Direct Crosstalk between Mast Cell-TNF and TNFR1-expressing Endothelia Mediates Local Tissue Inflammation

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Abstract

Signaling through tumor necrosis factor receptor 1 (TNFR1) controls bacterial infections and the induction of inflammatory Th1 cell-mediated autoimmune diseases. By dissecting Th1 cell-mediated delayed type hypersensitivity responses (DTHRs) into single steps, we localized a central defect to the missing TNFR1-expression by endothelial cells (ECs). Adoptive transfer and mast cell knock-in experiments into KitW/KitWv, TNF−/− and TNFR1−/− mice showed that the signaling defect exclusively affects mast cell-EC interactions but not T cells nor APCs. As a consequence, TNFR1−/− mice have strongly reduced mRNA and protein expression of P-selectin, E-Selectin, ICAM-1, and VCAM-1 during DTHR elicitation. In consequence, intravital fluorescence microscopy revealed up to 80% reduction of leukocyte rolling and firm adhesion in TNFR1−/− mice. As substitution of TNF−/− mice with TNF-producing mast cells fully restores DTHR in these mice, signaling of mast cell-derived TNF through TNFR1-expressing ECs is essential for the recruitment of leukocytes into sites of inflammation.
Introduction

TNF signaling through the TNF receptors (TNFR) TNFR1 and TNFR2 is critically involved in innate and adaptive T cell-mediated immune responses \(^1\). The crucial role of TNF in inflammation is demonstrated by the improvement of severe inflammatory autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, or psoriasis, during treatment with either anti-TNF antibodies or TNF-binding fusion proteins \(^2\). Concomitantly, inhibition of TNF increases susceptibility to infections with bacteria or mycobacteria, further emphasizing the essential role of TNF in critical steps of innate and adaptive immunity \(^2-5\). Comparison of TNFR1\(^{-/-}\) and TNFR2\(^{-/-}\) mice reveals that TNF-signaling through TNFR1 is essential in host defense against intracellular pathogens such as \textit{Listeria monocytogenes} \(^6\) and \textit{Mycobacterium tuberculosis} \(^7\), while TNF-signaling through TNFR2 increases susceptibility to intracellular infections \(^8\). Moreover, TNFR1\(^{-/-}\) mice show that signaling through TNFR1 is involved in early phases of acute graft versus host disease \(^9\), experimental autoimmune encephalitis (EAE) \(^10\), autoimmune diabetes \(^11\), or arthritis \(^12,13\).

Even though the cascade of TNF-signaling through TNFR1 has been studied extensively \(^14\), and even though it is established that TNF-signaling through TNFR1 plays an essential role in T cell-mediated inflammation \(^6,7,9-13,15\), the underlying mechanism remains unknown. This is especially true for the TNFR1-signaling pathway that translates local T cell responses into inflammation, which remains enigmatic. Various models have been proposed to explain the profound defect in early phases of T cell-mediated inflammation in the absence of TNFR1. The data focus on impaired activation of nuclear factor \(\kappa B\)-dependent genes, leading to inappropriate apoptosis induction \(^13,16\) or defective T cell priming \(^17\). Importantly, while CD4 T cell-mediated control of bacterial infections and CD4 T cell-mediated
inflammation depend strictly on signaling through TNFR1, CD8 T cell-mediated control of viral infections with choriomeningitis virus is TNFR1-independent. Together these data suggest that signaling through TNFR1 is necessary for the translation of CD4 T cell-mediated immune responses into local inflammation and pathogen control, while this signaling pathway is obviously not required for killing of virally infected target cells by CD8 T cells. To uncover the role of TNFR1 during T cell-mediated inflammation, we strictly studied T cell- and TNF-dependent delayed type hypersensitivity reactions (DTHR) in response to the hapten trinitrochlorobenzene (TNCB).

Three major haptens are used to induce contact hypersensitivity reactions (CHSRs) to evaluate DTHRs. TNCB-induced CHSRs are among the best studied model diseases to investigate hapten-induced DTHRs as these reactions strictly depend on Th1 or Tc1 cells and can be attenuated either by interleukin-4 (IL-4) or by IL-4-producing Th2 cells. In these types of reactions, IL-4 initially promotes the activation of IL-12 producing antigen presenting cells (APCs), yet, during periods of either T cell priming or T cell effector functions, IL-4 attenuates TNCB-induced CHSR.

A second, frequently used hapten is Oxazolon. In contrast to DTHR induced by TNCB, IL-4 seems to play a role in the generation of DTHR induced by Oxazolon. A third frequently used hapten is dinitrofluorobenzene (DNFB). DNFB exerts the strongest toxic reactions. DNFB-specific CHSRs also differ from TNCB-induced CHSRs as they also induce IL-10-producing mast cells that are required to attenuate local toxicity. Moreover, DNFB-induced CHSRs induce NK cell-mediated memory responses. As TNCB-induced CHSRs thus share most of the mechanisms characterized for conventional DTHRs, we studied the role of TNFR1 in
response to TNCB, using the TNCB-induced CHSR as a model for the DTHR that provides the unique possibility to individually investigate antigen presentation, priming, and differentiation of CD4 and CD8 T cells, cell migration, T cell-mast cell interactions, and mast cell-endothelia interactions with the help of mast cell knock-in experiments. Importantly, it allows the study of these effects largely independently of phenomena such as antigen processing by APCs. Here, we show that TNFR1-signalling is neither needed for \textit{in vivo} priming nor for the introduction of Th1 cells capable of inducing severe DTHRs.

Dissecting the single steps of CHSRs from the APC, T cell and mast cell function to the local vascular endothelia, we discerned that TNFR1-expression exclusively by ECs is necessary for the translation of T cell-mediated immune responses into local inflammation. TNFR1-expression by EC was critically needed for the expression of adhesion molecules required for leukocyte rolling, adhesion, and migration in response to locally produced mast cell TNF.
Materials and Methods

Animals. TNFR1⁻/⁻ C57BL/6 mice were from Technische Universität Munich, Germany and TNF⁺/+ C57BL/6 mice were from Charles River Laboratories, while TNF⁻/⁻ 129/SvxC57BL/6 mice and TNF⁺/+ 129/SvxC57BL/6 mice, mast cell deficient Kit⁺⁺/Kit⁻⁻ mice, and congenic WBB6F1⁺/+ (Kit⁺⁺/Kit⁺⁺) mice were bred under specific pathogen-free conditions at the German Research Center for Environmental Health (GmbH) in Munich. Kit⁺⁺/Kit⁻⁻ mice were originally from The Jackson Laboratory. All mice were between 8- and 12- wks old. Animal experiments were approved by the Bavarian ministry and the Regierungspräsidium Tübingen (HT 1/03).

