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TNF Production in Macrophages Is Genetically Determined and Regulates Inflammatory Disease in Rats

Alan Gillett,* Monica Marta,* Tao Jin,† Jonatan Tuncel,‡ Patrick Leclerc,§ Rita Nohra,* Stefan Lange,¶ Rikard Holmdahl,‡ Tomas Olsson,* Robert A. Harris,* and Maja Jagodic*

Dysregulation of TNF is an important pathophysiological phenotype for many diseases. Recently, certain genetically regulated loci have been identified to regulate several inflammatory diseases. We hypothesized that a region on rat chromosome 4 known to regulate experimental autoimmune encephalomyelitis, experimental arthritis and experimental autoimmune neuritis harbors a gene regulating central inflammatory molecules, such as TNF. We therefore mapped TNF production using linkage analysis in the 12th generation of an advanced intercross line between DA and PVG.AV1 rats, which differ in susceptibility to several inflammatory conditions. A single TNF-regulating quantitative trait locus with a logarithm of odds score of 6.2 was identified and its biological effect was confirmed in a congenic rat strain. The profound TNF regulation mapped in congenic strains to the macrophage population. Several TLR signaling cascades led to the same reduced proinflammatory phenotype in congenic macrophages, indicating control of a convergence point for innate inflammatory activity. The decreased TNF potential and reduced proinflammatory macrophage phenotype in congenic rats was also associated with reduced clinical severity in experimental autoimmune encephalomyelitis, pristane-induced arthritis and sepsis experimental models. Determination of genes and mechanisms involved in this genetically determined TNF regulation will be valuable in understanding disease pathogenesis and aid treatment development. The Journal of Immunology, 2010, 185: 000–000.

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a region of rat chromosome 4 known to regulate several inflammatory diseases, including experimental autoimmune encephalomyelitis (EAE) (20), collagen-induced arthritis (CIA) (21), pristane-induced arthritis (PIA) (22), and experimental autoimmune neuritis (23). We identified a quantitative trait locus (QTL) using the 12th generation of the AIL and confirmed our findings using congeneric rats that captured the biological phenotype. This differential TNF regulation was macrophage-driven and resulted in skewed immunocellular phenotypes. Several TLR signaling pathways led to similar differential TNF production, indicating a common convergence regulated by genetic variability within the congeneric insertion. The same genetically regulated pathway leading to a decrease in TNF production also reduced the severity of inflammatory diseases including EAE, PIA, and sepsis.

Materials and Methods

Animals

DA rats were originally obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany). MHC-identical PVG.A V1 rats were originally obtained from Harlan UK Limited (Blackdown, U.K.). Congenic R11 (DA.PVG-[D4Rat108-D4Rat233]) and R23 (DA.PVG-[D4Got60-D4Kin1]) rats were derived from the C4 congeneric strain described previously (24). Repeated backcrossing to the DA strain was performed and offspring genotyped to select for desired recombinants. The AIL F1 generation was established by two reciprocal breeding couples of DA and PVG.A V1. The F2 generation was produced from seven couples each of F1 rats with DA or PVG.A V1 female founders. Fifty F2 breeding pairs with both DA and PVG.A V1 female founders generated the third generation. Non-brother–sister random breeding of 50 males and females created the following generations. Animals were bred and housed at the Karolinska University Hospital (Stockholm, Sweden) or at Scanbur (Sollentuna, Sweden), in polystyrene cages containing aspen-wood shavings with ad libitum food and standard rodent chow and a 12-h light/dark cycle. The animals were routinely monitored for pathogens according to a health-monitoring program for rats at the National Veterinary Institute (Uppsala, Sweden). A total of 463 rats (239 males and 224 females) from the 12th generation were used for the AIL genotyping and TNF phenotyping. The septic arthritis model was performed at Sahlgrenska University Hospital (University of Gothenburg, Sweden). Congenic and control rats were mixed in cages and matched for sex and age. All experiments in this study were approved and performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and the European Community Directive (86/609/EEC).

