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Retinal degeneration in experimental coronavirus retinopathy (ECOR) is associated with increased TNF- α , soluble TNFR2 and altered TNF- α signaling

Laura C. Hooper^a, Marian S. Chin^a, Barbara Detrick^b, John J. Hooks^{a,*}

^aLaboratory of Immunology, Immunology and Virology Section, National Eye Institute, National Institutes of Health, NIH Bldg 10, Rm 6N228, 10 Center Drive, Bethesda, MD 20892, USA

^bDepartment of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, USA

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Abstract

Experimental coronavirus retinopathy (ECOR) is a virally triggered model of retinal degeneration composed of both genetic and autoimmune components. Since TNF- α plays a role in immune-mediated processes we evaluated the levels of TNF- α /TNF- α receptors and the downstream signaling molecule nitric oxide (NO) during disease in both retinal degeneration susceptible BALB/c and degeneration resistant CD-1 mice. Following coronavirus injection, TNF- α mRNA was detected at higher levels within the retinas, and concentrations of TNF- α ($p < 0.005$) and sTNFR1 ($p < 0.0005$) proteins were increased within the sera of BALB/c but not CD-1 mice. While concentrations of sTNFR2 proteins were elevated in both BALB/c ($p < 0.00005$) and CD-1 ($p < 0.005$) mice compared to controls, concentrations were higher in BALB/c mice ($p < 0.0005$). Gene expression of iNOS while initially high in BALB/c mice decreased during the acute phase of infection, while it increased in CD-1 mice. These trends are attributable to differences in monocyte TNFR2 release ($p < 0.0005$) between the strains since sTNFR2 decreased ($p < 0.01$) levels of NO production. These studies demonstrate that retinal degeneration following viral infection is associated with increased release of TNF- α /TNF receptors combined with a down-regulation of NO. Furthermore they suggest that these molecules are involved in alterations in immune response leading to autoimmune reactivity.

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Keywords: Viral; Cytokine receptors; Cytokines; Inflammation

1. Introduction

An animal model of retinal degeneration, referred to as experimental coronavirus retinopathy (ECOR), was established using the coronavirus, mouse hepatitis virus (MHV), to examine genetic and host immune responses that may contribute to retinal disease (Robbins et al., 1990). Coronaviruses are large enveloped positive strand RNA viruses that cause prevalent disease in both humans and animals and have recently been determined to be the cause of SARS. Inoculation of retinal degeneration susceptible

mice with MHV, strain JHM, by both direct (intravitreal, corneal scarification) and indirect (intracerebral) routes leads to retinal pathology (Robbins et al., 1991). In ECOR, mice are injected intravitreally, which produces a biphasic disease in degeneration susceptible BALB/c mice with inflammation in the early phase and retinal degeneration in the late phase (Wang et al., 1996). The early phase is characterized by the presence of infectious virus within the retina and retinal pigment epithelium (RPE), by infiltration of immune cells and release of pro-inflammatory mediators. This is followed by clearance of infectious virus to undetectable levels by day 8 post-infection (P.I.). The late phase is associated with a progressive loss of photoreceptors and ganglion cells, general thinning of the neuroretina and the production of retinal and RPE cell autoantibodies in the

* Corresponding author. Tel.: +1 301 496 6578; fax: +1 301 480 2988.

E-mail address: hooksj@nei.nih.gov (J.J. Hooks).

absence of infectious virus. In degeneration resistant CD-1 mice, only the early inflammatory phase is seen with viral infectivity titers and anti-viral antibody titers similar to those of degeneration susceptible BALB/c mice. Similar increases in viral and anti-viral titers between retinal degeneration susceptible and resistant mice as well as production of autoantibodies only in degeneration susceptible mice (Hooks et al., 1993) indicates that loss of retinal integrity is not due to viral amplification alone but also may be mediated in part by altered immune responses. We are now systematically evaluating host responses to the infection by studying cytokine, chemokine and adhesion molecule profiles. Recently we identified that IFN- γ is critically important in controlling virus clearance within the retina. Nevertheless, IFN- γ gene expression and production was similar in both resistant and susceptible mouse strains (Hooks et al., 2003). Since TNF- α can play a prominent role in immune mediated disease, we evaluated this cytokine in ECOR.

TNF- α is a pleiotropic cytokine that exerts its effects through binding with its receptors TNF receptor 1 (TNFR1, p55) and TNF receptor 2 (TNFR2, p75) on the cellular surface. Functions attributed to TNF- α signaling include both proinflammatory, such as cell proliferation of T and B cells (Pape et al., 1997), as well as anti-inflammatory processes, such as attenuation of T cell receptor signaling (Cope et al., 1997) and deletion of CD8⁺ T cells by interaction with both TNFR1 (Speiser et al., 1996) and TNFR2 (Zheng et al., 1995). Several studies have implicated TNF- α in autoimmune diseases. In insulin-dependent diabetes mellitus (IDDM), signaling of TNF- α via TNFR1 was shown by one group to contribute to β cell toxicity (Kagi et al., 1999) while another showed that administration of TNF- α delayed the onset and decreased the severity of IDDM (Jacob et al., 1990). In rheumatoid arthritis (RA), drugs that inhibit TNF- α are routinely given to reduce symptoms (Cope et al., 1994). In a multiple sclerosis (MS) model it was shown that TNF- α decreased disease severity in myelin oligodendrocyte glycoprotein (MOG)-induced demyelination (Liu et al., 1998) whereas systemic lupus erythematosus (SLE) may result from unregulated expression of the TNF- α homologue zTNF4 (Blys, BAFF, TALL-1, THANK) (Gross et al., 2000). Although the action of TNF- α seems contradictory, many of these studies do not consider the concentrations of TNF receptors present during disease. This may be an important variable since TNF receptors have been shown to interfere with the function of TNF- α , as well as prolong its activity.

In the present study we measured the levels of TNF- α and its receptors during the course of a viral infection and whether these levels correlated with the development of retinal degeneration. In addition, we wanted to determine the source of TNF- α /TNF receptors and whether their concentrations could alter the ability to produce downstream signaling molecules that may be important in inflammatory responses such as nitric oxide (NO).

2. Materials and methods

2.1. Animals

Male BALB/c (Harlan Sprague Dawley, Indianapolis, IN) and CD-1 (Charles River, Raleigh, NC) mice (8–13 weeks old, 25–30 g) were used. All experimental procedures conformed to the Association for Research in Vision and Ophthalmology (ARVO) resolution for the use of animals in ophthalmic and vision research.

2.2. Virus inoculations

Mouse hepatitis virus (MHV), strain JHM, was obtained from the American Type Tissue Collection (Manassas, VA) and was propagated in mouse 17CL1, 3T3 or mouse L2 cells. Viral titers were determined by plaque assay on mouse L2 cells. Eyes were injected intravitreally with 5 μ l of either 1.35×10^6 PFU/ml of MHV (virus-infected) or with media alone (mock-infected).

