Spontaneous onset of TNFα-triggered colonic inflammation depends on functional T lymphocytes, S100A8/A9 alarmins, and MHC H-2 haplotype

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Abstract

Recently, we established a doxycycline-inducible human tumor necrosis factor alpha (TNFα)-transgenic mouse line, ihTNFtg. Non-induced young and elderly mice showed low but constitutive expression of hTNFα due to promoter leakiness. The persistently present hTNFα stimulated endogenous pro-inflammatory mouse mS100A8/A9 alarmins. Secreted mS100A8/A9 in turn induced the expression and release of mouse mTNFα. The continuous upregulation of pro-inflammatory mTNFα and mS100A8/A9 proteins, due to their mutual expression dependency, gradually led to increased levels in colon tissue and blood. This finally exceeded the threshold levels tolerated by the healthy organism, leading to the onset of intestinal inflammation. Here, recombinant hTNFα functioned as an initial trigger for the development of chronic inflammation. Crossing ihTNFtg mice with S100A9KO mice lacking active S100A8/A9 alarmins or with Rag1KO mice lacking T and B lymphocytes completely abrogated the development of colonic inflammation, despite the still leaky hTNFα promoter. Furthermore, both the intensity of the immune response and the strength of immunosuppressive Treg induction was found to depend on the major histocompatibility complex (MHC) genetic composition. In summary, the onset of intestinal inflammation in elderly mice depends on at least four factors that have to be present simultaneously: TNFα upregulation, S100A8/A9 protein expression, functional T lymphocytes and genetic composition, with the MHC haplotype being of central importance. Only joint action of these factors leads to chronic intestinal inflammation, while absence of any of these determinants abrogates the development of the autoimmune disorder.

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Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are chronic remittent inflammatory diseases of the gastrointestinal tract that can lead to abdominal pain, bloody diarrhea, and fatigue in affected patients. The underlying pathophysiology is not completely understood. Accordingly, no curative medical options are available and chronic uncontrolled inflammation can result in disabling disease complications and the need for surgery [1]. Despite different phenotypes, both entities are characterized by intestinal inflammation, epithelial injury, and altered commensal microbiota, as well as by the upregulation of cytokines and immense infiltration of T lymphocytes, monocytes, and neutrophils into the mucosa [2–5]. In addition, numerous reports demonstrate impaired homeostasis between effector and regulatory T cells in patients with inflammatory bowel disease (IBD) [6].

The exact etiology of IBD is unknown, but it is commonly believed that genetic susceptibility, environmental conditions, and immunological disturbances are involved in the development of the disease. For example, based on monozygotic twin cohorts, it is estimated that 23% of the risk for UC and 50% of the risk for CD is due to genetic factors, while the remaining 77% and
50% of the risk is due to lifestyle and environmental factors [7–9]. Furthermore, some mutations, such as those in interleukin-10 receptor subunit 1 or interleukin-10 receptor subunit 2 (IL10R1 or IL10R2) would even be expected to be 100% concordant for CD in monozygotic twin cohorts [10]. Especially intriguing in this relation is the relationship between distinct allele variants of the major histocompatibility complex (MHC) locus, as for example DRB1*0103, and the emergence of CD [11–15]. MHC class II ligands are highly expressed on dendritic cells and present antigens to T cells, triggering activation of different T cell subsets [16–18].

In addition, several mouse experiments, but especially anti-tumor necrosis factor alpha (TNFα) therapy with humanized antibodies, have provided valid evidence that TNFα plays a central role in the etiology of IBD [3,19,20]. Nevertheless, many molecular as well as cellular immunological details of IBD development are still obscure. A further hallmark of autoimmune diseases, including IBD, is the upregulation of alarmins, such as S100A8/A9 proteins, locally at sites of inflammation in response to tissue damage or cellular stress, but also systemically in the blood. Furthermore, elevated levels of S100A8/A9 serve as a sensitive marker for the molecular detection of even subclinical disease activity in ongoing inflammatory processes [21,22]. Similar to TNFα, the released S100A8/A9 heterodimeric molecule acts like a cytokine and potentiates ongoing inflammation by recruiting neutrophils and monocytes and by inducing pro-inflammatory cytokines and chemokines like TNFα, IL-1β, IL-6, or (C-X-C motif) ligand-10 (CXCL-10) [21,22]. Furthermore, the protein complex increases leukocyte adhesion to and the permeability of blood vessels via activation of the microvascular endothelium [23,24].

In this study, we show that the development of colitis in mice depends on at least four different factors: low but persistent expression of TNFα, a functional S100A8/A9 protein complex, functional T lymphocytes, and a particular genetic composition with the H-2 MHC haplotype forming according to the manufacturer’s protocol.

Materials and methods

See Supplementary materials and methods for experimental details.

Animal studies

All animal experiments were approved by the local ethics committee and performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV-NRW), Germany. See supplementary material and methods for genotyping methods and primers. A list of genotype and numbers of mice analyzed is provided in supplementary material, Table S1.

Histology and IBD scoring

Paraffin-embedded tissue sections (4 μm) stained with hematoxylin & eosin (H&E) were evaluated independently by two blinded investigators for intestinal inflammation severity (none = 0; very mild = 1; mild = 2; mild to moderate = 3; moderate to severe = 4; severe = 5) and the percentage of affected area (0% = 0; <25% = 1; 25% = 2; 50% = 3; 75% = 4; >75% = 5). The whole colon was analyzed and the sum of both scores (scale 0–10) represents the final inflammation severity index.