Genotype analysis

Genomic DNA was prepared from ear clippings or tail tips using a standard protocol. Polymorphic microsatellite markers for genotyping were selected from The Rat Genome Database (http://rgd.mcw.edu). Primers were obtained from Sigma-Aldrich (St. Louis, MO) or Applied Biosystems (Foster City, CA). DNA amplification was performed with PCR using forward primers end-labeled with either [γ-33P]-ATP or a fluorescent dye (VIC, NED, FAM, and PET) (25). [γ-3P]-ATP-labeled PCR products were size-fractioned in 6% polyacrylamide gels and visualized by autoradiography. Fluorescently labeled products were run using an ABI 3730 capillary sequencer and analyzed using GeneMapper v3.7 software (Applied Biosystems) (26). All genotypes were evaluated manually and double checked.

Whole blood LPS stimulation

Blood samples were taken from tail tips of rats and collected in Falcon lithium heparin Microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). The 2 × 100 μl heparinized blood per rat was added in a 48-well Nunc Δ Surface plate (Nunc, Roskilde, Denmark) and diluted 1:4 with DMEM (Life Technologies-BRL, Grand Island, NY) containing 1% t-glutamine, 1% penicillin-streptomycin, and 1% sodium pyruvate (complete medium (CM), all from Life Technologies, Paisley, Scotland). Duplicate cultures were stimulated with LPS (Sigma-Aldrich) at a final concentration of 50

FIGURE 1. Identification of a genomic region regulating TNF production. Blood was collected from 463 G12 AIL rats and stimulated with LPS. TNF levels were determined using ELISA and linked to microsatellite genotypes. A, LOD plot versus marker position for a 39 Mb region of rat chromosome 4. Genotyped markers are listed along the x-axis. A single QTL with a LOD score of 6.2 at peak marker D4Mit12 was identified. The confidence interval was determined using external markers from a 1.5 LOD drop. Congenic rat strain insertions of PVG DNA in a DA background are illustrated (R11, white, external marker borders from 82.1 to 108 Mb; and R23, gray, external marker borders from 80.6 to 81.7 Mb). The threshold of family residuals (LOD = 2) is plotted as a dashed line. The F2 generation was produced from seven couples each of F1 rats with DA or PVG.A V1 female founders. Fifty F2 breeding pairs with both DA and PVG.A V1 female founders generated the third generation. Non-brother–sister random breeding of 50 males and females created the following generations. Animals were bred and housed at the Karolinska University Hospital (Stockholm, Sweden) or at Scanbur (Sollentuna, Sweden), in polystyrene cages containing aspen-wood shavings with ad libitum food and standard rodent chow and a 12-h light/dark cycle. The animals were routinely monitored for pathogens according to a health-monitoring program for rats at the National Veterinary Institute (Uppsala, Sweden). A total of 463 rats (239 males and 224 females) from the 12th generation were used for the AIL genotyping and TNF phenotyping. The septic arthritis model was performed at Sahlgrenska University Hospital (University of Gothenburg, Sweden). Congenic and control rats were mixed in cages and matched for sex and age. All experiments in this study were approved and performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and the European Community Directive (86/609/EEC).

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monocytes. Error bars represent STD. *p < 0.05 using nonparametric Kruskal-Wallis ranking test, followed by a Dunn’s posthoc test for B and Mann-Whitney U test on prenormalized values for C.
μg/ml or unstimulated (only CM) and incubated at 37°C with 5% CO₂, for 18 h. Supernatants were analyzed in duplicates using a commercial TNF ELISA kit (BioSite, Paris, France). Cytokine levels in unstimulated blood were below the detection limit (<32.5 pg/ml). Congenic rat experiments were normalized to DA controls by dividing the congenic average by the DA average, then multiplying by 100.

**Cell collection and stimulation**

Splenocytes were collected from extracted spleens by mechanical separation through a mesh screen followed by erythrocyte lysis using 0.84% NH₄Cl (Sigma-Aldrich). pH 7.3 and washing with CM plus 5% heat-inactivated FCS (Life Technologies-BRL). Sorting of T, B, and dendritic cells was performed using successive MACS separation columns and beads according the manufacturer’s instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The i.p. macrophages were collected from sacrificed rats by lavage with 50 ml sterile PBS into the peritoneal cavity. The fluid was collected and centrifuged for 7 min at 330 G for 7 min at 330 G. Supernatants were analyzed in duplicates using a commercial TNF production kit (R&D Systems). TNF production from BM (n = 4) and i.p. macrophages isolated from DA (n = 4) rats exhibited no difference compared with DA. Graphs are representative of two experiments.

