression and lymph node metastasis.