2.3. Plaque assays

Mouse L2 cells (a gift from Kathryn Holmes, University of Colorado, Denver) were seeded at a concentration of 1×10^5 cells/well in a 24-well plate. When the cells became confluent, medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non essential amino acids (NEAA) and antibiotic/antimycotic, (Invitrogen, Grand Island, NY) was replaced with 1ml of medium containing TNF- α (R&D Systems, Minneapolis, MN), IFN- α or IFN- γ (Biosource International, Camarillo, CA). After 24 h, medium was removed and MHV (40 PFU/well) was added. After a 2 h incubation, the inoculum was removed and cells were washed with PBS and refed with cytokine free medium containing 0.75% methylcellulose. After 36 h, the medium was removed and cells were washed twice with PBS, fixed with 95% ethanol and stained with Giemsa (Karyomax, Invitrogen, Grand Island, NY). Following staining, plates were rinsed with water, dried and plaques were counted.

2.4. Immunohistochemistry

On the days indicated, eyes were removed, embedded in OCT and frozen. Eight-micrometer thick sections were fixed with acetone/methanol (1:1), blocked and incubated overnight with a 1:500 dilution of antibody to either mouse TNFR1 (H-5) or TNFR2 (D-2) (Santa Cruz Biotechnologies, Santa Cruz, CA). Mouse anti I-A IgG was used as a control (Serotec, Raleigh, NC). Slides were washed, followed by incubation with a 1:1000 dilution of the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, Molecular Probes, Eugene, OR). After a 1 h incubation at room temperature, slides were washed and mounted with

Vectashield (Vector labs, Burlingame, CA) and then observed under a fluorescent microscope.

2.5. Reverse-transcriptase PCR

Retinas were dissected from enucleated eyes on the days indicated and six to twelve retinas were pooled. Total RNA was isolated from each pool using RNA Stat (Friendswood, TX) and each pooled sample (0.5 µg) was reverse transcribed to cDNA. It was then amplified using a GeneAmp RT-PCR kit (Applied Biosystems, Foster City, CA) and specific mouse TNF-α (upstream primer: 5' TTCTGTCT-ACTGAACTTCGGGGTGATCGGTCC 3', downstream primer: 5' GTATGAGATAGCAAATCGGCTGACG-GTGTGGG 3') or iNOS (upstream primer: 5' CCCTTC-CGAAGTTTCTGGCAGCAGC 3', downstream primer: 5' GGCTGTCAGAGCCTCGTGGCTTTGG 3') primers (Clontech, Palo Alto, CA). Following amplification, 10 µl of PCR products were electrophoresed in a 4% agarose gel (BioWhittaker Molecular Applications, Rockland, ME), denatured, neutralized and transblotted to a nylon membrane (Roche Diagnostics, Indianapolis, IN). Membranes were hybridized with a digoxigenin-labeled TNF-α or iNOS probe made using positive control DNA and a Genius kit (Roche Diagnostics, Indianapolis, IN). Equal loading of RNA was verified by amplification of the housekeeping gene actin. Densitometry analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

2.6. Enzyme-linked immunoabsorbant assays

Concentrations of TNF-α and TNF receptors were determined using ELISA kits from BD Pharmingen (San Diego, CA) and R&D Systems (Minneapolis, MN) respectively. Samples were processed according to the manufacturer's instructions.

2.7. Isolation of blood mononuclear cells

Whole blood was diluted 1:3 with PBS and mononuclear cells separated by density gradient separation using lymphoprep (Invitrogen, Grand Island, NY). Following centrifugation at 800 ×g, the interface layer containing the mononuclear cells was removed and washed twice with PBS. Remaining RBCs were lysed with ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD) and cells were washed twice with PBS and resuspended in RPMI 1640 with L-glutamine supplemented with 10% FBS, non-essential amino acids and antibiotic/antimycotic (Invitrogen, Grand Island, NY). Mononuclear cells were counted, plated at 2 × 10⁵ cells/well and were allowed to adhere for 2 h. At the end of 2 h, supernatant fluid with nonadherent (enriched for lymphocytes) cells was transferred to new wells and the

wells with adherent (enriched for monocytes) cells refed. After 72 h plates were centrifuged for 5 min at 250 ×g and supernatant fluids were tested for TNF-α receptors by ELISA. Viability both before and after culture was determined by trypan blue staining and no significant amount of death was seen.

2.8. NO assays

RAW cells (ATCC, Manassas, VA) were plated in 24 well plates with MEM minus phenol red supplemented with 10% FBS, 1X NEAA and antibiotic/antimycotic at a density of 5 × 10⁵ cells/ml using six wells per condition. After 48 h, TNF-α (10 ng/ml) from Biosource (Camarillo, CA) and IFN-γ (100 units) and sTNFR2 (1500 ng/ml) from R&D Systems (Minneapolis, MN) was added. After an additional 24 h incubation, culture supernatants were collected and NO was measured by a kit from R&D Systems.

2.9. Statistical analysis

Significance was determined using a two-tailed Student's *T*-test. Values less than 0.01 were considered significant.

3. Results

3.1. TNF-α gene expression within the retina

TNF-α gene expression within retinal tissue samples from virus-infected, mock-infected and untreated (control) mice was evaluated by RT-PCR analysis of RNA from retina. TNF-α mRNA was detected in retinas obtained from BALB/c mice at 4 and 8 days P.I. but not at 20 days P.I. (Fig. 1). TNF-α mRNA was detected at 4 and 20 days P.I. in CD-1 mice. TNF-α mRNA was absent in retinas from untreated and mock-infected BALB/c or CD-1 mice. These studies demonstrated that TNF-α gene expression was induced within retinal tissues during the acute phase of MHV infection in both mouse strains. However, the levels were higher and sustained longer in BALB/c than in CD-1 mice.

3.2. TNF-α protein concentrations in MHV-infected BALB/c and CD-1 mice

The concentration of TNF-α in the sera from BALB/c and CD-1 mice infected with MHV were evaluated by ELISA (Table 1). At day 4 P.I., 100% of serum samples from infected BALB/c mice were positive for TNF-α protein with an average value of 56 ± 15 pg/ml. No TNF-α protein was detected at day 8 or day 20 P.I. Moreover, TNF-α was not detected in any of the sera from 20 mock-infected BALB/c mice. Only one out of 6 sera from uninfected control BALB/c mice contained TNF-α at a concentration

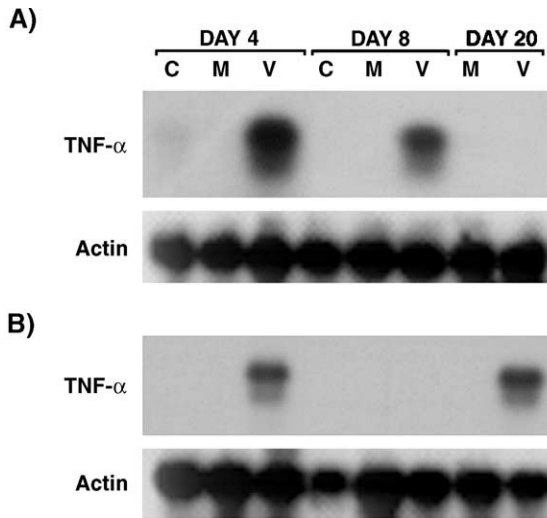


Fig. 1. Expression of TNF- α in BALB/c (A) and CD-1 (B) mouse retinas. Retinas from six to eight mice for each time point were pooled. RNA was extracted and RT-PCR performed using specific mouse TNF- α primers. Bands were confirmed using a mouse TNF- α probe. The letter C stands for control, M for mock-infected and V for virus-infected eyes. These data are representative of three separate experiments with similar results.