**Flow cytometry**

Splenocytes and blood cells were collected. RBCs were lysed and removed through successive washing with PBS. Cells were stained for 20 min at 4°C with the following Abs: CD3-APC, CD4-PE:Cy5, CD8a-PE, CD11b-FITC, and CD45RA-PE (all from BD Biosciences, San Jose, CA). Staining was visualized using a FACS Calibur (Becton Dickinston) with Cell Quest (version 3.2.1f, BD) and analyzed using FlowJo (version 8.8; TreeStar, Ashland, OR).

**Quantitative PCR**

Cultured BM macrophages were harvested after 1 and 24 h, respectively, and washed with PBS, resuspended in RLT buffer and RNA was isolated using an iScript kit (BioRad, Hercules, CA). Expression was determined using TaqMan low density arrays (TLDAs; Applied Biosystems, Ashland, OR). The targets of the TLDA are listed in Supplemental Table I. The geometric mean of 18S, hypoxanthine phosphoribosyltransferase, and GAPDH (27) was used as reference gene.

**Air pouch model**

Air pouches were formed in 8- to 10-week-old female rats according to methods described previously (28). Briefly, 20 ml of 0.2 μm filtered air was injected s.c. at the dorsal midline, caudal, to the scapulae. After 72 h, 10 ml sterile air was added to the pouches to reconstitute structure. The 10 μg LPS in 2 ml saline (Braun, Melsungen, Germany) was injected into the pouch on day 4 to induce cytokine production. Rats were sacrificed after 1 h and 2 ml of 5-4 mM EDTA (Sigma-Aldrich) solution was injected into the pouch and collected to harvest the inflammatory exudate. TNF concentration was determined using a TNF ELISA kit.

**PIA model**

PIA was induced by a single intradermal injection of 150 μl pristane (2,6,10,14-tetramethylpentadecane; ACROS Organics, Liège, Belgium) at the base of the tail in 8- to 10-week-old male rats anesthetized with isoflurane. Arthritis development was blindly monitored in all four limbs using a macroscopic scoring system. Briefly, 1 point was given for each swollen and red toe, 1 point for each affected midfoot, digit, or knuckle, and 5 points for a swollen ankle (maximum score 15 per limb and 60 per rat). Sera was collected from rats at day 20 postimmunization and α-1-acid glycoprotein levels were determined using a TNF ELISA kit.

**Figure 2.** Congenic macrophages have less TNF production than DA after TLR stimulation. TNF production from BM (A) and i.p. (B) macrophages differentiated or isolated from DA (n = 4) and R11 (n = 4) rats were stimulated with LPS or a combination of LPS and IFN-γ for 24 h. R11 macrophages produce less TNF compared with DA. Graphs are representative of three BM macrophage and two i.p. macrophage experiments. C. Splenocytes from DA (n = 4) and R11 (n = 4) rats exhibit no difference in TNF production after stimulation with LPS, LPS/IFN-γ, or Con A. D. R11 (n = 4) BM macrophages have reduced TNF production compared with DA (n = 4) after LPS, LPS/IFN-γ, zymosan, or poly(I:C) stimulation. A total of 1.5 × 10⁶ cells/well were used. Graphs are representative of two experiments. Unstimulated samples were below the detection limit. *p < 0.05 using nonparametric Mann-Whitney U test. Error bars represent SD.
glycoprotein (AGP) levels determined using ELISA according to the manufacturer’s protocol (Life Diagnostics, West Chester, PA).

