

Polymorphic Structure of the Tumor Necrosis Factor (TNF) Locus: An NcoI Polymorphism in the First Intron of the Human TNF- β Gene Correlates with A Variant Amino Acid in Position 26 and a Reduced Level of TNF- β Production

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Summary

Since a dysregulated synthesis of tumor necrosis factor α (TNF- α) may be involved in the pathogenesis of autoimmune diseases, it was of interest to precisely locate the recently reported NcoI restriction fragment length polymorphism (RFLP) of the TNF- α region. However, by mapping of 56.8 kb of overlapping cosmid clones and direct sequencing, we could localize the polymorphic NcoI restriction site within the first intron of the TNF- β gene and not in the TNF- α gene. To study whether regulatory mechanisms are affected by this polymorphism, we analyzed the TNF- α /TNF- β production of phytohemagglutinin-stimulated peripheral blood mononuclear cells of individuals homozygous for the TNF- β NcoI RFLP by ELISA and concomitant Northern blot analysis. On days 2–4 after stimulation with mitogen, the TNFB*1 allele corresponding to a 5.3-kb NcoI fragment presented with a significantly higher TNF- β response. A mRNA analysis demonstrated that higher protein levels of TNF- β correlate also with increased amounts of TNF- β transcripts. No allelic association was found in respect to TNF- α production. To further investigate a possible allelic influence on transcription, we determined the DNA sequence of 2 kb of the 5' portion of our cloned TNFB*2 allele and compared it with the available TNF- β sequences. By computer-aided recognition motif search of DNA binding factors, we report putative binding sites conserved between mouse and man in the 5' flanking region as well as in intron 1 of the TNF- β gene, found also in other cytokine promoter sequences. In addition, by polymerase chain reaction amplification and sequencing of 740 bp of the 5' part of TNF- β of individuals typed homozygously for the NcoI RFLP, we could show that amino acid position 26 is conserved as asparagine in the TNFB*1 and as threonine in the TNFB*2 sequence. A previously reported, EcoRI RFLP in the 3' untranslated region of TNF- β does not segregate with either of the two alleles. Thus, four TNFB alleles can be defined at the DNA level.

Tumor necrosis factor α (TNF- α) (cachectin) and TNF- β (lymphotoxin) are cytokines with numerous similar immunoregulatory effects binding to their target cells via cell surface receptor molecule types A and B with different affinities (see references 1–6). The tumoricidal and tumorstatic effects of TNF- α and TNF- β raised much interest and led to the rapid cloning of the genes in mouse and man, as well as to the production of rTNF- α and rTNF- β (7–10). The broadest range of bioactivities has been described for TNF- α (see for reviews references 1–3), including activation of the transcription factor NF- κ B (11, 12, 12a). The two cytokines are both produced by different activated PBMC. While TNF- β ap-

pears to be mainly produced by stimulated T cells, TNF- α is the product of activated macrophages, as well as of T, B, and NK cells (see references 13–15). The localization of the TNF genes in tandem, within the MHC between HLA-B and the HLA class III genes (16–19), has raised the suspicion that the TNF loci might play a role in the notable association between HLA and various autoimmune diseases. In a mouse model for SLE nephritis, both a decreased TNF- α production, and a RFLP have been correlated with the development of the disease (20). However, the linkage of a decreased TNF- α synthesis with a mutation in the TNF- α locus has been questioned on the basis of RFLP analysis in a large

number of inbred strains and wild mice, demonstrating the presumptive autoimmune TNF- α allele to be present in many mouse strains (21). The search for a possible involvement of TNF- α in human autoimmunity led to the detection of polymorphisms in the human TNF genes. Several groups described a NcoI RFLP for the TNF- α gene with the less frequent allele (5.5-kb NcoI fragment) being strongly linked with the HLA-A1/B8 haplotype (22, 23). The 10.5-kb NcoI allele was found to be negatively associated with primary biliary cirrhosis (24), and heterozygosity for the TNF alleles was significantly more frequent in patients with type I insulin-dependent diabetes mellitus (22). In addition, a loss of an EcoRI recognition sequence of low frequency in the 3' untranslated region of the TNF- β gene has been described (25).