Immunohistochemistry

Antigen retrieval was performed by heating the tissue sections in 1 mM EDTA (pH 8.0) solution for 20 min followed by cooling on ice for another 20 min. An ABC-AP kit (Vector Laboratories, Dossenheim, Germany) was used for visualization. See Supplementary materials and methods for details of antibodies used.

Determination of soluble cytokines and immunoglobulins

The amount of soluble human and mouse cytokines or mouse immunoglobulins in sera and cell culture supernatants were quantified using bead-based multiplex assays (LEGENDplex 13-plex mouse inflammation panel, LEGENDplex Human TNFα capture bead B3, and LEGENDplex 6-plex mouse immunoglobulin panel) and enzyme-linked immunosorbent assay (ELISA) (IgE ELISA MAXTM Standard Set) from BioLegend (Fell, Germany). All measurements were performed according to the manufacturer’s protocol.

Reverse-transcription quantitative PCR (RT-qPCR)

Colon samples were minced and immediately transferred into RNAlater solution, incubated overnight at 4 °C, homogenized, and RNA isolated using a Qiagen RNAsasy kit (Qiagen, Hilden, Germany). See Supplementary materials and methods for details.

Flow cytometry

Flow cytometry measurements were performed on a Gallios flow cytometer (Beckman Coulter, Krefeld, Germany) and data were analyzed with FlowJo software (version 10.0.8; Tree Star, Ashland, OR, USA). Antibody staining of cells was routinely performed with 1 μg/ml of each antibody used. See Supplementary materials and methods for details. Antibodies used are described in supplementary material, Table S2.
Enzymatic colon digestion for analysis of the immune microenvironment

A colon single cell suspension was obtained by enzymatic digestion using a mouse Lamina Propria Dissociation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) and was stained for surface and intracellular antigens for flow cytometry.

Isolation and differentiation of primary mouse bone marrow–derived dendritic cells (BMDCs)

Bone marrow cells were flushed from femurs and tibias of BALB/c (H-2d) mice with cold phosphate buffered saline (PBS)/1% fetal calf serum (FCS) and erythrocytes lysed. After filtering through a cell strainer (100 μm), cells were seeded onto six-well plates (non-tissue culture treated) in medium supplemented with 10% FCS, 1% NEAA, 1% HEPES buffer, 1% penicillin/streptomycin, 1% L-glutamine, and 0.5% gentamycin. Half the medium was replaced on day 3. On day 6, immature BMDCs were washed twice and stimulated with LPS (100 ng/ml), hTNFα (100 ng/ml), or S100A8 (250 ng/ml), either alone or in combination for 48 h. See Supplementary materials and methods for details.

Evaluation of CD4+ T cell proliferation

Spleens were passed through cell strainers (70 μm), erythrocytes lysed, and CD4+ cells enriched by magnetic-activated cell sorting (MACS) using the CD4-negative selection kit from Miltenyi Biotec. CD4+ cells were labeled with CFSE-fluorochrome (Invitrogen, Darmstadt, Germany) for 3 min at 37 °C; resuspended in medium supplemented with 10% FCS, 1% NEAA, 1% HEPES buffer, 1% penicillin/streptomycin, 1% L-glutamine, and 1% sodium pyruvate; and co-cultured with BMDCs from BALB/c (H-2d) mice for 5 days. The proportion of proliferating T cells was quantified by flow cytometry as CFSE-low-positive cells.

Activation of mouse CD4+ T cells

CD4+ splenocytes of ihTNFα (H-2b) and ihTNFα (H-2q) mice were sorted by an SH800S cell sorter and activated by culturing with anti-CD3/CD28-coated Mouse T-Activator Dynabeads for 4, 16, or 24 h. Cell supernatants were collected and analyzed for cytokine contents by the LEGENDplex 13-plex mouse inflammation panel kit.

Analysis of the composition of the intestinal microbiome

See Supplementary materials and methods.

Results

Colonic inflammation onset depends on TNFα expression and the H-2 MHC genetic background

Recently, we established an inducible human TNFα-transgenic mouse line (ihTNFα) that upon stimulation with doxycycline (Dox) expresses large amounts of the transgenic cytokine (up to 1000 pg/ml in serum) and develops psoriatic arthritis–like disease symptoms [25,26]. In addition, all untreated control mice express low hTNFα serum levels (3–20 pg/ml) and do not show abnormalities during the first 2 months of life. However, with increasing age, some adult animals develop signs of inflammation. More specifically, laparoscopy showed an enlarged abdomen that mirrors a dilated colon (Figure 1A, lower panel).

ihTNFα mice were obtained by crossing C57Bl/6 (H-2b) with FVB (H-2q) mice that carried the rtTA2S or the hTNFα transgene, respectively [26]. Consequently, ihTNFα mice have a mixed genetic background. Hence, we suggested that the development of the intestinal phenotype might be related to either variation in hTNFα expression with age or in the genetic background. In particular, certain MHC H-2 haplotypes are already known to increase the risk of autoimmune diseases [27]. Therefore, we analyzed ihTNFα mice homozygous for either H-2b or H-2q. Measuring levels of soluble hTNFα in blood samples did not reveal an age dependency (Figure 1B). In addition, none of the mice homozygous for H-2b showed a spontaneous onset of intestinal inflammation, but 6- to 12-month-old mice homozygous for the H-2q haplotype did (Figure 1C, Table S1). Notably, the original non-transgenic C57Bl/6 or FVB mice of any age did not develop any signs of intestinal inflammation, either macroscopically or microscopically (supplementary material, Figure S1A), indicating that the expression of low levels of hTNFα is crucial for the initiation of intestinal inflammation.