**Septic arthritis model**

Eight- to 10-week-old female rats received *Staphylococcus aureus* LS-1 (1 × 10^9 CFU/rat) i.v. (30). After each inoculation procedure viable counts were performed in the leftover suspension to confirm the actual number of administered bacteria. Rats were weighed and examined for arthritis regularly by a blinded observer. Arthritis was defined as erythema and/or swelling of the joints. To evaluate the severity of arthritis, a visual clinical scoring system of 0–3 for each limb was used: 0, normal; 1, mild swelling and/or erythema; 2, moderate swelling; and 3, marked swelling. The arthritis index was constructed by adding the scores from all limbs for each rat (31). Rats were sacrificed by exsanguination under anesthesia on day 5 after inoculation. Kidneys were aseptically removed, homogenized, diluted serially in PBS, and transferred to agar plates containing 5% v/v horse blood (Boule Nordic, Huddinge, Sweden). Bacteria were grown for 24 h and then counted as CFUs in serial dilutions of homogenate. A bioassay method using the murine hybridoma cell line B9, which is dependent on IL-6 for growth, was used to detect the serum levels of IL-6 (32). B9 cells were seeded into microtiter plates (5000 cells/well), and dilutions of the serum samples or IL-6 standard were added to the wells. After 68 h incubation, [³H]thymidine (Amersham, U.K.) was added; 6 h later the cells were harvested using a Wallac Tomtec (PerkinElmer, Waltham, MA) and isotype incorporation was measured using a Wallac TriLux 1450 Microbeta (PerkinElmer).

**Statistical analysis**

Linkage analysis was performed with the statistical software R/qtl version 2.6.2 (33). The natural log of TNF after LPS stimulation of whole blood was linked to microsatellite genotypes using a Haley-Knott model. No sex-specific effects were detected. The 95% CI for the identified QTL was defined by determining external flanking markers after a drop in a 10-base logarithm of odds (LOD) of 1.5 (34). Differences in allelic effects of the QTL were measured at D4Mit12 with the nonparametric Kruskal-Wallis ranking test using JMP version 6.0 (SAS Institute, Cary, NC).

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**FIGURE 3.** Congenic BM macrophages have a reduced proinflammatory phenotype on 24 h zymosan stimulation. A, Relative expression compared with the geometric mean of hypoxanthine phosphoribosyltransferase, GAPDH, and 18S for inflammatory targets with at least a 2-fold difference between DA (n = 4) and R11 (n = 4). All targets are listed in Supplemental Table I. B, Schematic diagram of zymosan stimulation of macrophages; left panel, adapted from the TLR signaling KEGG pathway (04620). Genes in red indicate upregulation in DA macrophages after stimulation. Genes in black indicate no difference between DA and R11. *p < 0.05 using nonparametric Mann-Whitney U test. Error bars represent SEM.
residuals were calculated by taking the individual values for each rat minus the family average. These values were then tested in R/qtl and the highest LOD score set as threshold (LOD = 2) (24). All other p values were calculated using the nonparametric Mann-Whitney U test, except for incidence that was determined using the Fischer exact test.

Results

Identification of a genetic region regulating TNF using a rat G12 AIL

We hypothesized that the induction of a TNF response is associated with a previously identified disease-regulating region on rat chromosome 4 and therefore fine-mapped the genetic region using linkage analysis in a high-resolution AIL derived from DA and PVG. AV1 rats. Blood from 463 rats from the 12th generation of the AIL was collected and subjected to LPS stimulation. The resulting TNF concentration was determined using commercial ELISA. The natural log of the values were calculated to approximate a normal data distribution and linked to genotypes of 17 markers between 78 and 117 Mb.

Linkage analysis identified a single QTL regulating TNF production upon whole blood LPS stimulation with a LOD score of 6.2 at the peak marker D4Mit12, located at 104.6 Mb (Fig. 1A). The confidence interval estimation using external markers to a 1.5 LOD drop spanned 3 Mb between D4Got215 (102.0 Mb) and D4Rat232 (105.0 Mb). A total of 45 annotated genes reside within this genomic region (Supplemental Table I). The peak marker score was well above the LOD 2 threshold calculated using family residual levels. DA alleles were linked to higher TNF production in a dominant manner (Fig. 1B).

Confirmation of phenotype in congenic rats

To confirm the linkage analysis we bred and tested congenic rat strains named R11 and R23, which contain PVG insertions of sections of chromosome 4 in a DA background (Fig. 1A). Both congenics were bred from a single, larger congenic allowing us to test the influence of a specific region, R23 or R11, while controlling for potential background contamination of donor genome. On LPS stimulation of whole blood, R11 produced reduced levels of TNF compared with DA; whereas, R23 exhibited no difference (Fig. 1C). We concluded that control of TNF specifically maps to the R11 insertion, which has external borders of 82.1 and 108.0 Mb, and that background genetic contamination does not contribute to this phenotype. Cellular population differences in blood did not contribute to the differences in TNF production. CD3^+CD4^+ and CD3^+CD8^+ T cell, CD45RA^+ B cell, and CD11b^+ monocyte proportions were equivalent between the R11 and DA strains (Fig. 1D).