In this paper, we show that the polymorphic NcoI restriction site is located in the first intron of the TNF- β gene by mapping overlapping cosmid clones and direct allelic sequencing. In addition, the EcoRI RFLP of the 3' untranslated region does not segregate with either NcoI allele, allowing at least four different alleles to be defined by RFLP typing. We further show that the two TNF- β alleles also differ by one amino acid at position 26. Functional tests demonstrate that the TNFB*1 allele is strongly associated with increased TNF- β production by PBL in response to PHA.

Materials and Methods

Isolation and Mapping of the Human TNF Region. The genomic DNA from the homozygous B cell line, established from an individual typed HLA-A3, -Bw47, -Cw6, and -DR7, was extracted, and a cosmid library in the vector pTCF was established as described (26). The human genes for TNF- α/β were isolated by crosshybridization to a murine TNF- α probe (kindly provided by Dr. Michael Steinmetz, Basel, Switzerland) in five overlapping cosmid clones covering 56.8 kb of genomic DNA and mapped with various restriction endonucleases, including NcoI and EcoRI. The restriction map of cosmid cah5 is shown Fig. 2.

Southern Blot Analysis. Genomic DNA of 70 healthy donors was analyzed according to standard protocols (27). The following DNA probes were used: a TNF- β 2.4-kb EcoRI, a TNF- α 2.75-kb EcoRI, a TNF- β 5' 700-bp BamHI, and a 1.4-kb BamHI fragment. By sequence analysis, the latter probe carries part of the human analogue to the murine B144 gene (28), which is located ~10 kb centromer of TNF- α . A 2.9-kb SalI/HindIII single copy probe, located 13 kb upstream of TNF- β , was also used. All probes were labeled by the random-priming method (29). Hybridization was performed with 5×10^6 cpm/ml according to the method of Church and Gilbert (30). All donors in this study were typed for HLA class I and most also for class II.

The following donors were tested homozygous for the NcoI 5.3-kb TNFB*1 allele: BC, A1 B8 Cw7 DR3/4 DQw2/w3; BK; A3/28 B35/w62 Cw3/w4 DRw11; DC; A2 B27/51 Cw2/w3; FB, A1/2 B8/w62 Cw3/w7; JC, A1/3 B7/51 Cw3/w7 DQw1/3; KP, A2 B35/w39 Cw4 DRw6/w8 DQw1/w3; KR, A2 B44 Cw5; LG2, A2 B27 Cw1; SA, A1 B8 Cw7 DR3 DQw2; SE, A1/3 B8 Cw1; WW, A2/3 B27/44 Cw2/w5. The following donors were typed homozygous for the 11-kb NcoI fragment (TNFB*2): BCA, A2/24 B35/w60 C3/w4 DR2/w8 DQw1/w4; BS, A2 B7/13 Cw6/w7 DR7/DQw2; BU; A24/30 B18/w61 Cw2/w5 DR3/5 DQw2/w3; EC, A3/29 B7/44 Cw5/w7 DR2/3 DQw1/2; GB,

A11/24 B27/35 Cw2/w4; GRU, A1/29 B7/44 DR2/7 DQw1/w2; HP, A2/26 Bw62 Cw3; KW, A2/3 Bw60/w62 Cw3 DR3/4; MG, A2/11 B7/49 Cw7 DR2/3; MS, A11 B51/w62 Cw3 DR2/4 DQw1/3; PG, A2/3 B7/w62 Cw3/w7 DR4 DQw3; PH, A1/3 B7/w55 Cw3/w7 DR2/5; PS, A2/32 Bw22/44 Cw3/w4 DR5/7 DQw2/w3; RK, A3/w33 B7/w64 Cw7 DR1/2 DQw1; RO, A2/28 B51 DR2/7 DQw1/w2; SD, A2/3 B35/37 Cw4/bl DR1/2 DQw1; SG, A2 B49/w62 Cw4 DR4; SM, A1/2 B27/49 Cw2 DR2/5; SU, A24/32 B18 Cw3 DR2/4 DQw1/w3; WE, A2/30 B13/w62 Cw3/w6 DR2/4.