To confirm the dominance of the H-2q over the H-2b haplotype in the induction of intestinal inflammation, we analyzed H-2b/q heterozygous ihTNFαg mice. Despite the codominant expression of MHC loci, only the half of twelve, 6-month-old H-2 heterozygous animals developed bowel inflammation, with only one mouse showing inflammation intensity comparable to their H-2b/q counterparts (Figure 1D), indicating that the FVB genetic background is important but the H-2q haplotype is critical. Thus, permanent exposure to limited pro-inflammatory stress in transgenic hTNFα mice drives the development of intestinal inflammation, with the H-2q MHC haplotype being a critical factor. Of note, expression of hTNFα happens without Dox induction or any other apparent stimulation. It is notable that all mice were kept under stringent specific-pathogen-free conditions and that no changes in the gut microbiota were observed between ihTNFα (H-2b/q) mice of different ages (supplementary material, Figure S2).

Histological evaluation of H&E-stained gut sections of ihTNFα mice confirmed the intestinal inflammation...
Figure 1. Colonic phenotype of ihTNF<sup>tg</sup> (H-2<sup>b</sup>) and ihTNF<sup>tg</sup> (H-2<sup>q</sup>) mice. (A) Macroscopic colon images of 2-, 6-, and 12-month-old ihTNF<sup>tg</sup> (H-2<sup>b/b</sup>) and ihTNF<sup>tg</sup> (H-2<sup>q/q</sup>) mice. (B) Serum levels of soluble recombinant hTNFα cytokine in ihTNF<sup>tg</sup> (H-2<sup>b/b</sup>) and ihTNF<sup>tg</sup> (H-2<sup>q/q</sup>) mice of different ages. (C) Intestinal inflammation severity of ihTNF<sup>tg</sup> (H-2<sup>b/b</sup>) and ihTNF<sup>tg</sup> (H-2<sup>q/q</sup>) mice. (D) hTNFα levels in sera and intestinal inflammation severity of 6-month-old heterozygous ihTNF<sup>tg</sup> (H-2<sup>b/q</sup>) mice. Individual animal and mean scores (red lines) are shown. Each group contained five to seven mice. In (D), the group of (H-2<sup>b/q</sup>) mice contained 12 animals. ***p < 0.001 (U-test). (E) H&E-stained colon tissue of ihTNF<sup>tg</sup> (H-2<sup>b/b</sup>) and ihTNF<sup>tg</sup> (H-2<sup>q/q</sup>) mice of different ages. (F) H&E images of 12-month-old ihTNF<sup>tg</sup> (H-2<sup>b/b</sup>) mice. Note the transmural disease manifestation with thickened muscularis propria (hatch mark), crypt abscesses (black arrowheads), lymphoid aggregates (asterisks), granuloma-like lesions (arrow), and severe crypt damage (red arrowheads). Scale bars = 200 μm. Note that images of mice with colitis were taken at lower magnification than images of healthy mice to demonstrate the thickened muscularis propria and enlarged crypts, which are enlarged due to dilated colon tissue of mice with colitis (see the lower panel of A).
of animals homozygous for H-2q but not for mice with H-2b MHC haplotype (Figure 1E), despite H-2b^th ihTNFtg mice having a mixed C57Bl/6 and FVB genetic background similar to H-2q^q mice. Diseased mice demonstrated a severe inflammatory reaction. A transmural disease manifestation with a thickened muscularis propria and edematous segments was observed. The bowel tissue was heavily infiltrated by immune cells consisting of lymphocytes, neutrophils, and accumulated macrophages, which formed several lymphoid aggregates as well as a few granuloma-like lesions. Furthermore, severe crypt damage was observed with partial loss of the epithelial cell layer, accompanied by deep ulcerations and crypt abscesses (Figure 1F). Finally, a high proliferative activity of all crypt cell layers was observed in ihTNF^tg mice homozygous for H-2^q but not in mice of H-2^b haplotype (Figure 2A). In the latter, only basal crypt cells revealed Ki67-positive staining. All but one of the affected H-2^b^q heterozygous ihTNFtg mice showed a moderate phenotype, with only short bowel stretches being distended (Figure 1D and supplementary material, Figure S1B). Of interest, no inflammatory changes were found in the small intestine, including the terminal ileum in mice homozygous for H-2^q (supplementary material, Figure S1C). In following experiments, only mice homozygous for H-2^q and H-2^b haplotypes were analyzed, since they best reflect the TNFα-triggered onset of gut inflammation.

Colonic inflammation onset depends on a functional adaptive immune system and S100A8/A9 protein expression

The development of IBD is believed to be accompanied by a dysregulated intestinal T lymphocyte response and increased expression of the S100A8/A9 alarmin proteins. The latter have been shown to act in a concerted manner with TNFα and are increasingly used as biomarkers for mucosal inflammation in human IBD patients [28–31]. Immunohistochemical analysis of the colonic wall of ihTNF^tg mice with homozygous H-2^q at different ages unraveled a massive number of CD3^+ T cells and S100A9-positive cells, although this was not observed in mice of H-2^b haplotype (Figure 2B,C). T lymphocytes were located in large numbers along the colonic crypts, but also often at apical sites or in the underlying mucosal layer, indicating that the basal membrane was damaged. S100A9 protein synthesis was observed in infiltrating cells, but S100A9 was also present in its soluble form within colonic tissue. Noteworthy, only samples of 6- and 12-month-old but not of 2-month-old ihTNF^tg mice with H-2^q haplotype showed features of manifested inflammation such as immune cell infiltration and crypt damage. Staining of affected H-2^b^q heterozygous mouse samples for CD3- and S100A9-positive cells confirmed the rather mild inflammation in these mice (supplementary material, Figure S1B).