of 8 pg/ml (data not shown). Although, TNF- α protein was detected in virus-infected CD-1 mice, it was lower than that of BALB/c mice. At day 4 only 25% of serum samples were positive, with a mean value of 22 ± 20 pg/ml. At day 8 P.I., 43% of infected CD-1 mice were positive for TNF- α protein with an average value of 35 ± 20 pg/ml. At day 20 P.I., one animal was positive, with a value of 11 pg/ml. TNF- α was detected in sera from 1 out of 21 mock-infected CD-1 mice (Table 1). Only one out of 6 sera from control CD-1 mice contained TNF- α at a concentration of 20 pg/ml (data not shown). There was a significant increase in TNF- α protein concentration in the early phase of MHV infected BALB/c compared to mock-infected ($p < 0.005$) and un-injected animals ($p < 0.01$), while there was no significant difference in MHV infected CD-1 mice compared to mock-infected or un-injected animals.

Table 1
Identification of TNF- α protein levels in the sera of MHV infected mice by ELISA

Mouse strain	Day P.I.	MHV-injected		Mock-infected	
		Pos/total ^a	(pg/ml) ^b	Pos/total ^a	(pg/ml) ^b
BALB/c	4	9/9	56 ± 15^c	0/8	0
	8	0/7	0	0/7	0
	20	0/11	0	0/5	0
CD-1	4	2/8	22 ± 20	0/7	0
	8	3/7	35 ± 20	1/7	11 ± 11
	20	1/9	1 ± 1	0/7	0

The sensitivity of the ELISA is 5 pg/ml thus values above this are considered to be positive.

^a The number of animals where TNF- α was detected over the number tested.

^b Average TNF- α levels \pm the standard error of the mean.

^c $p < 0.005$ compared to mock-infected controls.

3.3. The effect of TNF- α , IFN- α and IFN- γ on MHV replication

TNF- α has been shown to have direct antiviral, as well as proviral, effects. Since the concentrations of TNF- α were higher in retinopathy-susceptible BALB/c mice, we wanted to determine the effect TNF- α had on the replication of MHV. The effect of varying concentrations of TNF- α , IFN- α and IFN- γ on MHV replication was evaluated using a plaque assay system. IFN- α and IFN- γ inhibited MHV replication in a dose dependent manner (Fig. 2). Treatment of cells with TNF- α over a wide range of concentration (0.5 to 50,000 pg/ml) did not significantly decrease the replication of MHV. These studies indicate that MHV replication was not directly inhibited by TNF- α in mouse L2 cells.

3.4. Immunohistochemical staining for TNF receptor in the retina

To determine if TNF receptors were present in the infected retinas of BALB/c and CD-1 mice, immunohis-

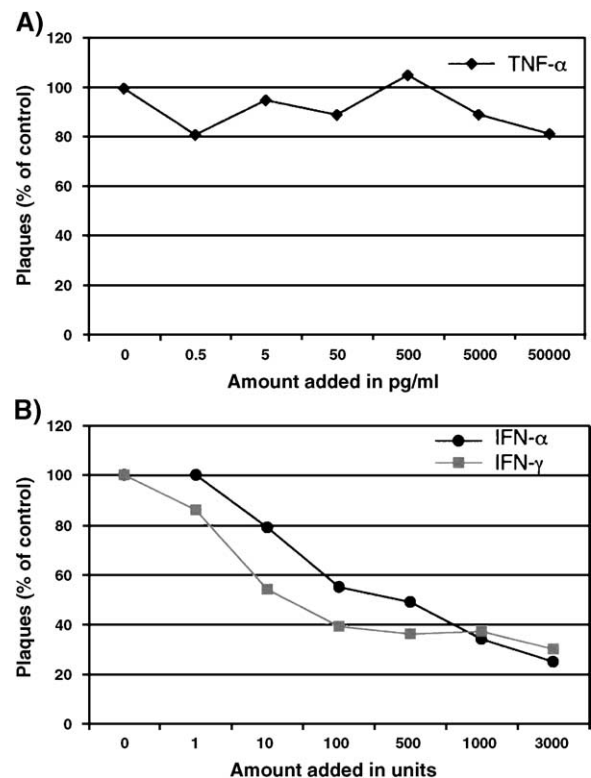


Fig. 2. MHV plaque formation in the presence of cytokines. Mouse L2 cells were plated at a concentration of 1×10^5 cells/well and allowed to adhere. After 24 h, cells were incubated with the indicated cytokine. After 24 h, cytokine media was removed and cells inoculated with approximately 40 PFU of MHV per well. Inoculum was removed after 90 min and cells refed with media containing methycellulose. Plaques were stained and counted after 36 h. The graphs above represent two separate experiments done in triplicate. The average number of plaques is represented as a percentage of control.

Table 2
TNF receptor staining in retinal tissue

	BALB/c			CD-1		
	Untreated	Virus-infected		Untreated	Virus-infected	
		Day 4	Day 8		Day 4	Day 8
Media	–	–	–	–	–	–
IgG	–	–	–	–	–	–
TNFR1	–	–	+	–	–	+
TNFR2	–	–	++	–	+	++

tochemistry was performed using antibodies to TNFR1 and TNFR2. TNFR1 was detected in both BALB/c and CD-1 virus-infected retinas at day 8 P.I. but not at day 4 P.I. (Table 2). TNFR2 was also detected at day 8 P.I. within the virus-infected retinas of both mouse strains and at day 4 P.I. in CD-1 virus-infected retinas. No staining was observed with media or control IgG antibodies. TNFR1 and TNFR2 staining was not detected in control or mock-infected mice. Fig. 3 shows the location of TNF receptor staining. At day 8 P.I., TNFR1 and TNFR2 can be seen throughout the neuroretina of infected BALB/c mice. In contrast, TNFR1 and TNFR2 staining within the CD-1 infected retina are confined to the outer nuclear and ganglion cell layers. These patterns were similar throughout the length of the retina and were not confined to the injection site.

3.5. Concentrations of TNF receptor 1 in BALB/c and CD-1 mice infected with MHV

TNF- α signal transduction can be inhibited by soluble forms of TNFR1 (sTNFR1) and TNFR2 (sTNFR2) that have been released from the cellular surface through the action of metalloproteinases. Therefore we evaluated sera concentrations of TNFR1 and TNFR2 in untreated, mock-

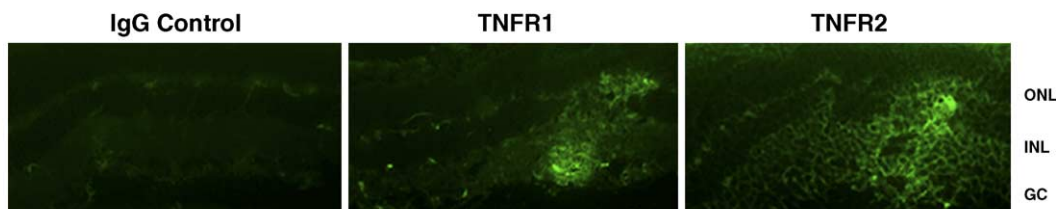
infected and virus-infected BALB/c and CD-1 mice. Average concentrations of sTNFR1 in untreated BALB/c and CD-1 mice were 685 and 1103 pg/ml ($p < 0.05$) (data not shown). A significant increase in the average sTNFR1 concentration was observed in infected BALB/c mice compared to the mock-infected control animals. At day 4, sTNFR1 concentrations increased from 668 (mock-infected) to 969 pg/ml (virus-infected) ($p < 0.0005$) and at day 8 they increased from 776 (mock-infected) to 1051 pg/ml (virus-infected) ($p < 0.01$). (Fig. 4A). There were no significant differences between mock-infected and control mice on any of the days sampled (4 and 8).