In vivo experiments. We sensitized TNFR1⁻/⁻ C57BL/6 mice, TNF⁻/⁻ 129/SvxC57BL/6 mice with 5% TNCB (80 µl of a 4:1 mixture of acetone/olive oil), or 0.5% DNFB (80 µl of a 4:1 mixture of acetone/olive oil), with the exception of experiments involving Kit⁺⁺/Kit⁻⁻ mice, where we used 2% TNCB (20 µl of a 4:1 mixture of acetone/olive oil) on both sides of one ear. This modified protocol is required as Kit⁺⁺/Kit⁻⁻ mice do not tolerate abdominal sensitization using higher concentrations of TNCB. Thus they were sensitized at the non-reconstituted ear. In order to establish equal conditions for all experiments, we always analyzed only the right ear. The negative control is always a non-sensitized mouse, challenged with the hapten at the right ear. Sensitization at the ear induced equivalent DTHR in Kit⁺⁺/Kit⁻⁻ and wild-type mice. One week after sensitization, we challenged mice with 1% TNCB (20 µl; 1:9 in acetone/olive oil) or 0.2% DNFB (20 µl of a 4:1 mixture of acetone/olive oil) on both sides of the previously untreated ear. Specific ear swelling was determined by measuring ear thickness with a micrometer (Oditest; Kroepelin, Germany) at the indicated time points before and after TNCB challenge. An irritant reaction, caused
by 1% TNCB in naïve animals, was used as the control. Kit<sup>W</sup>/Kit<sup>W-<i>v</i></sup> mice were locally reconstituted with mast cells (derived from wild-type and TNF<sup>-/-</sup> mice) by injecting intracutaneously 5x10<sup>5</sup> bone marrow-derived mast cells (BMMC), 5 wks before sensitization, exclusively into the ear selected for the elicitation of a DTHR. Similarly, TNFR1<sup>-/-</sup> and TNF<sup>-/-</sup> mice were locally reconstituted with BMMC derived from syngeneic TNF<sup>+/+</sup> mice by injecting intracutaneously 5x10<sup>5</sup> cultured BMMC, 5 wks before sensitization, exclusively into ear selected for elicitation of a DTHR. For adoptive T cell transfer experiments, 2x10<sup>4</sup> cultured Th1, Tc1, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells were locally injected exclusively into one ear of either naïve TNFR1<sup>+/+</sup> or TNFR1<sup>-/-</sup> C57BL/6 mice, 0.5 h prior to TNCB-challenge.

**Histology.** Hematoxylin and eosin staining were performed according to standard procedures.<sup>20</sup>

**Immunofluorescence staining and confocal microscopy.** Frozen sections were fixed with periodate-lysine-paraformaldehyde. Sections were blocked using donkey serum and then incubated with primary rat monoclonal antibodies (mAbs) kindly provided by Prof. Vestweber, Münster, Germany: anti-P-selectin RB40 mAb (dilution 1:40), anti-E-selectin 10E9 mAb (1:50), anti-ICAM-1 KAT-1 mAb (1:200), and anti-VCAM-1 6C7.1 mAb (1:50). For each primary antibody double staining was performed using goat anti Typ-IV-Collagen Ab (1:20, Biozol, Eching, Germany). Bound mAb was visualized using Cy3-donkey anti-rat Ab and Cy5-donkey anti-goat Ab (Dianova, Hamburg, Germany). For nuclear staining we used Yopro (1:2000 Invitrogen, Karlsruhe, Germany). Sections were analyzed using a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems, Wetzlar, Germany); original magnification: x630.
RT-PCR. Mouse ears were directly frozen in liquid nitrogen without or 4 h after TNCB challenge and homogenized in lysis buffer (RNeasy kit; Qiagen, Hilden, Germany) as well as mouse heart endothelial cells and brain endothelial cells. During RNA purification, genomic DNA was digested with RNase-free DNase (Qiagen). Two micrograms of total RNA were reverse transcribed (Omniscript RT-kit, Qiagen). For relative quantification by RT-PCR, 20 ng of each cDNA was analyzed in a LightCycler Real Time PCR System (Roche Diagnostics, Mannheim, Germany). For each primer pair, a standard curve was developed. Relative mRNA expression levels of E-selectin, P-selectin, ICAM-1 and VCAM-1 were normalized with the expression level of aldolase. The following primers were used: Aldolase (amplicon length 571 bp): 5’-AGCTGTCTTGACACATCGCTCACC; RP, 5’-
CACATACTGGGAGCGCTTCAAG; E-selectin (632 bp): 5’-GCTGTCCAGTGTGAAGCCTTATC; RP, 5’-GCAATGAGGACGATGTCAGGA;
P-selectin (300 bp): 5’-GGTTCAGGACAATGGACAGC; RP, 5’-
CTTTCTTAGCAGAAGCCAGGAGTG; ICAM-1 (463 bp): 5’-
GGAGACGACAGGACCTTAACAG; RP, 5’-CATCTCTGGCTTGGACAGACTTCACC;
VCAM-1 (320 bp): 5’-AGAGAAACCATTTATTGTTGACATCTCCC; RP, 5’-
CAAGTGGGCCACTCATTATAATTACTGG; TNFR1 (197 bp): 5’-
CAGTCTGCAAGGAGTGTGAA; RP, 5’-CACGCACCTGGAAGTGTTCT.

Protein analysis. MPO activity was determined in protein extracts from ear tissues and directly frozen in liquid nitrogen 24 h after TNCB challenge. Tissue was homogenized in extraction-buffer. MPO activity was expressed as units per gram.