To investigate the impact of the adaptive immune system and the pro-inflammatory S100A8/A9 protein in the development of TNFα-triggered and H-2^q haploptype-supported intestinal inflammation, ihTNF^tg (H-2^q) mice were crossed with Rag1^ KO mice lacking mature T and B lymphocytes or with S100A9^KO mice deficient for the functional S100A8/A9 protein complex. Both double-mutant mouse lines were homozygous for the MHC H-2^q haplotype and expressed the recombinant hTNFα cytokine, the level of which in serum was constant over time and similar to that of ihTNF^tg mice (supplementary material, Figure S3A). However, analysis of colon tissue by histology did not reveal any signs of inflammation. Neither 2-, 6- nor 12-month-old mice were affected (supplementary material, Figure S3B), and no inflammation could be detected when colon sections were stained with H&E or with antibodies recognizing the CD3 T cell surface marker or the S100A9 protein (supplementary material, Figure S3C–E). Thus, the spontaneous onset of intestinal inflammation in ihTNF^tg mice depends on at least four different factors: the presence of rather low but permanent levels of TNFα, the H-2^q MHC haplotype, a functional adaptive immune system, and a functional S100A8/A9 alarmin protein complex. Absence of one of these factors abrogated the development of intestinal inflammation.

Colonic inflammation in ihTNF^tg mice coincides with activation of T cells and enhanced expression of endogenous TNFα and S100A8/A9

A detailed analysis of the bowel immune status by flow cytometry revealed an increased colon infiltration by CD3^+ , CD4^+ , and CD8^+ T cells in 6- and 12-month-old mice compared to 2-month-old animals, but only in ihTNF^tg mice with an H-2^q haplotype (Figure 3A). It is interesting to note that the number of CD4^+Foxp3^+ Treg cells in the colon of diseased ihTNF^tg with the H-2^q haplotype also increased with age, but their relative number was constantly lower than in ihTNF^tg or ihTNF^tg/S100A9^KO mice homozygous for H-2^q or H-2^q, respectively (Figure 3B). This was especially obvious when the portion of Foxp3-positive cells was evaluated among CD4^+ T cells only (Figure 3B, middle and right images). In agreement with these data, expression analysis of inflammation-associated genes such as transcription factors for activated Th1 (Tbx21), Th2 (Gata3), and Treg (Foxp3) lymphocytes revealed an upregulation with age only in ihTNF^tg mice with H-2^q haplotype (Figure 3C). Especially interesting was the enhanced activity of the mouse Tnf and S100a9 genes only in 6- and 12-month-old ihTNF^tg mice with H-2^q haplotype but not in the other tested mouse lines, including H-2^q homozygous but non-transgenic FVB mice (Figure 3D and supplementary material, Figure S6C). These data accentuate the importance of colon endogenous TNFα and S100A8/A9 proteins in the spontaneous onset of intestinal inflammation. The complete immune status analysis at the local intestinal and systemic levels is described in supplementary material, Figures S4–S6. Taken together, our data show that only mice that develop intestinal inflammation with age showed an altered colonic immune status.

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TNFα and S100A8 have an additive effect on dendritic cell stimulation

The development of intestinal inflammation clearly depends on the expression of TNFα and S100A8/A9 pro-inflammatory proteins and the levels of these mediators increased with age and accompanied the development of intestinal inflammation in ihTNFtg mice with H-2q haplotype. Hence, we wondered whether these factors stimulate the activation of antigen presenting cells in an additive manner, thus supporting the activation of immunogenic T lymphocytes. To support this assumption, BALB/c bone marrow cells of H-2d MHC
were isolated and mature dendritic cells (DCs) were generated by incubation with GM-CSF and IL-4 for 6 days. DCs were then activated by stimulation with hTNFα and mS100A8 proteins alone or in combination for an additional 2 days. Of note, mS100A8 homodimeric protein was used in the stimulation experiments instead of the heterodimer S100A8/A9 since the in vitro activity of the heterodimer is short-lived [32]. Lipopolysaccharide (LPS) served as a positive control stimulus. Activated DCs were then tested for their ability to stimulate the proliferation of allogeneic ihTNFtg (H-2b) and ihTNFtg (H-2q) CD4+ T lymphocytes (Figure 4A). The combined action of hTNFα and mS100A8 was indeed able to enhance T cell activation over the basal GM-CSF + IL-4 stimulus, while the action of both factors alone hardly activated DCs under the conditions tested. Of note, CD4+ cells of both mouse lines were increasingly activated by DCs simultaneously stimulated with hTNFα and S100A8. However, CD4+ cells with an H-2q MHC background demonstrated stronger activation than T

Figure 3. Immunological status of the colon of 2-, 6-, and 12-month-old ihTNFtg (H-2b), ihTNFtg (H-2q), ihTNFtg/Rag1KO (H-2q), and ihTNFtg/S100A9KO (H-2q) mice. (A) Percentage of (CD3+, CD4+, CD8+) T cells. (B) Percentage of Treg cells of viable (CD4+FoxP3+) and of CD4+ (Foxp3+) cells. (C) RT-qPCR analysis of mRNA levels of transcription factors characteristic for activated sets of Th1, Th2, and Treg lymphocytes. (D) mRNA levels of mouse Tnf and S100a9. Mean values per group (red lines), with n = 5 to 6 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 (U-test).
Cells with the H-2^b haplotype. Of interest, this difference was also observed when LPS-stimulated DCs were used, suggesting that T cells of the H-2^q haplotype are generally more sensitive to activation by DCs than T cells with the H-2^b genetic background.