In contrast, when sera from virus-infected CD-1 mice were evaluated, sTNFR1 concentrations were not significantly increased during the entire observation period (Fig. 4B). In addition, no significant differences were seen among untreated, mock-infected or virus-infected animals. These data demonstrated that during the inflammatory phase of infection serum levels of sTNFR1 were significantly elevated in BALB/c mice but not in CD-1 mice.

3.6. Concentrations of TNF receptor 2 in BALB/c and CD-1 mice infected with MHV

There was no significant difference in concentrations of sTNFR2 in untreated BALB/c and CD-1 mice (2407 vs. 2967 pg/ml) (days 4 and 8, data not shown). When sTNFR2 concentrations in infected and mock-infected BALB/c mice were compared, a 3.1 fold increase at day 4 ($p < 0.00005$) and a 2.6 fold increase at day 8 ($p < 0.00001$) were observed in infected mice. In contrast, no significant differences were seen between mock-infected and virus-infected mice at day 20 (2067 vs. 2286 pg/ml) or between mock-infected mice and untreated mice on any of the days tested.

A) BALB/c (day 8 PI)



B) CD-1 (day 8 PI)

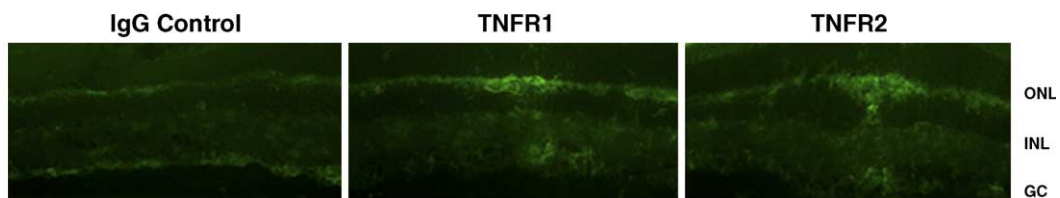


Fig. 3. Detection of TNF receptor 1 and 2 in infected mouse retinas. Eyes from BALB/c (A) and CD-1 (B) MHV-injected mice were harvested 8 days post-infection (P.I.), sectioned and incubated with antibodies to mouse TNFR1, TNFR2 or I-A, as a control. Retinal orientation is shown at the right with the layers represented as follows: outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GC).

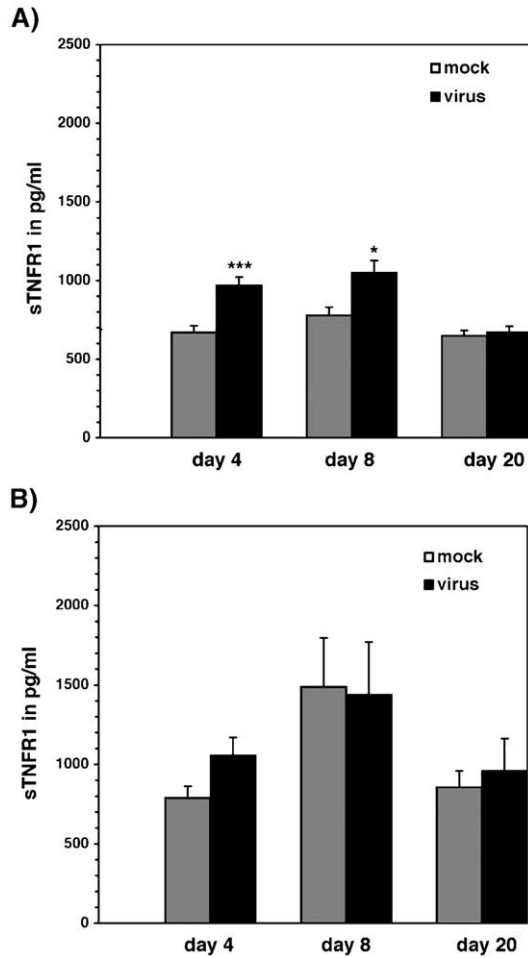


Fig. 4. Amount of soluble TNF receptor 1 present in BALB/c (A) and CD-1 (B) mice. Mice were injected intravitreally with 5 μ l of media (mock) or media with MHV (virus). On the days indicated, serum from six to eleven mice per group was collected and tested for the presence of soluble TNF receptor 1 by ELISA. The bars represent the mean concentration of soluble receptor \pm se for each group. Significant increases of sTNFR1 were seen in virus-infected BALB/c mice, compared to mock-infected controls (A), on days 4 (** p < 0.0005) and 8 (* p < 0.01) post-injection. No differences were noted between mock- and virus-infected CD-1 mice. No differences were seen between the two strains of mice. These data are representative of two separate experiments with similar results.

In CD-1 mice, significant increases of sTNFR2 in virus-infected compared to mock-infected mice were observed only at day 4 (p < 0.005) (Fig. 5B). Less than 2 fold differences were noted between control and mock-infected animals or between control and virus-infected animals on days 4 and 8 and these differences were not statistically significant.

When comparing the concentrations of sTNFR2 between the two virus-infected mouse strains, the concentration of sTNFR2 at day 4 was almost 2 times higher in BALB/c mice (7149 pg/ml) than in CD-1 mice (3851 pg/ml) (p < 0.0005). Significant differences were not noted on days 8 or 20 or in uninfected controls. These studies demonstrated that BALB/c mice released more sTNFR2 than CD-1

mice in response to virus in the early inflammatory phase of the infection (day 4).