Cell cultures. For adoptive transfer experiments, T cells were isolated from lymph nodes derived from either TNCB-sensitized or naive TNFR1+/+ or TNFR1-/- mice.
CD4⁺ and CD8⁺ T cells were purified by negative selection. Lymph node cells were pre-incubated with anti-CD4 ³⁷ or anti-CD8 mAbs ³⁸ and purified using a mouse T cell purification column (Mouse T,Celllect™, Alberta, USA). CD4⁺ and CD8⁺ T cells were cultured for 12-14 days with anti-IL4 mAb ³⁹ (10 μg/ml), 5 U/ml (after day 3 with 50 U/ml) IL-2, and hapten-modified T cell-depleted splenocytes (5x10⁵; APC) in a total volume of 200 µl medium in 96 well plates in a incubator (37°C, 7.5% CO₂). After 3 days T cells were cultured in 24 well plates and after another 5 days in 100 ml culture bottles; IL-2 (50 U/ml) was added every 3 days. Femoral TNF⁺/⁺ and TNF⁻/- bone marrow cells were cultured in the presence of 10-20 U/ml IL-3 ⁴⁰ and 200 ng/ml c-kit ligand ⁴¹. After 4 wks of culture, BMMC were determined to be >97% pure for mast cells and were used for adoptive transfer ⁴².

**Proliferation and cytokine assays.** For cytokine assays, T cells (1x10⁵) were cultured with either irradiated unmodified or hapten-modified APCs (5x10⁵) in 96-well plates in a total volume of 200 µl of medium. Supernatants were harvested for cytokine analysis after 48 h. IFN-γ was assayed by ELISA (PharMingen, Heidelberg, Germany). To analyze T cell proliferation, T cells isolated from draining lymph nodes derived from either TNFR1⁺/⁺ or TNFR1⁻/- mice (1.25x10⁵ or 2.5x10⁵ T cells) were stimulated with either irradiated unmodified or hapten-modified APC (5x10⁵) in a total volume of 200 µl of medium. After 72 h of culture in DMEM containing 10% FCS (PAA, Cölbe, Germany), 2-mercaptoethanol (Sigma, Steinheim, Germany) and 2 mM glutamine (Gibco, Eggenstein, Germany) at 37°C in a humidified atmosphere with 5% CO₂ cells were pulsed with [³H] thymidine for the final 6-8 h. For ELISpot analysis, we coated 96-well plates (Multiscreen-Millipore, Schwalbach, Germany) with rat anti-mouse-IFN-γ mAb (4 μg/ml). T cells (5.0 and 2.5 x10⁵ well⁻¹), and unmodified or hapten-modified irradiated APC (1x10⁵ well⁻¹) were cultured with 5
U/ml IL-2 for 48 h. Cytokine-producing T cells were stained with secondary mAb and the number of dots was analyzed with an ELISpot-reader (Biosys, Karben, Germany). As positive controls, cells stimulated with 10 mg/ml ConA were used.

**In vivo neutralization of adhesion molecules.**

Rat RB40.34 mAb \(^{43}\) (anti-P-selectin; BP Pharmingen; 50 μg), 40 μg rat YN1/1.7.4 mAb \(^{44}\) (anti-ICAM-1; Biozol, Eching, Germany), and 100 μg rat M/K-2 mAb \(^{44}\) (anti-VCAM-1; Chemicon, Asperg, Germany) or 100 μg rat IgG1 isotype control (BD Pharmingen, Heidelberg, Germany), and 40 μg rat IgG2b isotype control (Biozol, Eching, Germany) were injected i.v. 1.5-2.0 h after ear challenge. Rat UZ4 \(^{45}\) mAb (anti-E-Selectin; kindly provided by Prof. Hallmann, Münster, Germany; 200 μg) or 100 μg of rat IgM isotype control (BD Pharmingen, Heidelberg, Germany) were injected i.p., 1.0-1.5 h after ear challenge.

**Non-invasive intravital fluorescence microscopy.** Leukocyte rolling and adhesion dynamics during DTHR were monitored *in vivo* by video fluorescence microscopy. First, mice were anaesthetized with ether (Sigma Aldrich, Seelze, Germany) to cannulate the tail vein to administer rhodamine. Mice were anaesthetized by inhalation of isoflurane-O\(_2\) (1.8 %; Forene, Abbott GmbH, Wiesbaden, Germany) and fixed on a heating pad to maintain body temperature between 36° and 37°C. The hapten-treated mouse ear was used to study the behavior of leukocytes in the microcirculation. Ears were fixed with two peripheral sutures (7-0, Prolene; Ethicon GmbH; Norderstedt; Germany) on a black box. Leukocytes were stained *in vivo* by intravenous injection of 100 μl 0.02% Rhodamine-6G (Molecular Probes, Eugene, USA). Subsequently, skin venules were visualized 2.5 – 4.5 h after TNCB-challenge using a Zeiss Axiootech microscope (water immersion objective: 20X, W 20X/0.5;
Zeiss) with a 100 W HBO mercury lamp for epi-illumination. All images were videotaped and evaluated off-line, and single unbranched skin venules (15 - 60 μm diameter) were selected. The number of rolling and adherent leukocytes was determined offline during video playback analysis. Rolling leukocytes were defined as cells crossing an imaginary perpendicular line through the vessel at a velocity significantly lower than the centerline velocity. The number of adherent leukocytes was assessed by counting cells that did not move or detach from the endothelial surface within 20 s. Cells were quantified as the number of cells mm⁻², calculated from the diameter and the length of the vessel segment observed, assuming cylindrical geometry ⁴⁶.

**Statistical analysis.** Differences in ear swelling responses, myeloperoxidase concentration and relative mRNA expression were compared using a two-sample Student’s t test. Differences in leukocyte adhesion were analyzed by the Wilcoxon test. Data are represented by mean ±SEM, and p values below 0.05 were regarded as significant.
Results