Immune cells of the H-2^q haplotype have a lower activation threshold

To support this assumption, CD4^+ cells from ihTNFtg mice with H-2^b and H-2^q haplotypes were co-cultured with DCs from BALB/c (H-2^d) mice, and their proliferation was measured as reduction of CFSE-labeling by flow cytometry. Although only about 40% of H-2^b T cells were triggered into proliferation under these conditions, the proportion of CFSE-low-positive H-2^q cells was over 60%. It is notable that despite the higher proliferation of H-2^q CD4^+ T cells, the proportion of Foxp3-positive lymphocytes among them was significantly lower than in the proliferating pool of the H-2^b cell population (Figure 4B,C).

These data are in good accordance with our colon tissue analysis for different T cell populations (supplementary Figure 4).
material, Figures S4 and S5) and additionally emphasize the disturbed relationship between the activation of autoimmune reactive T cells and the generation of Tregs during IBD.

To exclude the notion that the recombinant hTNF transgene is responsible for the observed differences in T cell activation, CD4+ cells from C57Bl/6 (H-2b) and FVB (H-2q) mice that do not express the hTNFα transgene were tested for their activation capacity by DCs from allogeneic BALB/c (H-2b) mice (Figure 4D). Similarly, T cells with an H-2q MHC background were more strongly activated than T cells bearing the H-2b haplotype. As with ihTNFα mice (Figure 4C), proliferating lymphocytes with H-2q haplotype (FVB) contained a lower proportion of Foxp3+ Tregs (Figure 4D). Because ihTNFα/S100A9KO mice did not develop intestinal inflammation and had no increased intestinal immune cell infiltration and T cell activation despite permanent expression of hTNFα and the H-2q haplotype, we wondered whether S100A8/A9 proteins influence T cell activation. A comparison of CFSE-low-positive cells of ihTNFα/S100A9KO mice with the H-2q and H-2b haplotypes after co-culture with stimulated allogeneic DCs showed that S100A9-deficient T cells homozgyous for the H-2q MHC allele proliferated to a greater extent than their counterparts bearing the H-2b MHC (Figure 4E). However, the proportion of Foxp3+ cells among proliferating ihTNFα/S100A9KO CD4+ cells with the H-2q haplotype was not decreased but increased, indicating that S100A8/A9 does not influence the antigenic activation of CD4+ cells per se, but inhibits their development toward Tregs.

Finally, to confirm that T cells with the H-2q haplotype are generally more responsive to antigenic stimuli, CD4+ cells from ihTNFα mice with the H-2b and H-2q haplotypes were co-cultured with anti-CD3 and anti-CD28 coated Dynabeads for 4, 16, and 24 h, and the amount of inflammatory cytokines produced by these cells was quantified (Figure 4F). Indeed, T cells with the H-2q genetic background produced significantly more proinflammatory cytokines after 4 h of stimulation, and the difference between T cells from H-2q to H-2b further increased over time. Thus, T lymphocytes with H-2q MHC are significantly more sensitive to antigenic stimulation than T cells with H-2b MHC; in other words, the activation threshold of H-2q T cells is significantly lower than that of H-2b T cells.

Discussion

Our data suggest that the spontaneous onset of colonic inflammation in recombinant ihTNFα mice depends on: (1) permanent expression of low amounts of recombinant hTNFα, (2) active S100A8/A9 alarmin protein complexes, (3) functional T lymphocytes, and (4) the genetic background, with H-2q MHC being of central importance. Removal of any of these factors abrogated the onset of intestinal inflammation and only their joint action led to chronic infiltration of colonic tissue with different sets of T lymphocytes and antigen-presenting cells, ultimately resulting in severe colonic crypt damage accompanied by deep ulcerations and crypt abscesses. In addition, our results emphasize the disturbed relationship between the ability to activate T lymphocytes and to generate Tregs and show that this disturbance mainly depends on the MHC H-2 haplotype and S100A8/A9 proteins. Colon tissue from mice with the H-2q haplotype that developed IBD along with chronic expression of low amounts of hTNFα was abundantly infiltrated by T cells, but proportion of Tregs among them was significantly lower than in the T cell population of mice with the H-2b haplotype that did not develop IBD. Of note, both mouse lines were homozygous for the H-2 locus but had a mixed C57Bl/6 and FVB genetic background. Moreover, the generation of Tregs depended not only on the MHC haplotype but also on the presence of functional S100A8/A9 alarmins.