3.7. Gene expression of iNOS in BALB/c and CD-1 mice after infection with MHV

We have shown that increased concentrations of soluble TNFR2 and to a lesser extent TNFR1 were seen in degeneration susceptible BALB/c mice during the early phase of the infection. Based on these observations we next determined if increased sTNFR was associated with altered TNF- α driven signaling events within the retina. We evaluated inducible nitric oxide synthase (iNOS), since the iNOS gene is controlled by TNF- α signaling (Saura et al., 1999). At day 4 and 8 P.I. iNOS mRNA was only found in

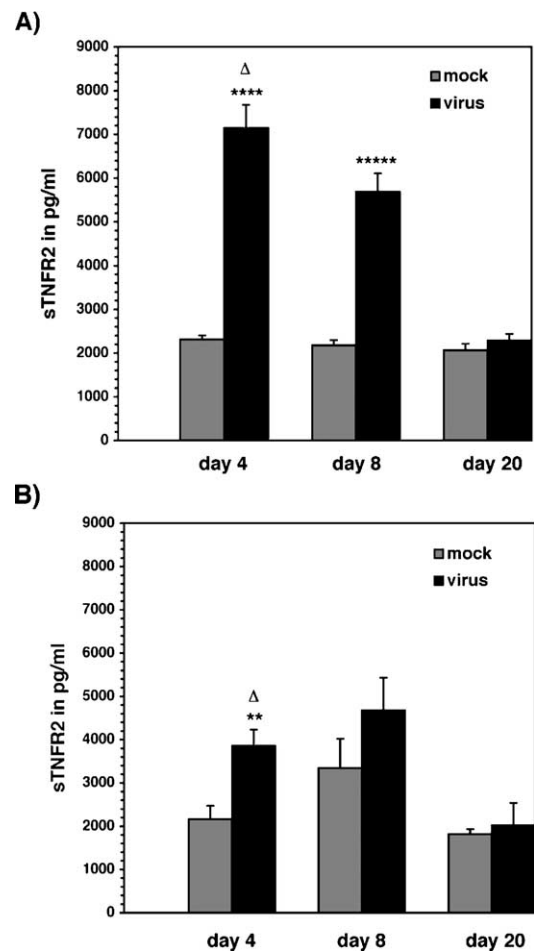


Fig. 5. Amount of soluble TNF receptor 2 present in BALB/c (A) and CD-1 (B) mice. Mice were injected intravitreally with 5 μ l of media (mock) or media with MHV (virus). On the days indicated, serum from six to eleven mice per group was collected and tested for the presence of soluble TNF receptor 2 by ELISA. The bars represent the mean concentration of soluble receptor \pm se for each group. Significant increases of sTNFR2 were seen in virus-infected BALB/c mice compared to mock-infected controls (A) on days 4 (**** p < 0.00005) and 8 (**** p < 0.00001) and between virus-infected CD-1 mice compared to mock-infected controls on day 4 (** p < 0.005). When comparing the two strains of mice significant differences were only seen on day 4 (Δ p < 0.0005). These data are representative of two separate experiments with similar results.

retinas of infected animals (both strains), but not in untreated or mock-infected animals, of either strain. In addition, gene expression of iNOS decreased from day 4 to 8 in BALB/c mice while it increased in CD-1 mice during this same time period (Fig. 6A). We confirmed these trends by performing densitometry on the blots using the public domain program, NIH image. The number of pixels was determined for each iNOS and actin band and these are expressed as a ratio for the virus-infected samples (Fig. 6B). The ratios confirm the trend indicated in Fig. 6A, namely that iNOS decreased from days 4 to 8 P.I. in BALB/c mice while it increased in CD-1 mice during this same time frame. Based on this data increased levels of soluble TNFR2 correlate with decreased levels of iNOS expression within the retinas of degeneration susceptible BALB/c mice. It is possible that soluble TNFR2 interferes with TNF- α signaling within the retina.

3.8. Release of TNFR2 by BALB/C and CD-1 mononuclear cells

Previous studies have shown that the monocyte predominates among infiltrating cells within the retina during ECOR (Vinores et al., 2001). We postulated that through the action of metalloproteinases, TNFR2 was released from monocyte cell surfaces. The soluble TNFR2 then prevented

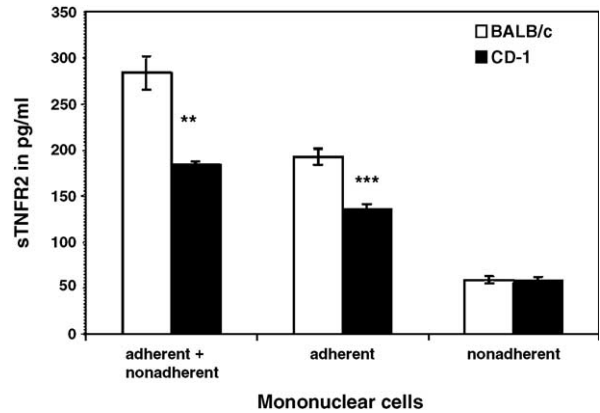


Fig. 7. Release of TNF Receptor 2 by mononuclear cells. Populations of mixed (adherent+nonadherent), adherent (enriched for monocytes) or nonadherent (enriched for lymphocytes) mononuclear cells were plated at densities of 20×10^4 , 18×10^4 or 2×10^4 , respectively. After 72 h supernatants were collected and assayed for the presence of sTNFR2 by ELISA. Average values for six wells per condition are shown above. Significant differences in release of sTNFR2 between BALB/c and CD-1 mononuclear cells were seen in both mixed (** $p < 0.005$) and adherent mononuclear cell populations (*** $p < 0.0005$).

TNF- α signaling and hence iNOS expression. The levels of soluble TNFR2 in BALB/c and CD-1 mononuclear cells were measured by ELISA (Fig. 7). Unstimulated mononuclear cells obtained from BALB/c mice secreted significantly more sTNFR2 ($p < 0.005$) than those isolated from CD-1 mice. To further define the population of cells responsible for TNFR2 release, mononuclear cells were separated into adherent and nonadherent fractions. Adherent mononuclear cells (enriched for monocytes) from BALB/c mice released significantly more TNFR2 ($p < 0.0005$) than those from CD-1 mice. No difference was noted in the release of TNFR2 in the nonadherent cell populations between the two strains of mice. These data indicated that the adherent fraction of mononuclear cells were responsible for increased TNFR2 release observed in mononuclear cells from BALB/c mice. This suggests that release of TNFR2 from the monocyte cell surface may play a role in decreasing iNOS expression within the retinas of infected BALB/c mice.

3.9. NO production from mouse macrophages

To further determine whether increased release of sTNFR2 could decrease iNOS expression and hence NO production, we stimulated the mouse macrophage cell line, RAW, with TNF- α and IFN- γ in the presence or absence of sTNFR2. Although incubation of RAW cells with TNF- α , or sTNFR2 produced 45 and 50 $\mu\text{mol/l}$ of NO, respectively, they were not statistically different from RAW cells alone (43 $\mu\text{mol/l}$) (Fig. 8). The addition of IFN- γ or IFN- γ plus TNF- α significantly increased production of NO to 101 and 99 $\mu\text{mol/l}$, respectively, compared to control ($p < 0.0000005$). However, there was no significant difference between these two groups. This may be due to endogenous

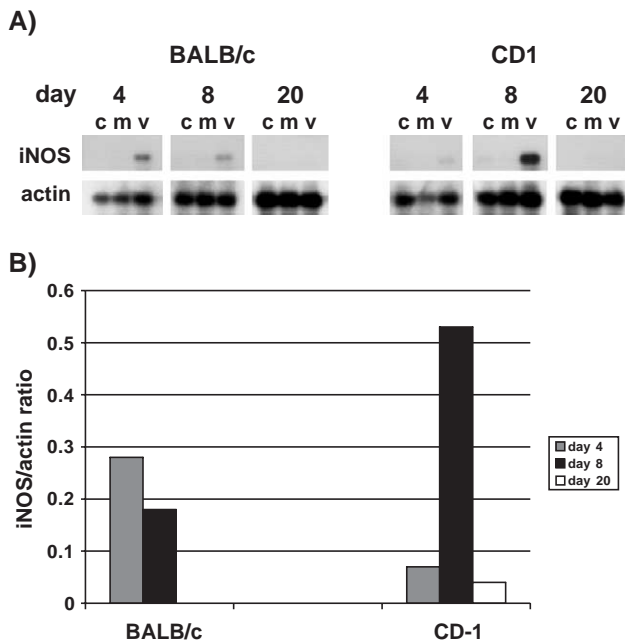


Fig. 6. Expression of iNOS in BALB/c and CD-1 mouse retinas. (A) Retinas from seven to twelve mice for each time point post infection were pooled. RNA was extracted and RT-PCR performed for iNOS and the housekeeping gene actin using specific mouse primers. Bands were confirmed by hybridization with digoxigenin labeled probes. C stands for control, M for mock-infected and V for virus-infected eyes. (B) The number of pixels in the iNOS and actin cDNA bands from BALB/c and CD-1 infected retinas shown in panel (A) was determined using NIH image and was expressed as a ratio.