Differential TNF regulation maps to the macrophage population

We next sought to determine whether TNF production differed at a cellular level and also to determine the cell type responsible for this difference. The i.p.- and BM-derived macrophages, which may represent varied phenotypes and activation states, were respectively stimulated with LPS or a combination of LPS and IFN-γ, followed by assessment of TNF levels using ELISA. At 6 h (data not included) and 24 h, both DA BM (Fig. 2A) and i.p. (Fig. 2B) macrophages had increased production of TNF compared with R11 macrophages. Heterogeneous splenocytes were subjected to stimulation with either LPS, a combination of LPS and IFN-γ or Con A, but exhibited no difference in TNF production (Fig. 2C). To determine whether isolated T, B, or dendritic cells were capable of differentially producing TNF, we sorted these cell types from spleens. T cells exhibited no difference on Con A stimulation (data not included). Sorted B cells and dendritic cells yielded no difference in TNF levels after LPS stimulation (data not included). We therefore concluded that genetic polymorphisms between DA and PVG, which lie within the R11 congenic profoundly and specifically control macrophage TNF production on LPS stimulation.

Several TLR signaling pathways differentially regulate TNF

We next aimed to determine whether the TNF regulation was LPS-specific. We targeted several innate immune pathways that use different intracellular signaling molecules and are known to regulate TNF levels. We tested activation of TLR2/6 (zymosan), TLR3 [poly (I:C)], TLR4 (LPS), and the IFN-γR. DA BM macrophages had increased TNF production after stimulation with LPS, LPS/IFN-γ, poly(I:C), or the yeast cell wall component zymosan, respectively (Fig. 2D). Zymosan stimulation induced the greatest levels of TNF and was used for further mRNA experiments. IFN-γ alone caused no TNF production (data not included). We concluded that a convergence point of several TLR signaling pathways is genetically regulated and leads to differential regulation of TNF in DA and R11 macrophages.

The proinflammatory cascade in macrophages is differentially regulated

Uregulation of the inflammatory cytokine TNF is likely to coincide with an altered macrophage phenotype through direct signaling and indirect cascade effects. To investigate the macrophage phenotype on stimulation, we analyzed 45 selected inflammatory targets for mRNA expression using TLDA (Supplemental Table I). Expression was tested using cDNA from DA and R11 BM macrophages stimulated with zymosan for 1 and 24 h, respectively. After 1 h, macrophages were characterized by regulated expression of several inflammatory receptors, including ICAM, decay accelerating factor (DAF1/CD55), FcγR2b, and FcεR2a (Supplemental Fig. 1). After 24 h, a more pronounced phenotype was detected. DA macrophages exhibited >2-fold increases in TNF, VCAM, STAT1, inducible NO synthase, metallothionein 1a, matrix metalloproteinase (MMP)-9, MMP-12, IFN-γR1, FcγR2b, CXCL10, CD86, CCR7, and CCL22 compared with R11 (Fig. 3A). A range of proinflammatory molecules representing cytokines, chemokines, receptors, channel proteins, enzymes, and enzyme inhibitors were upregulated in association with TNF production in DA macrophages (Fig. 3B). Interestingly, TIMP-3, a known inhibitor of MMP-9 (35), was upregulated in R11 macrophages early after activation.

TNF regulation in vivo

We next tested the TNF production capacity of DA and R11 rats in vivo using an LPS-injected air pouch, which is a model of local inflammation. TNF production measured by ELISA of exudate collected 1 h after LPS injection into air pouches of DA (n = 14) and R11 (n = 11) rats. Results are representative of two experiments. Error bars represent SEM. p < 0.05, using nonparametric Mann-Whitney U test.
inflammation. On introduction of LPS into the air pouch TNF protein levels increase dramatically by 30 min and remain high for several hours (36). Our results after 1 h revealed that R11 rats produced less TNF on stimulation compared with DA rats with R11 air pouches having a 25% reduction in TNF levels (Fig. 4).