TNFα and S100A8/A9 are pro-inflammatory proteins that not only accompany inflammation, but also actively stimulate and enhance the activation of immune cells [19,21]. Furthermore, TNFα has been shown to act in a concerted manner with S100A8/A9. TNFα upregulates S100A8/A9 expression and S100A8/A9 upregulate TNFα expression in a functional feedback loop [19,21,32]. Hence, TNFα and S100A8/A9 represent a self-sustaining inflammatory loop. The ihTNFα mouse continuously expresses low levels of recombinant hTNFα, apparently due to the leakiness of the Tet-dependent promoter. Thus, we hypothesized that the persistently present recombinant hTNFα in ihTNFα mice stimulates the induction of endogenous pro-inflammatory mS100A8/A9 alarmins. The synthesized mS100A8/A9 in turn induces the expression of endogenous mTNFα. The recombinant human TNFα gene is controlled by an artificial promoter and cannot be upregulated by S100A8/A9. Our data indicate that the continuous upregulation of endogenous pro-inflammatory mTNFα and mS100A8/A9 proteins, due to their mutual expression dependency, gradually increased their amounts in blood, which finally exceeded the threshold levels tolerated by the healthy organism and led to the development of colonic inflammation.

Both bowel tissue and serum levels of mouse TNFα and S100A8/A9 gradually increased with age and intestinal inflammation was not present in 2-month-old mice but appeared in older mice, thus supporting our hypothesis. In further accordance with this are the in vitro data showing that stimulation of DCs with S100A8 and TNFα was much more efficient than stimulation with either stimulus alone. Thus, it is conceivable that TNFα, in conjunction with S100A8/A9, stimulates colonic antigen-presenting cells, which are in permanent contact with gut microflora antigens, providing a permanent activation stimulus to DCs. Activation of DCs increases with time due to rising amounts of mTNFα and S100A8/A9. Stimulated DCs activate T cells that in turn infiltrate the colon tissue, inducing damage. Recombinant hTNFα functions here as an initiation trigger for chronic
inflammation. Indeed, no increase in mTNFα and no colitis was seen in elderly FVB mice with no hTNFα transgene, despite their homozygosity for the H-2b haplotype. The anti-inflammatory arm, that is, the number of Tregs, also increased during the development of IBD, but this was obviously not enough to restrict the ongoing T cell–mediated intestinal inflammation.

A further key finding of this work is that immune tolerance as well as the intensity of the immune response and the ability to induce suppressive T cells strongly depend on the MHC genetic composition. Only ihTNFα/KO mice with the H-2b but not with H-2b<sup>-</sup> MHC haplotype were able to develop intestinal inflammation. Notably, not only were increased infiltration of colonic tissue with T lymphocytes and increased activity of Th1 and Th2 cells (according to the expression of Tbx21 and Gata3 transcription factor mRNAs) characteristic for ihTNFα/KO (H-2b<sup>-</sup>) mice, but a generally low level of Treg cells was also found in their colonic tissue. Despite the existing activation capacity of Tregs with the H-2<sup>b</sup> haplotype (increased Foxp3 mRNA) and increased Treg cell numbers in the inflamed intestine of ihTNFα/KO (H-2b<sup>-</sup>) mice, the relative amount of Tregs in colonic tissue was always lower than in ihTNFα<sup>+</sup> (H-2<sup>-</sup>) and in ihTNFα<sup>+</sup>/S100A9<sup>-/-</sup> (H-2<sup>-</sup>) mice.

Our <i>in vitro</i> experiments confirmed that CD4<sup>+</sup> T cells with the H-2<sup>b</sup> haplotype, independently of the hTNFα transgene, were more prone to the stimulatory action of DCs than their counterparts with the H-2<sup>b</sup> haplotype. Furthermore, our experiments indicate that differentiation of CD4<sup>+</sup> T cells toward Foxp3<sup>+</sup> Tregs also depends on the MHC H-2 haplotype and that the presence of functional S100A8/A9 alarmins inhibits this process. Indeed, proliferating CD4<sup>+</sup> T cells with H-2<sup>b</sup> differentiated toward Foxp3<sup>+</sup> Tregs significantly more efficiently than CD4<sup>+</sup> T cells with the H-2<sup>-</sup> haplotype, independently of the presence of the hTNFα transgene (Figure 4C, D). However, in mice deficient for S100A9, the situation changed. The number of Foxp3<sup>+</sup> cells among proliferating CD4<sup>+</sup> lymphocytes in ihTNFα<sup>+</sup>/S100A9<sup>-/-</sup> mice with the H-2<sup>-</sup> haplotype was significantly higher than in ihTNFα<sup>+</sup>/S100A9<sup>-/-</sup> lymphocytes with the H-2<sup>-</sup> MHC haplotype (Figure 4E). These data indicate that S100A8/A9 alarmins regulate the capacity of T helper cells to differentiate toward Tregs, probably in a MHC-dependent manner. The molecular mechanism of this intriguing phenomenon is still unclear. These <i>in vitro</i> data, however, correspond to the <i>in vivo</i> data showing an increased proportion of intestinal Treg cells in ihTNFαtg/S100A9 mice, despite their H-2<sup>-</sup> haplotype.

Generally, the results of this work are in agreement with numerous publications showing that intestinal inflammation is a disease with disturbed peripheral tolerance or unbalanced regulation of T helper cell subsets. That MHC genotype plays a significant role in the development of autoimmune diseases is known. For example, DBA/1 mice, bearing the H-2<sup>b</sup> haplotype, are known to be susceptible to spontaneously developing psoriatic arthritis [33]. Mouse lines with H-2<sup>a</sup> and H-2<sup>b</sup> MHC haplotypes have been described as being sensitive to collagen-induced arthritis [34]. It is also well-known that, in humans, persons with particular HLA genetic loci have a higher risk of developing rheumatoid arthritis as well as autoimmune IBDs, including CD and UC [9,35]. Furthermore, induction of higher numbers of Tregs in mice with H-2<sup>b</sup> haplotype might be a significant factor providing increased resistance of C57Bl/6 mice to autoimmune diseases [36–38].