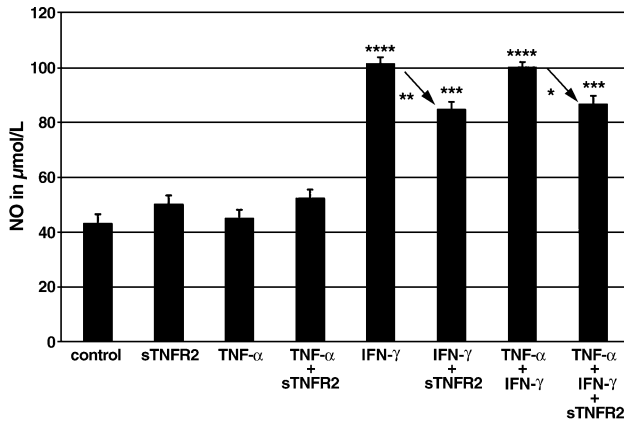


Fig. 8. Average NO production from RAW cells. Cells, 5×10^5 , were incubated with TNF- α (10 ng/ml), sTNFR2 (1500 ng/ml) and/or IFN- γ (100 units). After 24 h supernatants were collected and tested for the presence of NO. Production of NO was significantly increased in all wells given IFN- γ alone or plus TNF- α (**** $p < 0.000005$) compared to control. A significant decrease in the production of NO was observed when sTNFR2 was added to wells with IFN- γ alone (** $p < 0.005$) or plus TNF- α (* $p < 0.01$) although NO production in these wells was still significantly higher than control (*** $p < 0.000005$). These data are representative of three separate experiments using serum and serum-free media as well as different sources of TNF- α with similar results.

release of TNF- α (2 ng/ml) by these cells. Upon addition of sTNFR2 to those cells which had been stimulated with IFN- γ alone or with IFN- γ plus TNF- α , a significant decrease of NO to 84 $\mu\text{mol/l}$ ($p < 0.005$) or to 86 $\mu\text{mol/l}$ ($p < 0.01$) respectively, was noted. Since these cells were noted to release TNF- α constitutively we propose that sTNFR2 is decreasing NO by interfering with the signaling by endogenous TNF- α . Analysis of the mRNA from these cells showed that addition of sTNFR2 results in decreased iNOS gene expression (data not shown) suggesting that the decrease in NO was at the level of iNOS gene expression. These data indicate that sTNFR2 can interfere with NO production by macrophages via interference with TNF- α signaling.

4. Discussion

In this study we showed that both TNF- α and soluble TNFR2 (sTNFR2) were increased during coronavirus infection and that this upregulation correlated with the development of retinal degeneration. Furthermore we observed the secretion of these molecules by the macrophage and demonstrated that release of sTNFR2 altered TNF- α signaling and hence NO production.

TNF- α is produced predominantly by monocyte/macrophages. Within the retina, TNF- α gene expression may also be associated with neurons or glial cells since studies indicate that both cell types are able to secrete TNF- α (Cheng et al., 1994). In this study, the detection of TNF- α gene expression in the virus-infected retina and the absence of TNF- α gene expression in control and mock-infected

animals indicate that this response was virus triggered. Infected BALB/c retinas were found to have higher gene expression levels of TNF- α early in the disease (day 4) and TNF- α gene expression continued to be observed at day 8. In contrast, the gene expression of TNF- α in CD-1 mice is evident at day 4 but not at day 8. Differences in serum TNF- α protein expression were also seen between BALB/c and CD-1 mice. Protein levels in virus-infected BALB/c were, in fact, significantly higher ($p < 0.005$) than those of BALB/c mock-infected controls and were also higher than those of infected CD-1 mice at day 4. These results mirror the gene expression data seen at day 4 P.I. At later time points (day 8 in BALB/c and day 20 in CD-1 mice) we did not see a direct correlation between mRNA in retinal tissue and protein levels in sera. At day 8, lower levels of mRNA were detected in the absence of TNF- α protein in the sera. At day 20 in CD-1 mice TNF- α mRNA was detected in the absence of serum protein. This may be explained by the fact that mRNA was pooled from six to eight animals for RT-PCR analysis. This assay may have amplified low mRNA levels in a small percentage of animals. The TNF- α protein analysis was performed on individual serum samples for the 5 animals. As is seen in Table 1, at day 20, 1 out of 9 sera from CD-1 infected mice contained TNF- α in the serum. These studies indicate that during the acute phase of the disease, TNF- α gene expression and protein production were not only increased but also sustained longer in BALB/c mice than in CD-1 mice.

The precise role that TNF- α and its receptors play in ECOR is still unclear. In diseases with an autoimmune component, TNF- α has been shown to contribute to both the pathogenesis as well as the resolution of disease (O'Shea et al., 2002). Many studies however do not take into account the concentrations of sTNFRs present during disease that may alter TNF- α signaling and therefore outcome. Here we have shown not only increased TNF- α but also increased sTNFRs, specifically sTNFR2, during viral infection and have correlated higher concentrations with the development of ECOR, a disease in which anti-retinal antibodies are produced. Several studies have identified increased concentrations of sTNFR2 during viral infection (Bartholdy et al., 1999; Sippy et al., 1995; Marinos et al., 1995; Realdon et al., 2001) or autoimmune disease (StudnickaBenke et al., 1996; Heilig et al., 1993; Limb et al., 1999; Cope et al., 1992). Moreover, investigators have correlated increased sTNFR2 levels with increased disease severity either during viral infection (Godfried et al., 1995; Itoh et al., 1999) or autoimmune disease (Kulseng et al., 1999; Robak et al., 1998; Heilig et al., 1992). Here we link increased levels of sTNFR2 during viral infection with autoimmune reactivity. These studies indicate that sTNFR2 is important in the control of TNF- α signaling and hence disease resolution.

The importance of TNFR2 in the ocular microenvironment has recently been investigated in ACAID and corneal allograft survival. Niederkorn and associates evaluated TNFR2 KO mice and found that the absence of TNFR2

increased the risk of corneal graft rejection. These studies indicate that expression of TNFR2 on corneal cells conveys protection against immune rejection of corneal grafts by a mechanism independent of ACAID (Niederhorn et al., 2004).