A critical role for the TNFR1 in hapten-induced tissue damage

To address the role of TNFR1-signaling in DTHRs that are independent of infectious agents or antigen processing, we sensitized TNFR1−/− and TNFR1+/+ mice with DNFB and challenged mice seven days later with the hapten to elicit a DNFB-specific DTHR. Hapten-specific DTHRs are dependent on IFN-γ-producing T cells. In agreement with previous studies, we found that TNFR1−/− mice had rather increased ear swelling in response to DNFB when compared to TNFR1+/+ mice (Figure 1 A). This finding is surprising in view of the overwhelming number of data showing that TNFR1 is critical for the promotion of proinflammatory effects in DTHR in diseases leading to EAE, autoimmune diabetes, or arthritis. Therefore, we compared this DTHR in response to DNFB to the more widely used DTHR in response to TNCB, a hapten that also causes CHSRs that are dependent either on polarized Th1 cells or polarized Tc1 cells. In sharp contrast to DNFB-induced DTHR, TNCB-specific DTHRs were significantly diminished in TNFR1−/− mice when compared to TNFR1+/+ mice (Figure 1B). Both haptens, TNCB or DNFB, induced T cell-dependent CHSR that are dependent on IFN-γ-producing CD4+ Th1 and CD8+ Tc1 cells. To analyze why DNFB induced increased ear-swelling, whereas TNCB induced diminished ear swelling in TNFR1−/− mice, we determined tissue damage in TNFR1−/− and TNFR1+/+ mice by histopathology. In TNFR1+/+ mice, topical application of 0.5% DNFB to the skin induced severe necrosis, expanding into the mid-dermis (Figure 1C, upper right). In sharp contrast, little or no tissue necrosis occurred in TNFR1−/− mice exposed to 0.5% DNFB (Figure 1C, lower right). As a consequence, increased ear swelling in TNFR1−/− mice after DNFB application resulted from the reduced tissue damage that allowed the development of edema. This is in line with data showing that TNFR1 promotes cell death in mouse keratinocytes. In contrast...
to 0.5% DNFB, 5.0% TNCB induced very little cell death, but it did induce significant edema, a concomitant increase in dermal and epidermal thickness and a strong inflammatory infiltrate (Figure 1C, upper left). In TNFR1⁻/⁻ mice, all three TNCB-induced parameters of tissue damage, edema, infiltrate and keratinocyte apoptosis were significantly reduced (Figure 1C, lower left).

**Reduced TNCB-induced DTHR in TNFR1⁻/⁻ mice**

We next analyzed the TNCB-specific DTHR to investigate the mechanisms underlying the phenomenon of strongly reduced DTHR in TNFR1⁻/⁻ mice. We first examined the dynamics of ear swelling in TNFR1⁻/⁻ and TNFR1⁺/⁺ mice. TNFR1⁻/⁻ mice developed significantly reduced TNCB-specific DTHR at 12, 24, 48, and 72 h when compared to TNFR1⁺/⁺ litter mates (Figure 2A). To uncover the central mode of action, we compared hematoxylin and eosin stained sections of ear tissue from TNFR1⁻/⁻ to those from TNFR1⁺/⁺ mice. In TNFR1⁺/⁺ mice, TNCB-induced DTHRs resulted in strong edema, keratinocyte apoptosis, and abundant polymorphonuclear leukocyte (PMN) infiltrates with dermal and epidermal microabscesses. In sharp contrast, PMN infiltrates, keratinocyte apoptosis, and edema were virtually absent from TNFR1⁻/⁻ mice (Figure 2B). Quantification of myeloperoxidase (MPO) activity in ear tissues confirmed that, in TNFR1⁻/⁻ mice, PMN recruitment was reduced to <35% of TNFR1⁺/⁺ litter mice (Figure 2C). In contrast to the significant reduction in PMN, numbers of CD3⁺ cells was similar in TNFR1⁻/⁻ and TNFR1⁺/⁺ mice (Suppl. Figure 1A) and the number of CD11c⁺ dendritic cells increased in TNFR1⁻/⁻ mice. TNFR1⁻/⁻ dendritic cells are largely apoptosis resistant⁵¹ (Suppl. Figure 1B).
TNF^+/+ mast cells reconstitute DTHR in TNF^-/- mice, but not in TNFR1^-/- mice

Mast cells secrete both TNF and the chemoattractant macrophage inflammatory protein 2 (MIP-2). Both are critically needed for PMN recruitment and DTHR development. Neither T cells, macrophages, nor keratinocytes are capable of substituting for mast cells during this T cell-mediated DTHR in mast cell-deficient Kit^W/Kit^W-v mice. To formally prove the need for mast cell TNF for PMN recruitment, we first substituted mast cell deficient Kit^W/Kit^W-v mice with either TNF^+/+ or TNF^-/- mast cells and quantified PMN recruitment during DTHR by determining MPO activity in ear tissues. As such, TNF^+/+ and TNF^-/- mast cells repopulate the ear tissue of mice with similar density. In line with previous data, Kit^W/Kit^W-v mice reconstituted with TNF^+/+ mast cells developed strong DTHR and recruited large numbers of PMN, while mice substituted with TNF^-/- mast cells failed to develop DTHR. As a consequence, MPO activity remained at background levels in mice reconstituted with TNF^-/- mast cells (Figure 3).

In TNFR1^-/- mice, DTHRs were reduced to the same extent as in mast cell-deficient Kit^W/Kit^W-v or TNF^-/- mice (Figure 4A and B). This finding is most easily understood in a model where TNFR1-expression is needed downstream of the T cell–mast cell cascade and is responsible for the induction of adhesion molecules that are required for PMN recruitment.

To experimentally address this point, we reconstituted TNF^-/- and TNFR1^-/- mice with mast cells from normal C57Bl/6 mice. In the absence of mast cell reconstitution, neither TNF^-/- nor TNFR1^-/- mice developed significant DTHRs. Following mast cell reconstitution, TNF^-/- mice developed strong TNCB-specific DTHR, equivalent to DTHRs in wild-type mice. In sharp contrast, mast cell reconstitution did not restore
DTHRs in TNFR1−/− mice (Figure 4A and B) even though 0.5x10^6 directly (intracutaneously) into the right ear of naïve TNFR1−/− mice transplanted mast cells engrafted selectively to the site of injection (Figure 4C, Suppl. Figure 2). Together, these data demonstrate that TNFR1 expression by resident cells is needed for the development of strong, T cell-mediated inflammation in response to mast cell TNF.

**TNFR1 expression by endothelia is needed for appropriate expression of adhesion molecules**

Endothelia of different origin express variable amounts of TNFR1 mRNA. TNFR1 mRNA is also expressed in normal ear tissue (Figure 5A). P-selectin and E-selectin are the first molecules expressed during DTHRs, followed by VCAM-1 and ICAM-1. The two selectins responsible for leukocyte rolling show peak expression after 2 to 4 h, while the two major adhesion molecules responsible for leukocyte recruitment are strongly expressed starting at 3-4 h of DTHR, with expression increasing over time. As TNF is capable of inducing all four adhesion molecules on EC and as TNF is essential for PMN-recruitment during TNCB-induced CHSR, TNFR1 expression by endothelia might provide the critical link that establishes cross-talk between somatic cells and the immune system in DTHRs.