Only a few animal models for IBD are based on TNFα dysregulation, like the TNFα<sup>ARE</sup> mouse model. These mice were generated by deletion of the mouse <i>Tnf</i> AU-rich element of the 3′-untranslated region, which led to greater stability of the <i>Tnf</i> mRNA and consequently to an overproduction of this cytokine. TNFα<sup>ARE</sup> homozygous animals develop a severe rheumatoid-like arthritis and intestinal inflammation that affects mostly the terminal part of the small intestine (ileum) [1,39]. In contrast, in our ihTNFα<sup>+</sup> (H-2<sup>-</sup>) mice, only the colon was severely affected. This disease restriction may be caused by the much lower expression of TNFα in ihTNFα<sup>+</sup> (H-2<sup>-</sup>) mice compared to TNFα<sup>ARE</sup> mice. In addition, TNFα<sup>ARE</sup> mice develop IBD shortly after birth, while it appeared only in older in ihTNFαtg mice, resembling more spontaneous onset of the disease. We also cannot exclude that differences in the development of intestinal inflammation in TNFα<sup>ARE</sup> and ihTNFα<sup>+</sup> mice, especially in the disease pattern, are due to genetic differences in MHC. TNFα<sup>ARE</sup> mice have a C57Bl/6 background (H-2<sup>K</sup>). Indeed, a recent genetic association study that included 29 838 patients (16 902 with CD, 12 597 with UC) revealed that IBD location is in part genetically determined by and related to MHC expression [12]. Molecular studies have also suggested that variants in MHC alleles, such as DRB1*0103, are associated with the development of CD that mostly affects the colon [14,40]. Finally, hTNFα binds only to type II, not type I, mouse TNF receptors [39], which might have different consequences.

In summary, the existence of weak but chronic hTNFα-mediated inflammatory stress provokes the induction of the endogenous mS100A8/A9 alarmins and mTNFα. Their levels gradually increase due to their mutual upregulation, resulting in the activation of different sets of CD4<sup>+</sup> Th lymphocytes and gut epithelial injury. However, the intensity of both inflammatory and immune tolerance response depends on MHC genetic composition and the expression of S100A8/A9.

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Author contributions statement

RLD, DB, GV, and TW designed, performed, and analyzed the experiments. HTW performed specific experiments. UD, JR, TV, and SL designed the study. VW supervised the project, designed and analyzed the experiments, and wrote the paper. All authors critically read and approved the manuscript text.