**TNF and inflammatory mediator production regulates disease susceptibility**

Finally, we sought to investigate if this genetically regulated differential TNF production and altered macrophage phenotype had an impact on inflammatory diseases in vivo. We hypothesized that a decreased capacity to produce TNF may render rats less susceptible to experimental models of disease, as TNF is known to be an important cytokine in many inflammatory conditions (37). The R11 congenic was protected in a MOG-induced EAE model (Fig. 5A), confirming our previous study (24). There was a reduced cumulative EAE score (Fig. 5B) and maximum EAE score (Fig. 5C), which concords with our previous findings that R11 should control EAE severity phenotypes.

We then determined that R11 rats were protected from PIA (Fig. 5D). Congenic rats had reduced daily scores, but no difference was determined for incidence, onset, or maximum score (data not included). We also tested subclinical serum concentration of the acute phase protein α-1–AGP and determined elevated levels in DA rats at day 20 postimmunization (Fig. 5E). AGP levels correlated strongly with clinical disease score (Fig. 5F). No difference was determined between R11 and DA rats for susceptibility or severity of another arthritis model, CIA (data not included).

We next investigated DA and R11 rats in a sepsis model. There was no difference in clinical septic arthritis scores (Fig. 6A). However, R11 rats had less weight loss (Fig. 6B) during sepsis and reduced serum IL-6 production (Fig. 6C) and bacterial kidney load (Fig. 6D) at 5 d postsepsis induction. Our findings demonstrate that macrophage TNF levels determine severity of multiple inflammatory disease models.

**Discussion**

TNF was originally praised for its ability to kill tumor cells (1) and described as a mediator of the wasting disease cachexia (38).

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**FIGURE 5.** R11 congenic rats are protected from EAE and PIA. A, EAE plot of mean score over time. R11 rats (n = 40) have reduced EAE compared with DA rats (n = 29) from day 17 onward. R11 congenic rats have a decreased cumulative EAE score (B) and maximum EAE score (C). D, PIA plot of mean score over time. R11 (n = 9) rats have reduced scores compared with DA (n = 12) after day 19. E, DA rats have elevated day 20 serum α-1–AGP levels. F, AGP levels strongly correlate with clinical disease score. *p < 0.05; **p < 0.01; ***p < 0.001 using nonparametric Mann-Whitney U test. Error bars represent SEM.

**FIGURE 6.** R11 congenic rats are protected against sepsis. A, No difference between R11 (n = 10) and DA (n = 10) rats in sepsis induced arthritic score. B, R11 rats have reduced weight loss after sepsis induction compared with DA. R11 rats have reduced serum IL-6 levels (C) and kidney bacterial load (D) 5 d postsepsis induction. Error bars represent SEM. Results are representative of two experiments. *p < 0.05; ***p < 0.001 using nonparametric Mann-Whitney U test.
However, the understanding of its role in inflammation has greatly expanded; TNF can induce apoptosis, cause activation of inflammatory cells as well as orchestrate immune resolution (37). This may be attributed to different types of TNF (membrane-bound versus secreted), two signaling receptors, localization of the protein, timing of release, and coactivation with other external and internal molecules. Our study explored genetically regulated TNF production in rats using linkage analysis in a high-resolution AIL, congenic breeding, in vitro cellular phenotyping, and in vivo disease models. We have determined important differences between parental DA and congenic R11 rats that regulate TNF levels and disease severity due to the allele substitution at a single QTL.

We mapped TNF production after LPS stimulation, which could be considered a subphenotype of several diseases with a pathophysiology that includes dysregulated TNF levels. The use of a simple subphenotype allowed for efficient testing and permitted us to use a single set of AIL rats to define a common genetic region regulating inflammatory diseases. Several successful human studies have also used subphenotype mapping. Polymorphisms within IL2RA, a type 1 diabetes risk gene, are correlated with cellular protein levels (39) and a strong association exists with the subphenotype of circulating levels of the soluble IL2RA biomarker (40). In addition, low-density lipid levels are used as a subphenotype for cardiovascular and cerebrovascular diseases and provide information on disease progression and treatment targets (41). Similarly, subphenotypes can be used to distinguish subgroup within a disease, as is the case with anticitrulline Ab levels in RA, which are associated with different genetic determinants (42).