To directly address this point we first analyzed the mRNA expression of P-selectin, E-selectin, VCAM1, and ICAM1 in TNFR1−/−, TNFR1+/−, and TNF−/− (data not shown) mice. TNCB-induced DTHRs in TNFR1−/− mice resulted in significantly lower mRNA expression of P-selectin (p=0.0037), ICAM-1 (p=0.0039), or VCAM-1 (p=0.018) (Figure 5B). E-selectin showed a clear tendency toward reduced mRNA expression at 4 h (Figure 5B) after elicitation of DTHR. Importantly, mRNA expression of P-selectin, E-selectin, VCAM-1, and ICAM-1 was reduced to the same degree in TNF−/−
mice as it was TNFR1\(^{-/-}\) mice directly supporting the central role of TNF and TNFR1 in the expression of these adhesion molecules (4 h after challenge, data not shown). Equivalent findings for TNF\(^{-/-}\) mice were reported by Nakae et al. \(^{64}\). Immunofluorescence staining of P-selectin, E-selectin, ICAM-1, and VCAM-1 expression 2.5 h and 4.0 h after TNCB-ear challenge in TNFR1\(^{+/+}\) and TNFR1\(^{-/-}\) mice (Figure 5C) and western blot analysis (Suppl. Figure 3) confirmed strongly reduced expression of all four adhesion molecules in TNFR1\(^{-/-}\) mice (Figure 5C). Differences in protein expression were highest for P-selectin, E-selectin, and ICAM-1 (Figure 5C). Immunohistology underlined that only endothelial cells express E-selectin; moreover, endothelial cells and platelets are the only cells capable of producing P-selectin \(^{58,62}\). Thus, our data can only be explained by a direct response of TNFR1-expressing endothelial cells to mast cell-derived TNF.

**Impaired leukocyte adhesion to TNFR1\(^{+/+}\) endothelia in vivo**

To formally prove that TNFR1-expressing EC are needed to induce functionally active P-selectin, E-selectin, ICAM-1 or VCAM-1 protein during DTHR, we analyzed leukocyte rolling and leukocyte adhesion in TNFR1\(^{-/-}\) mice and TNFR1\(^{+/+}\) mice using intravital fluorescence microscopy \(^{46}\). At 2.5 h, median P- and E-selectin-dependent leukocyte rolling was determined to be at 475±112 (±SEM) cells mm\(^{-1}\)min\(^{-1}\) in TNFR1\(^{+/+}\) mice, but only 210±91 cells/mm\(^{-1}\)min\(^{-1}\) in TNFR1\(^{-/-}\) mice, which was close to background (114±65 cells mm\(^{-1}\)min\(^{-1}\)). At 3.5 h, this difference in rolling was lost (TNFR1\(^{+/+}\) mice: 384±73 cells mm\(^{-1}\)min\(^{-1}\); TNFR1\(^{-/-}\) mice: 417±92 cells mm\(^{-1}\)min\(^{-1}\)). While rolling differed only initially, TNFR1\(^{-/-}\) mice had a persistent defect in firm leukocyte adhesion. Firm leukocyte adhesion is a prerequisite for extravasation of PMN and T cells into the skin. Firm adhesion requires ICAM-1 and VCAM-1 interactions with lymphocyte function associated antigen-1 (LFA-1, \(\alpha_1\beta_2\) integrin) and
very late antigen-4 (VLA-4, $\alpha 4\beta 1$ integrin)\textsuperscript{60,65,66}. At 3.5 h and 4.5 h, when leukocytes had already infiltrated the connective tissue in TNFR1$^{+/+}$ mice, adherent leukocytes were still strongly reduced to levels $\leq 12\%$ in TNFR1$^{-/-}$ mice ($p=0.015$) (Figure 5D and E). Differential blood counts in TNFR1$^{-/-}$ mice and TNFR1$^{+/+}$ mice confirmed a similar number of lymphocytes and PMNs in both strains (data not shown).

To definitely address the role of TNFR1-dependent expression of the adhesion molecules in TNCB-induced DTHR, we injected E-selectin, P-selectin, ICAM-1, and VCAM-1 blocking mAbs or corresponding isotype controls into TNFR1$^{+/+}$ mice, 0.5-1.5 hours after challenge. Intravital fluorescence microscopy at 2.5 and 3.5 h after the challenge demonstrated a nearly complete inhibition of leukocyte rolling when all four adhesion molecules were blocked; similar results were obtained by blocking P- and E-selectin only (Figure 5F). In contrast, P- and E-selectin mAb had no effect on late adhesion. Only the combination of all four mAb severely inhibited firm adhesion at 3.5 h (Figure 5G); the difference was even significant when compared to mice injected with P- and E-selectin mAb only (Figure 5G).

**TNFR1 expression is neither needed for adequate T cell priming nor for induction of T cell effector functions**

Others have suggested that TNFR1$^{-/-}$ mice develop deficient DTHR because of impaired T cell priming due to defects in the functioning of T cells and dendritic cells\textsuperscript{17,67}. Therefore, we first determined the impact of TNFR1-expression on T cell priming and Th1 cell differentiation. We analyzed freshly isolated CD4$^+$ T cells from draining lymph nodes of TNCB-sensitized mice from both TNFR1$^{+/+}$ and TNFR1$^{-/-}$ mice and stimulated the cells for T cell proliferation and cytokine production using the Enzyme Linked Immuno Spot (ELISpot) technique\textsuperscript{68}. TNCB-specific CD4$^+$ T cells from either TNFR1$^{-/-}$ mice or TNFR1$^{+/+}$ mice proliferated equivalently in response to
TNCB-modified APCs (Figure 6A), and both groups of mice had equal numbers of IFN-γ producing TNCB-specific CD4+ T cells in draining lymph nodes (Figure 6B). This shows that TNFR1 expression was not needed for effective in vivo priming of CD4+ T cells as determined by their capacity to proliferate or to produce IFN-γ in response to specific stimulation by APCs. Yet, the signaling required for the induction of DTHR in vivo might be impaired.