References

1. Cominelli F, Arseneau KO, Rodriguez-Palacios A, et al. Uncovering pathogenic mechanisms of inflammatory bowel disease using mouse models of Crohn’s disease-like ileitis: what is the right model? Cell Mol Gastroenterol Hepatol 2017; 4: 19–32.
2. Kim DH, Cheon JH. Pathogenesis of inflammatory bowel disease and recent advances in biologic therapies. Inflamm Bowel Dis 2017; 23: 33–51.
3. Neurath MF. New targets for mucosal healing and therapy in inflammatory bowel disease. Nat Rev Gastroenterol Hepatol 2016; 2097274.
4. Silva FAR, Rodrigues BL, Ayrizono MLS, et al. The immunological basis of inflammatory bowel disease. Gastroenterol Res Pract 2016; 2016: 2097274.
5. Torres J, Mehandru S, Colombel JF, et al. Crohn’s disease. Lancet 2017; 389: 1741–1755.
6. Bogner S, Izumi T, Sanai N, et al. Disrupted regulatory T cell homeostasis in inflammatory bowel diseases. World J Gastroenterol 2016; 22: 974–995.
7. Brant SR. Update on the heritability of inflammatory bowel disease: the importance of twin studies. Inflamm Bowel Dis 2017; 23: 1–5.
8. Huang H, Fang M, Jostins L, et al. Fine-mapping inflammatory bowel disease loci to single-variant resolution. Nature 2017; 547: 173–178.
9. Parkes M. The genetics universe of Crohn’s disease and ulcerative colitis. Dig Dis 2012; 30(Suppl 1): 78–81.
10. Gallagher K, Boucher G, Jostins L, et al. Inherited determinants of Crohn’s disease and ulcerative colitis phenotypes: a genetic association study. Lancet 2016; 387: 156–167.
11. de la Concha EG, Fernandez-Arquero M, Lopez-Nava G, et al. Susceptibility to severe ulcerative colitis is associated with polymorphism in the central MHC gene IFNL3. Gut 2016; 65: 859–868.
12. Pruenster M, Gardiner E, Janssen A, et al. Macrophage-mediated psoriasis can be suppressed by regulatory T lymphocytes. J Pathol 2016; 240: 366–377.
13. Retse T, Schied T, Skyabin BV, et al. Inducible expression of transgenic human TNFalpha in adult mice develops psoriatic-like arthritis. Arthritis Rheum 2013; 65: 2290–2300.
14. Fernandez MM, Stevens CR, Walsh EC, et al. Defining the role of the MHC in autoimmunity: a review and pooled analysis. PLoS Genet 2008; 4: e1000024.
15. Vezzoni D, Jetel T, Schramm J, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. Blood 2005; 105: 2955–2962.
16. Leite Dantas R, Masemann D, Schied T, et al. Macrophage-mediated psoriasis can be suppressed by regulatory T lymphocytes. J Pathol 2016; 240: 366–377.
17. Retse T, Schied T, Skyabin BV, et al. Inducible expression of transgenic human TNFalpha in adult mice develops psoriatic-like arthritis. Arthritis Rheum 2013; 65: 2290–2300.
18. Fernandez MM, Stevens CR, Walsh EC, et al. Defining the role of the MHC in autoimmunity: a review and pooled analysis. PLoS Genet 2008; 4: e1000024.
19. Foell D, Witkowski H, Roth J. Monitoring disease activity by stool analyses: from occult blood to molecular markers of intestinal inflammation and damage. Gut 2009; 58: 859–868.
20. Fonseca-Camarillo G, Yamanoto-Furusho JK. Immune-regulation pathways involved in inflammatory bowel disease. Inflamm Bowel Dis 2015; 21: 2188–2193.
21. Smids C, Horjus Talabur Horje CS, Dreyewicz J, et al. Intestinal T cell profiling in inflammatory bowel disease: linking T cell subsets to disease activity and disease course. J Crohns Colitis 2018; 12: 465–475.
22. Zhang X, Wei L, Wang J, et al. Suppression colitis and colitis-associated colon cancer by anti-S100a9 antibody in mice. Front Immunol 2017; 8: 1747.
23. Vogl T, Stratis A, Wixler V, et al. Autoinhibitory regulation of S100A8/S100A9 alarmin activity locally restricts sterile inflammation. J Clin Invest 2018; 128: 1852–1866.
24. Lories RJ, Matthys P, de Vlam K, et al. Ankylosing spondylitis, dactylitis, and onychoperoistitis in male DBA/1 mice: a model of psoriatic arthritis. Ann Rheum Dis 2004; 63: 595–598.
25. Walter W, Loos M, Maeurer MJ. H2-M polymorphism in mice susceptible to collagen-induced arthritis involves the peptide binding groove. Immunogenetics 1996; 44: 19–26.
26. Kolho K-L, Paakkanen R, Lepisto A, et al. Novel associations between major histocompatibility complex and pediatric-onset inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2016; 62: 567–572.
27. Borm ME, He J, Kessell B, et al. A large quantitative trait locus on mouse chromosome 3 is involved in disease susceptibility in different colitis models. Gastroenterology 2005; 128: 74–85.
28. Esworthy RS, Kim BW, Larson GP, et al. Colitis locus on chromosome 2 impacting the severity of early-onset disease in mice deficient in Gpx1 and Gpx2. Inflamm Bowel Dis 2011; 17: 1373–1386.
29. Girardi M, Lewis J, Glusac E, et al. Resident skin-specific gamma-delta T cells provide local, nonredundant regulation of cutaneous inflammation. J Exp Med 2002; 195: 855–867.
30. Kontoyiannis D, Pasparakis M, Pizarro TT, et al. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements.
implications for joint and gut-associated immunopathologies. *Immunity* 1999; 10: 387–398.
40. Cleynen I, Vazeille E, Artieda M, et al. Genetic and microbial factorsmodulating the ubiquitin proteasome system in inflammatory bowel disease. *Gut* 2014; 63: 1265–1274.
41. Katsantoni EZ, Angelescu NE, Rottier R, et al. Ubiquitous expression of the rtTA2s-M2 inducible system in transgenic mice driven by the human hnRNPA2B1/CBX3 CpG Island. *BMC Dev Biol* 2007; 7: 108.
42. Leite Dantas R, Brachvogel B, Schied T, et al. The LIM-only protein 4 and a half LIM domain protein 2 attenuates development of psoriatic arthritis by blocking Adam17-mediated tumor necrosis factor release. *Am J Pathol* 2017; 187: 2388–2398.
43. Petersen B, Wolf M, Austermann J, et al. The alarmin Mrp8/14 as regulator of the adaptive immune response during allergic contact dermatitis. *EMBO J* 2013; 32: 100–111.
44. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009; 75: 7537–7541.
45. Kozich JJ, Westcott SL, Baxter NT, et al. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013; 79: 5112–5120.
46. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013; 41: D590–D596.
47. Cole JR, Chai B, Farris RJ, et al. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* 2007; 35: D169–D172.
48. McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 2013; 8: e61217.

References 41–48 are cited only in the supplementary material.

**SUPPLEMENTARY MATERIAL ONLINE**

**Supplementary materials and methods**

**Supplementary results**

**Figure S1.** Histology and immunohistochemistry of non-transgenic and transgenic mice

**Figure S2.** Evaluation of colon microbiota composition

**Figure S3.** Serum hTNFα and intestinal inflammation severity of ihTNFβ/Rag1KO and ihTNFβ/S100A9KO mice

**Figure S4.** Immunological status of the colon of ihTNFβ (H-2b/b), ihTNFβ (H-2q/q), ihTNFβ/Rag1KO (H-2q/q), and ihTNFβ/S100A9KO (H-2q/q) mice

**Figure S5.** RT-qPCR analysis of colon tissues of ihTNFβ (H-2b/b), ihTNFβ (H-2q/q), ihTNFβ/Rag1KO (H-2q/q), and ihTNFβ/S100A9KO (H-2q/q) mice

**Figure S6.** Serum inflammatory cytokines and immunoglobulins of ihTNFβ (H-2b/b), ihTNFβ (H-2q/q), ihTNFβ/Rag1KO (H-2q/q), and ihTNFβ/S100A9KO (H-2q/q) mice

**Table S1.** List of mice analyzed in this work

**Table S2.** Monoclonal antibodies used for flow cytometry analyses