The presence of TNF- α , sTNFR2 and NO may mechanistically be contributing to retinal tissue damage by two separate pathways. First, TNF- α induction of NO at the peak of virus replication directly leads to retinal tissue damage and as a result sTNFR is released as a by-product of this induction. Second, the presence of sTNFR interferes with TNF- α receptor signaling and thereby alters immune reactivity and contributes to autoimmune responses.

In our model we found that, at day 4, iNOS was present within the retinas of BALB/c mice but was almost absent in CD-1 mice. This occurs at the peak time of virus replication within the retina. Both TNF- α and IFN- γ gene expression (Hooks et al., 2003) are detected within the retina at this time point. Several earlier studies have shown that increases in iNOS transcription can be attributed to signaling through TNF- α to produce NF- κ B, and then through IFN- γ to produce IRF-1, which together have been shown to be necessary for iNOS transcription within the nucleus of macrophages (Saura et al., 1999). NO in high concentration causes damage in the photoreceptor layer of the retina in an experimental model of autoimmune uveoretinitis (EAU) and administration of TNFR-Ig abrogates this damage (Robertson et al., 2003). Therefore, it is possible that NO functions similarly in our ECOR model, predisposing BALB/c mice at an early stage to the progressive loss of photoreceptors seen during the degenerative phase of disease.

Alternatively release of sTNFRs may actually contribute to retinal degenerative processes by altering cell numbers/responses in T cells. Recently, it has been shown that overproduction of NO serves to limit the Th1 response in viral infection (Akaike and Maeda, 2000). This may be through the effect that NO has on NF- κ B activity. Connelly et al. (Connelly et al., 2001) showed that NO had a biphasic effect on NF- κ B enabling it to control host defense proteins such as iNOS, cyclooxygenase-2 and IL-6. In addition, low concentrations of NO have been shown by Niedbala et al. to increase the induction and differentiation of Th1 but not Th2 cells (Niedbala et al., 1999) by selectively upregulating the IL-12 receptor b2 via cGMP (Niedbala et al., 2002). Here we have shown that sTNFRs decrease the amount of NO released from macrophages. Therefore in ECOR overproduction of NO seen initially at day 4 may limit Th1 responses during the height of viral replication. In contrast the underproduction of NO at day 8 may increase the number and differentiation of Th1 cells at a time when disease is subsiding leading to the development of autoreactivity. This is not the case in CD-1 mice in which NO is initially low at day 4 and high at day 8 P.I. Therefore in CD-1 mice Th1 number and differentiation would be increasing during peak viral replication and decreasing as viral infection diminishes. These studies suggest that

underproduction or improper control of NO may therefore alter Th1/Th2 responses leading to increased reactivity with cellular proteins and production of autoantibodies. This may be particularly relevant in ECOR, because MHV-infected BALB/c mice have been shown to develop anti-retinal antibodies (Hooks et al., 1993) while CD-1 mice do not.

Soluble TNFRs may also play a role in the maintenance of mature CD8+ populations as well. TNF- α has been shown to mediate apoptosis of mature activated T cells by signaling through both TNFR1 (Speiser et al., 1996) and TNFR2 (Zheng et al., 1995). In our model, excessively released sTNFRs from macrophages could bind available TNF- α thereby inhibiting apoptosis of activated T cells. This would increase the number of CD8+ cells within the retinas of BALB/c mice and thereby increase the likelihood of retinal damage via cytotoxic T cells. Evidence to support this possibility comes from immunohistochemical studies which show the presence of CD8+ T cells at later time points (20 days) in the retinas of BALB/c compared to CD-1 mice (data not shown).

In conclusion, our findings indicate that coronavirus infection of the retina augments TNF- α expression, TNF receptor signaling and NO production within the retina. These factors may contribute to retinal degeneration in a multifaceted way. By one mechanism, the virus infects retinal cells and induces the production of NO that can directly trigger degeneration of photoreceptor cells. Using a second mechanism, the virus triggers sTNFR2 release that can directly modify T cell reactivity and autoimmunity. During the acute phase of the disease, both TNF- α gene expression and sTNFR2 release are higher in the retinal degeneration susceptible BALB/c mice. Future studies will try to delineate between these two possibilities.

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References

- Akaike, T., Maeda, H., 2000. Nitric oxide and virus infection. *Immunology* 101, 300–308.
- Bartholdy, C., Nansen, A., Marker, O., Thomsen, A.R., 1999. Soluble tumour necrosis factor (TNF)-receptor levels in serum as markers of anti-viral host reactivity. *Clin. Exp. Immunol.* 116, 299–306.
- Cheng, B., Christakos, S., Mattson, M.P., 1994. Tumor necrosis factors protect neurons against metabolic–excitotoxic insults and promote maintenance of calcium homostasis. *Neuron* 12, 139–153.
- Connelly, L., Palacios-Callender, M., Ameixa, C., Moncada, S., Hobbs, A.J., 2001. Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J. Immunol.* 166, 3873–3881.
- Cope, A.P., Aderka, D., Doherty, M., Engelmann, H., Gibbons, D., Jones, A.C., Brennan, F.M., Maini, R.N., Wallach, D., Feldmann, M., 1992. Increased levels of soluble tumor-necrosis-factor receptors in the sera