Adequate activation of resident cells, namely mast cells by Th1 and Tc1 cells, is essential for the recruitment of inflammation amplifying cells from the circulation and for the manifestation of DTHRs. Thus, priming T cells for specific proliferation and IFN-γ production is a prerequisite for inducing DTHRs, but it is not sufficient. To test the role of TNFR1 during the in vivo imprinting of T cells for the induction of effector cells capable of inducing tissue inflammation, we isolated Th1 or Tc1 effector cells from sensitized TNFR1−/− or TNFR1+/+ mice, expanded these cells in vitro, and tested their capacity to elicit DTHR. To overcome the defective expression of adhesion molecules in TNFR1−/− mice, we adoptively transferred the T cell populations directly into the ears of either TNFR1−/− or TNFR1+/+ mice. Prior to injection, TNCB-specific Th1 or Tc1 cells proliferated equivalently and produced similar amounts of IFN-γ in response to hapten-modified APC, irrespective of whether they were derived from TNFR1+/+ or TNFR1−/− mice (Figure 7A-D). Thirty minutes after transfer of these Th1 or Tc1 cells into the ears of either naïve TNFR1−/− or naïve TNFR1+/+ mice, we challenged their ear skin with TNCB. Following transfer, T cells from either TNFR1−/− or TNFR1+/+ mice caused significant a DTHR in TNFR1+/+ mice. Th1 cells induced stronger DTHRs than Tc1 cells, independently of whether or not the T cells expressed TNFR1 (Figure 7E and F). Thus, TNFR1-expression was not required, neither for in vivo priming of T cells for Th1 development nor for the differentiation of...
naïve T cells into a functionally, fully efficient Th1 or Tc1 effector phenotype. In sharp contrast, when transferred into the ears of TNFR1^{−/−} mice, none of the Th1 or Tc1 cell lines, whether derived from TNFR1^{−/−} or TNFR1^{+/+} mice, elicited TNCB specific effector functions, further underlining that establishment of local inflammation needed TNFR1 expression exclusively by resident cells (Figure 7E and F).
Discussion

Using knock in-experiments with selective reconstitution of either distinct T cell populations (Th1, Tc1) or distinct mast cell populations into either control, TNFR1⁻/⁻, TNF⁻/⁻, or KitW+/KitW⁻⁻ mice, we previously found that differentiation of IFN-γ-producing Th1 or Tc1 cells was normal in TNFR1⁻/⁻ mice. Th1 or Tc1 cells from TNFR1⁻/⁻ mice were also fully capable of inducing DTHR. Yet, TNFR1⁻/⁻ mice had a profound defect in developing Th1- or Tc1-mediated DTHR, as reported for other disease models of T cell-dependent DTHR 6,10-13. Dissecting the single steps of DTHR starting with in vivo T cell priming through T cell differentiation and T cell effector functions, we localized the defect downstream of the T cell-mast cell interaction. Importantly, TNF-producing mast cells were unable to translate T cell responses into DTHRs in TNFR1⁻/⁻ mice exclusively. This defect was associated with defective PMN recruitment and reduced expression of the four critical adhesion molecules at the site of inflammation. In contrast to the other adhesion molecules analyzed, P-selectin and E-selectin are highly specific for endothelia 58. We propose that P-selectin and E-selectin expression resulted from endothelial cells, as the only other cells capable of expressing P-selectin mRNA or protein are platelets 58. This was confirmed by immunohistology. To directly determine whether TNFR1⁻/⁻ endothelia were capable of inducing functional adhesion molecule expression, we performed intravital fluorescence microscopy. Knock-out experiments have shown that only the combined loss of E-selectin and P-selectin impairs hapten-induced T cell-mediated skin inflammation 69. In line with this, we found strongly impaired leukocyte rolling and adhesion in TNFR1⁻/⁻ mice directly underlining that E-selectin and P-selectin were not only reduced at the mRNA-level, but were also functionally ineffective. Thus, direct crosstalk between TNF produced by mast cells in response to locally activated Th1...
cells and TNFR1, expressed by endothelia, ultimately controlled PMN recruitment into the site of inflammation.

These findings help to explain various biologically important questions which arose during the characterization of TNFR1^−/− mice and the introduction of anti-TNF mAb or soluble TNF receptors into therapy for autoimmune disease in humans.

Elimination of lymphocytic choriomeningitis virus (LCMV)-infected cells, which strictly depends on the functioning of CD8 T cells, is completely unaffected in TNFR1^−/− mice. While others have suggested that T cell priming would be impaired in TNFR1^−/− mice, the analysis of CD8 T cells in LCMV infection and the analysis of either CD4 or CD8 T cells in adoptive T cell transfer clearly show that T cell priming and T cell effector function develop normally in TNFR1^−/− mice. While TNFR1-signalling does not seem to be needed for these direct T cell-mediated immune functions, TNFR1-signalling is obviously crucial for the development of DTHR effector functions where, in addition to T cells, macrophages or PMN are involved. Thus, TNFR1^−/− mice infected with intracellular bacteria such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* are highly susceptible and cannot cope with these infections. Similarly, the acute phases of collagen induced arthritis or EAE are attenuated in TNFR1^−/− mice. Importantly, comparing disease development in TNFR1^−/− and TNFR2^−/− mice demonstrates that efficient DTHR in infectious and autoimmune diseases depend on TNFR1, as TNFR2-deficient mice show a normal phenotype in these models of human disease.

Here, we have shown that T cell-mediated inflammation strictly requires a direct crosstalk between mast cell-TNF and TNFR1-expressing endothelia, as TNFR1-
signaling by endothelial cells is essential for PMN recruitment into sites of inflammation. This seems to be of great relevance for understanding the mode of action of anti-TNF therapy in humans. Blocking TNF-action in humans with either anti-TNF mAbs or TNFR fusion proteins is highly effective in diseases that critically involve PMN recruitment. In consequence, blocking TNF aggravates the course of sepsis \(^\text{71}\), but improves PMN-associated autoinflammatory diseases like rheumatoid arthritis \(^\text{2,72-74}\), psoriasis, psoriasis arthritis \(^\text{2,74-77}\), or inflammatory bowel disease \(^\text{2,78}\). In sharp contrast, no consistent effects have been reported for PMN-independent inflammation, such as chronic phases of multiple sclerosis \(^\text{2,74}\). This central role of TNF in PMN recruitment is underlined by reports showing that concomitant infections with bacteria or mycobacteria may be severely aggravated and difficult to treat in patients treated with TNF-blocking agents \(^\text{2,72-78}\), while defense against viral diseases seems to be largely unaffected \(^\text{2,72-78}\).