We described differential TNF production that is genetically regulated between parental DA and congenic R11 macrophages. The TNF locus is genetically identical between the strains and therefore does not contribute to differential regulation. In vitro experiments stimulating multiple TLR signaling cascades demonstrated that a common intersection point is affected by the TNF-regulating alleles at the innate immune system level. MyD88-dependent (TLR2 and 4) and -independent (TLR3 and 4) stimulations (43) were under similar genetic regulation, with DA macrophages producing more TNF compared with their R11 counterparts. Thus, activation of endosomal TLR3 by poly(I:C) leading to IFN response factor 3 activation (44), as well as signaling through other TLRs causing activation and translocation of AP-1 and NF-κB (45), all similarly determined TNF production levels. We speculate that the responsible gene is either a signaling modulator or a transcription factor that is part of a cascade with widespread inflammatory effects. Alternatively, posttranscriptional modifications of TNF mRNA could equally determine differential protein levels between the strains, as has previously been demonstrated in activated macrophages (46). Furthermore, the initial differences in TNF production by macrophages may result in altered patterns of stimulation for other cell types, such as T cells, which could exacerbate the skewed mRNA and protein cytokine levels in vivo.

The differences in TNF levels and altered macrophage phenotypes affected severity of disease but not susceptibility. The biological effect of differential TNF production could be at the peripheral priming or target restimulation stages when macrophages act as APCs to T cells and dictate the effector T cell subset, at the macrophage effector stage when tissue destruction occurs and/or during resolution of ongoing inflammation. With clinical differences in EAE and PIA but not CIA, it seems that T cells may be an important component determining disease severity (47, 48), as the role of B cells and Abs in CIA is more relevant (49). In addition, IFN-γ levels in T cells are regulated during experimental autoimmune neuritis by the same locus, which argues for adaptive immune system regulation (23). At the same time, the proinflammatory macrophage phenotype in DA rats would indicate their role as effector cells contributing to disease severity through damaging MMP expression as well as recruitment of other cells through chemokine release. In support of this theory, macrophages are found in MS and EAE lesions as well as RA joints (50–52). The sepsis experiments determined interesting results; increased TNF associated with both weight loss, likely due to subclinical disease and cachexia, and unexpectedly reduced bacterial clearance, possibly due to macrophage autophagy (53).

This genetically regulated pathway leading to higher TNF production and proinflammatory activation in macrophages conforms well to previously demonstrated pathogenic roles of TNF. Genetic studies on TNF in the mouse have employed transgenic and knockout strategies to evaluate the role of TNF in several diseases. Overproduction of TNF in the pancreas of the nonobese diabetic mouse increases the rate of diabetes (54). The TNF<sup>−/−</sup> mouse has reduced arthritis (55) and EAE (56); whereas, the TNF<sup>R1</sup>−/− mouse is protected from EAE (57) and endotoxin-induced shock (58), which corroborates our findings that reduced TNF signaling is protective. Furthermore, neutralization of TNF prevents the development of septic shock in animal models (59), which indicates that reduced TNF in R11 rats may lead to less severe infection. However, it is important to note that TNF is not always detrimental and that the role of TNF may change during disease course. T cell-specific TNF ablation results in increased bacterial infection, indicating that TNF is required to mount an effective immune response (60). In addition, dual beneficial and harmful roles of TNF have been proposed for experimental diabetes (61) and EAE (62). Interestingly, TNF blockade in MS was an ineffective therapeutic option (13) and some patients treated with TNF blockade have developed demyelinating conditions (63). Therefore, it is important to dissect different TNF pathways and their roles in different cell compartments, which can be performed genetically as demonstrated here.

Individualized tailored therapy may be achieved through prediction of individuals who are predisposed to high TNF production. It has been proposed that genetics may determine TNF blockade response in patients; however, to date no single genetic factor is unequivocally associated with this difference (64). We have used unbiased linkage analysis to define a region regulating TNF production in macrophages. The identification of the responsible gene(s) will not only provide a potential therapeutic target, but also enable dissection of mechanism(s) controlling inflammation for development of new therapies.

Disclosures
The authors have no financial conflicts of interest.

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