- and synovial-fluid of patients with rheumatoid arthritis. *Arthritis Rheum.* 35, 1160–1169.
- Cope, A.P., Londei, M., Randall-Chu, N., Cohen, S.B.A., Eliot, M.J., Brennan, F.M., Maini, R.N., Feldmann, M., 1994. Chronic exposure to tumor necrosis factor (TNF) in vitro impairs the activation of T cells through the T cell receptor/CD3 complex: reversal in vivo by anti-TNF antibodies in patients with rheumatoid arthritis. *J. Clin. Invest.* 94, 749–760.
- Cope, A.P., Liblau, R.S., Yang, X.-D., Congia, M., Laudanna, C., Schreiber, R.D., Probert, L., Kollias, G., McDevitt, H.O., 1997. Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling. *J. Exp. Med.* 185, 1573–1584.
- Godfried, M.H., Vanderpoll, T., Mulder, J.W., Weverling, G.J., Endert, E., Lange, J.M.A., Sauerwein, H.P., 1995. Soluble tumor-necrosis-factor receptors as surrogate markers for the assessment of zidovudine treatment in asymptomatic HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* 10, 531–539.
- Gross, J.A., Johnston, J., Mudri, S., Enselman, R., Dillon, S.R., Madden, K., Xu, W., Parrish-Novak, J., Foster, D., Lofton-Day, C., Moore, M., Littau, A., Grossman, A., Haugen, H., Foley, K., Blumberg, H., Harrison, K., Kindsvogel, W., Clegg, C.H., 2000. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* 404, 995–999.
- Heilig, B., Wermann, M., Gallati, H., Brockhaus, M., Berke, B., Egen, O., Pezzutto, A., Hunstein, W., 1992. Elevated TNF receptor plasma-concentrations in patients with rheumatoid-arthritis. *Clin. Investig.* 70, 22–27.
- Heilig, B., Fiehn, C., Brockhaus, M., Gallati, H., Pezzutto, A., Hunstein, W., 1993. Evaluation of soluble tumor-necrosis-factor (TNF) receptors and TNF receptor antibodies in patients with systemic lupus erythematoses, progressive systemic-sclerosis, and mixed connective-tissue disease. *J. Clin. Immunol.* 13, 321–328.
- Hooks, J.J., Percopo, C., Wang, Y., Detrick, B., 1993. Retina and retinal pigment epithelial cell autoantibodies are produced during murine coronavirus retinopathy. *J. Immunol.* 151, 3381–3389.
- Hooks, J.J., Wang, Y., Detrick, B., 2003. The critical role of IFN-gamma in experimental coronavirus retinopathy. *Invest. Ophthalmol. Vis. Sci.* 44, 3402–3408.
- Itoh, Y., Okanoue, T., Ohnishi, N., Sakamoto, M., Nishioji, K., Nakagawa, Y., Minami, M., Murakami, Y., Kashims, K., 1999. Serum levels of soluble tumor necrosis factor receptors and effects of interferon therapy in patients with chronic hepatitis C virus infection. *Am. J. Gastroenterol.* 94, 1332–1340.
- Jacob, C.O., Aiso, S., Michie, S.A., McDevitt, H.O., Acha-Orbea, H., 1990. Prevention of diabetes in non-obese diabetic mice by tumor necrosis factor (TNF)-similarities between TNF α and interleukin-1. *Proc. Natl. Acad. Sci. U. S. A.* 87, 968–972.
- Kagi, D., Ho, A., Odermatt, B., Zakarian, A., Ohashi, P.S., Mak, T.W., 1999. TNF receptor-1-dependent b cell toxicity as an effector pathway in autoimmune diabetes. *J. Immunol.* 162, 4598–4605.
- Kulseng, B., Vatten, L., Espevik, T., 1999. Soluble tumor necrosis factor receptor in sera from patients with insulin-dependent diabetes mellitus: relations to duration and complications of disease. *Acta Diabetol.* 36, 99–105.
- Limb, G.A., Soomro, H., Janikoun, S., Hollifield, R.D., Shilling, J., 1999. Evidence for control of tumour necrosis factor-alpha (TNF-alpha) activity by TNF receptors in patients with proliferative diabetic retinopathy. *Clin. Exp. Immunol.* 115, 409–414.
- Liu, J., Marino, M.W., Wong, G., Grail, D., Dunn, A., Bettadapura, J., Slavin, A.J., Old, L., Bernard, C.C.A., 1998. TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat. Med.* 4, 78–83.
- Marinos, G., Naumov, N.V., Rossol, S., Torre, F., Wong, P.Y.N., Gallati, H., Portmann, B., Williams, R., 1995. Tumor-necrosis-factor receptors in patients with chronic hepatitis-B virus-infection. *Gastroenterology* 108, 1453–1463.
- Niedbala, W., Wei, X.Q., Campbell, C., Thomson, D., Komai-Koma, M., Liew, F.Y., 1999. Effects of nitric oxide on the induction and differentiation of Th1 cells. *Eur. J. Immunol.* 29, 2498–2505.
- Niedbala, W., Wei, X.Q., Campbell, C., Thomson, D., Komai-Koma, M., Liew, F.Y., 2002. Nitric oxide preferentially induces type 1 T cell differentiation by selectively up-regulating IL-12 receptor beta 2 expression via cGMP. *Proc. Natl. Acad. Sci.* 99, 16186–16191.
- Niederhorn, J.Y., Mayhem, E., Mellon, J., Hegde, S., 2004. Role of tumor necrosis factor receptor expression in anterior chamber-associated immune deviation (ACAID) and corneal allograft survival. *Invest. Ophthalmol. Vis. Sci.* 45, 2674–2679.
- O'Shea, J.J., Ma, A., Lipsky, P., 2002. Cytokines and autoimmunity. *Nat. Rev. Immunol.* 2, 37–45.
- Pape, K.A., Khoruts, A., Mondino, A., Jenkins, M.K., 1997. Inflammatory cytokines enhance the in-vivo clonal expansion and differentiation of antigen-activated CD4+ T cells. *J. Immunol.* 159, 591–598.
- Realdon, S., Pontisso, P., Adami, F., Trentin, L., Noventa, F., Ferrari, A., Migliorato, I., Gatta, A., Alberti, A., 2001. High levels of soluble tumor necrosis factor superfamily receptors in patients with hepatitis C virus infection and lymphoproliferative disorders. *J. Hepatol.* 34, 723–729.
- Robak, T., Gladalska, A., Stepień, H., 1998. The tumour necrosis factor family of receptors/ligands in the serum of patients with rheumatoid arthritis. *Eur. Cytokine Netw.* 9, 145–154.
- Robbins, S.G., Hamel, C.P., Detrick, B., Hooks, J.J., 1990. Murine coronavirus induces an acute and long-lasting disease of the retina. *Lab. Invest.* 62, 417–426.
- Robbins, S.G., Detrick, B., Hooks, J.J., 1991. Murine coronavirus is retinotropic when introduced by several direct routes and one indirect one and long-lasting disorder can result after coronavirus infection. *Invest. Ophthalmol. Vis. Sci.* 32, 1883–1893.
- Robertson, M., Liversidge, J., Forrester, J.V., Dick, A.D., 2003. Neutralizing tumor necrosis factor-alpha activity suppresses activation of infiltrating macrophages in experimental uveoretinitis. *Invest. Ophthalmol. Vis. Sci.* 44, 3034–3041.
- Saura, M., Zaragoza, C., Bao, C., McMillan, A., Lowenstein, C.J., 1999. Interaction of interferon regulatory factor-1 and nuclear factor kB during activation of inducible nitric oxide synthase transcription. *J. Mol. Biol.* 289, 459–471.
- Sippy, B.D., Hofman, F.M., Wallach, D., Hinton, D.R., 1995. Increased expression of tumor-necrosis-alpha receptors in the brains of patients with AIDS. *J. Acquir. Immune Defic. Syndr.* 10, 511–521.
- Speiser, D.E., Sebada, E., Ohteki, T., Bachmann, M.F., Pfeffer, K., Mak, T.W., Ohashi, P.S., 1996. Tumour necrosis factor receptor p55 mediates deletion of peripheral cytotoxic T lymphocytes in vivo. *Eur. J. Immunol.* 26, 3055–3060.
- StudnickaBenke, A., Steiner, G., Petera, P., Smolen, J.S., 1996. Tumour necrosis factor alpha and its soluble receptors parallel clinical disease and autoimmune activity in systemic lupus erythematosus. *Br. J. Rheumatol.* 35, 1067–1074.
- Vinore, S.A., Wang, Y., Vinore, M.A., Derevjani, N.L., Shi, A., Klein, D.A., Detrick, B., Hooks, J.J., 2001. Blood–retinal barrier breakdown in experimental coronavirus retinopathy: association with viral antigen, inflammation, and VEGF in sensitive and resistant strains. *J. Neuroimmunol.* 119, 175–182.
- Wang, Y., Burnier, M., Detrick, B., Hooks, J.J., 1996. Genetic predisposition to coronavirus-induced retinal disease. *Invest. Ophthalmol. Vis. Sci.* 37, 250–254.
- Zheng, L., Fisher, G., Miller, R.E., Peshon, J., Lynch, D.H., Leonardo, M.J., 1995. Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature* 377, 348–351.