The critical role for TNF-mediated PMN recruitment in host protection against bacteria was originally unexpected and first observed in humans that received TNF-blockade as a treatment for sepsis \(^\text{71}\). In this study, TNFR fusion protein was given to reduce fever and TNF-induced symptoms of septic shock in the hope of reducing morbidity and lethality. TNFR fusion protein, however significantly increased the lethality of septic shock \(^\text{71}\). The critical role of TNF-mediated PMN recruitment for the control of sepsis was also shown in mice with either bacterial pneumonitis or septic peritonitis. In the absence of TNF the mice failed to recruit PMN, failed to control expansion of the bacteria and succumbed to these bacterial infections \(^\text{4,79}\). In sharp contrast, viral diseases seem to be well controlled in anti-TNF treated humans \(^\text{2,72-78}\). These data showed that TNF induces many symptoms of sepsis, like fever or hypotonia, but is essential for efficient control of bacterial expansion in patients.
Even though previous data have independently shown that TNF and TNFR1-signaling are essential for establishing DTHR that involve PMN and macrophages, the underlying biological mechanism remained enigmatic. Most authors on the subject have speculated that TNFR1-signaling may either be involved in T cell priming or macrophage functioning. Here, we show that the priming of effector T cells capable of inducing DTHR is TNFR1-independent and that the central TNFR1-expressing cell required for PMN-extravasation and development of DTHR is the EC. Thus, TNFR1-expressing EC establish the molecular cross-talk between the intravascular leukocytes and TNF-producing mast cells. As TNFR1-expressing EC respond to mast cell-TNF by expressing adhesion molecules required for leukocyte rolling and firm adhesion, TNFR1-signalling is responsible for the critical step that initiates local inflammation in protective and harmful T cell-mediated immune responses. As such, the data provided here not only unravel the long sought after molecular basis for understanding the well described defects of TNFR1−/− mice in developing efficient DTHR, but they also propose a concept to explain the risks and opportunities for anti-TNF therapy in humans.

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**Authorship**

and B.J.P performed the experiments. M.K., R.M., L.H., T.S., K.F., M.S., D.B., S.M.,
M.K., T.B., and M.R. wrote the manuscript. All authors checked for the final version of
the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interest.

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Phone (+49) 70712986870, Fax: (+49) 7071294405
References

Figure Legends

Figure 1.

**DNFB induces strong tissue necrosis in TNFR1**+/+** mice.**

(A) DNFB-sensitized and challenged TNFR1**+/+** mice developed reduced ear swelling responses 24 hours after ear challenge compared to TNFR1**−/−** mice (n = 5-7). (B) TNCB –sensitized and challenged TNFR1**−/−** mice developed reduced ear swelling responses 24 hours after ear challenge compared to TNFR1**+/+** mice (n = 16-27). (C) Enhanced tissue necrosis in TNFR1**+/+** mice after DNFB-application. Hematoxylin and eosin-stained abdominal skin sections from TNCB- (upper left) or DNFB (upper right) treated TNFR1**+/+** and from TNCB (lower left) or DNFB (lower right) -treated TNFR1**−/−** mice at day 5 after hapten-application on the abdomen (n = 3-5).

Figure 2.

**Impaired TNCB-specific DTHR in TNFR1**+/−** mice.**

(A) TNCB-sensitized and naïve TNFR1**+/−** (gray bars) and TNFR1**+/+** (black bars) mice were challenged with TNCB. Ear thickness was measured before and at the indicated time points after TNCB-challenge. Differences in ear thickness between TNFR1**+/−** (gray bars) and TNFR1**+/+** (black bars) were significant (p < 0.05) 24 h, 48 h, and 72 h after ear challenge; (24 h: n = 16-27; 48 h and 72 h: n = 6-7). (B) Reduced PMN infiltrates, tissue necrosis, and edema in ear tissue from TNFR1**+/−** mice 24 h after TNCB challenge. Hematoxylin and eosin-stained ear sections from TNFR1**+/+** (left: top = overview, bottom = detail) and TNFR1**+/−** mice (right: top = overview, bottom = detail); (n = 13-15). (C) PMN-recruitment is TNFR1-dependent. MPO activity in protein extracts from ear tissue from TNFR1**+/−** and TNFR1**+/+** mice (n = 3).
Figure 3.

Only TNF$^{+/+}$ mast cells reconstitute PMN-recruitment in Kit$^{W/Kit^{W-v}}$ ears.

MPO activity in protein extracts of ear tissue from Kit$^{W/Kit^{W-v}}$ mice reconstituted with either TNF$^{-/-}$ (gray bars) or TNF$^{+/+}$ (black bars) mast cells. Ear tissue was harvested 24 h after TNCB challenge from sensitized and naive Kit$^{W/Kit^{W-v}}$ mice (n= 2).

Figure 4.

TNF$^{+/+}$ mast cells reconstitute DTHR in TNF$^{-/-}$ ears, but not in TNFR1$^{-/-}$ ears.

(A) Representative visualization of PMN infiltrates, tissue necrosis, and edema. Ear sections from TNF$^{-/-}$ (upper left), TNFR1$^{-/-}$ (upper right), mast cell-reconstituted TNF$^{-/-}$ mice (lower left) and TNFR1$^{-/-}$ mice (lower right) were fixed 24 h after ear challenge and then stained with hematoxylin and eosin. (B) Hapten-specific DTHR in C57BL/6 wild-type, TNF$^{-/-}$, TNFR1$^{-/-}$, mast cell-reconstituted TNF$^{-/-}$, or mast cell-reconstituted TNFR1$^{-/-}$ mice. Ear swelling was measured 24 h after TNCB challenge (n = 4-6).

(C) Fluorescence microscopy of intracutaneously engrafted Cy5 (left) and RFP (right) stained mast cells in ears of TNFR1$^{-/-}$ mice 5 days after mast cell engraftment (top = overview, bottom = detail).

Figure 5.

Altered adhesion molecule expression and reduced leukocyte adhesion in TNFR1$^{-/-}$ mice.

(A) mRNA expression of TNFR1 in mouse heart endothelial cells, brain endothelial cells, and mouse ear (data were normalized to aldolase expression; relative mRNA expression was calculated by dividing TNFR1 mRNA expression of TNFR1$^{+/+}$ mice, mouse heart endothelial cells, and brain endothelial cells though the background TNFR1 mRNA in TNFR1$^{-/-}$ mice, corresponding to the detection limit, set as 1).
(B) mRNA expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 in TNFR1+/+ mice and TNFR1−/− mice, 4 h after elicitation of DTHR (data was normalized to aldolase expression). Data are given in a decade log scale (n = 9-10). (C) Immunofluorescence staining of P-selectin, E-selectin, ICAM-1, and VCAM-1 (red) 2.5 h and 4.0 h after TNCB challenge in TNFR1+/+ and TNFR1−/− mice (green: nuclei; blue: type IV collagen; n = 3). (D) Leukocyte adhesion to vascular endothelia in TNFR1+/+ and TNFR1−/− mice 3.5 h after elicitation of DTHR as determined by intravital fluorescent microscopy (n = 6-7). (E) Non-invasive intravital microscopy images of leukocyte adhesion to vascular endothelia in TNFR1+/+ and TNFR1−/− mice, 4.5 h after elicitation of DTHR (supplementary video 1 and 2). (F) Leukocyte rolling and (G) firm adhesion of rhodamin-stained leukocytes to vascular endothelia in TNFR1+/+ mice after application of P-selectin, E-selectin, ICAM-1, and VCAM-1 blocking Abs or isotype control 2.5 and 3.5 h after elicitation of DTHR. Analysis was performed by intravital fluorescent microscopy (n = 5-7).

Figure 6.

TNFR1-expression is not needed for T cell priming in vivo.

(A) Proliferation of 2.5x10^5 (gray and black bars) or 1.25x10^5 (white and striped bars) CD4+ T cells from either naive TNFR1+/+ or TNFR1−/− mice, or primed TNFR1+/+ or TNFR1−/− mice were stimulated for 72 h with 5x10^5 of either unmodified (gray and white bars) or TNBS-modified (black and striped bars) APC; [³H] thymidine was added for the final 6 h. (B) Frequency of IFN-γ producing TNCB-specific CD4+ T cells; 5x10^5 (black bars) or 2.5x10^5 (gray bars) T cells from either naive or sensitized TNFR1+/+ or TNFR1−/− mice were stimulated on anti-IFN-γ mAb coated 96 well plates with 5x10^5 either unmodified APC (striped bars, third column) or TNBS-modified APC (black and gray bars). After 48 h of incubation ELISPOT assay was developed.
Control T cells were stimulated with 10 μg/ml Con A and APC (white bars); Three independent experiments.

**Figure 7.**

TNFR1-expression is needed neither for T cell imprinting nor for T cell effector functions.

(A-D) Proliferation and IFN-γ production by TNFR1+/+ or TNFR1−/− Th1 and Tc1 cells. (A, B) TNFR1+/+ (black bars) or TNFR1−/− (grey bars) TNCB-specific T cells (10⁵) of the indicated origin were stimulated for 24 h with 5x10⁵ hapten-modified APC. [³H] thymidine was added for the final 6 h. (C, D) TNFR1+/+ (black bars) or TNFR1−/− (grey bars) T cells (10⁵) from the indicated origin were stimulated for 24 h with 5.0 x 10⁵ hapten-modified APC. Supernatants were harvested after 24 h and the IFN-γ content was determined by ELISA. (E, F) Efficient DTHR require TNFR1-expressing resident cells. Th1 or Tc1 cell lines that were either TNFR1−/− or TNFR1+/+ were transferred intracutaneously into ears of naïve TNFR1−/− or TNFR1+/+ mice, 0.5 h prior to challenge with TNCB (n= 3-7). Ear swelling was determined 24 h later.
Fig. 1

A

Sensitization

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B

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Ear swelling (μm)

C

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200 μm
**Fig. 2**

**A**

![Graph showing ear thickness over time](image)

- Ear thickness (μm) over time (hours)
- Strain Sensitization:
  - TNFR1^+/^ +
  - TNFR1^−/− +
  - TNFR1^+/^ −
  - TNFR1^−/− −

- *p < 0.05

**B**

- TNFR1^+/^ vs. TNFR1^−/−
  - Images of tissue sections with magnification markers (200 μm)

**C**

- Graph showing MPO (U/g) levels:
  - p = 0.027

- Comparison of MPO levels for TNFR1^+/^ Control, TNFR1^+/^ Sens., TNFR1^−/− Control, and TNFR1^−/− Sens.
Figure 3

The figure shows a bar graph comparing MPO (U/g) levels in Kit^W/Kit^W-/- mice under different sensitization and reconstitution conditions.

- **Sensitization**:
  - TNF^-/-
  - TNF^+/+
  - TNF^+/+

- **Mast cell Reconstitution**:
  - TNF^-/-
  - TNF^+/+

The graph indicates a significant increase in MPO levels in Kit^W/Kit^W-/- mice following sensitization and mast cell reconstitution, compared to other conditions.

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A

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Fig. 5

A

log (relative mRNA expression)

Heart endothelial cells
Brain endothelial cells
Ear tissue C57BL/6 mice

B

p = 0.0037

p = 0.0039

p = 0.018

not significant

P-Selectin
ICAM-1
VCAM-1
E-Selectin

log (mRNA)

TNFR1−/− TNFR1+/+
TNFR1−/− TNFR1+/+
TNFR1−/− TNFR1+/+
TNFR1−/− TNFR1+/+
Fig. 5

F

Leukocyte Rolling mm\(^2\)

- **2.5h**
  - Isotype Abs
  - anti-E-Selectin
  - anti-P-Selectin
  - anti-ICAM-1
  - anti-VCAM-1

- **3.5h**
  - Isotype Abs
  - anti-E-Selectin
  - anti-P-Selectin
  - anti-ICAM-1
  - anti-VCAM-1

G

Leukocyte Adhesion mm\(^2\)

- **3.5h**
  - anti-E-Selectin
  - anti-P-Selectin
  - anti-ICAM-1
  - anti-VCAM-1

- **p = 0.028**
Direct crosstalk between mast cell-TNF and TNFR1-expressing endothelia mediates local tissue inflammation

Manfred Kneilling, Reinhard Mailhammer, Lothar Hultner, Tanja Schonberger, Kerstin Fuchs, Martin Schaller, Daniel Bukala, Steffen Massberg, Christian A. Sander, Heidi Braumuller, Martin Eichner, Konrad L. Maier, Rupert Hallmann, Bernd J. Pichler, Roland Haubner, Meinrad Gawaz, Klaus Pfeffer, Tilo Biedermann and Martin Rocken