PCR Amplification of Genomic DNA. Genomic DNA was extracted from PBL, and amplification of TNF gene regions (see for lower bars in Fig. 2) was performed as described by Saiki et al. (31), using the Hybaid thermocycler (Biometra, Göttingen, FRG). 30 cycles encompassing 1 min of denaturation, annealing, and extension were run. The initial denaturation time was 6 min. Specific oligonucleotide primers for the NcoI/amino acid 26 reaction are depicted in Fig. 3, annealing temperature was 55°C. All oligonucleotides were synthesized by the phosphoramidite method using a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and used directly after deprotection. For amplification of the EcoRI polymorphic region, a 5' primer (TCCTCAGCCCTAGTACTGTC) and the sequence CCTGCAGGTGGACAAGACC as 3' primer were chosen; the annealing temperature was 59°C. For direct genomic DNA sequencing of the 5' flanking TNF- α region, 312 bp of DNA were amplified by priming with the oligonucleotides CAGCATATGAGTCTCCGGGTC and CTAACCTCCAGACAGGATGC (annealing temperature 57°C). 1.6 U of Taq-polymerase (United States Biochemical Corp., Cleveland, OH) were used per reaction. The amplification products were directly digested either with NcoI or EcoRI and analyzed on 1.5% agarose gels. In all amplification experiments, DNA of the homozygous B cell line CAH from which the cosmid cah5 has been isolated was incubated as internal control.

DNA Sequence Analysis. Single-stranded M13 subclones, as well as double-stranded pUC subclones, were sequenced by the dideoxy chain termination method described by Sanger et al. (32), using either a T7 sequencing kit (Pharmacia Fine Chemicals, Freiburg, FRG) or the Taq-Track method (Promega Biotec, Madison, WI). The PCR-amplified DNA products were purified by agarose gel electrophoresis and phenol/chloroform extraction and sequenced as described for the subclones. No differences were found between sequences determined from the isolated cosmid cah5 or those obtained from amplicate of genomic CAH DNA. The denaturation step was performed at 96°C for 5 min and the annealing procedure was shortened to 10 min at 37°C.

Stimulation of T Lymphocytes. PBMC were separated from heparinized venous blood on a Ficoll-Hypaque density gradient (Pharmacia Fine Chemicals). PBMC were kept in RPMI 1640 supplemented with 2% L-glutamine, 1% penicillin/streptomycin (both from Gibco Laboratories, Grand Islands, NY). For analysis of lymphokines, 2×10^6 cells were stimulated by 2 μ g/ml PHA (Sigma Chemical Co., Deisenhofen, FRG) and harvested after 1, 2, 3, or 4 d. All supernatants were centrifuged and frozen immediately at -80°C. Control experiments with unstimulated cells were always performed in parallel.

Quantitation of TNF- α and TNF- β Protein Titer. TNF- α and - β were measured by a sandwich ELISA in polystyrene microtiter plates (Flow Laboratories, Meckenheim, FRG), coated with anti-TNF- α mAb 199-1 or anti-TNF- β mAb 9B9, respectively (5 μ g/ml in sodium bicarbonate buffer at pH 9.5). The plates were blocked with 1% BSA (Sigma Chemical Co.). 100 μ l of a serially diluted test sample or recombinant lymphokines at various concentrations as external standards were added to each well and incubated over-

night. Bound lymphokine was detected by biotinylated anti-TNF- α mAb 195-8 or biotinylated polyclonal anti-TNF- β antiserum, followed by incubation with streptavidin coupled to horseradish peroxidase (both from Boehringer Mannheim Biochemicals, Mannheim, FRG). Tetramethylbenzidine (Serva, Heidelberg, FRG) in acetic acid buffer (pH 4.9) was used as chromogenic substrate. Extinctions were read in an ELISA spectrophotometer (SLT, Grödig, Austria) at 450 nm and were evaluated using a four-parameter analysis. rTNF- α and rTNF- β , as well as the mAbs 199-1, 195-8, and 9b9 and the polyclonal anti-TNF- β antiserum, were kindly provided by Dr. A. Möller (BASF AG, Ludwigshafen, FRG). The sensitivity of the ELISA was 10 pg/ml for each lymphokine.

RNA Isolation and Northern Blot Analysis. RNA was isolated by the method of Chirgwin et al. (33). To quantitate the TNF response upon PHA stimulation of PBMC, the TNF protein levels after the indicated time were measured in the culture supernatants. For RNA isolation, 8×10^6 cells were harvested from a parallel stimulation experiment, started simultaneously but treated for 3 h with 10 μ g/ml of cycloheximide (Sigma Chemical Co.) before harvesting. Totally lysed cells in the extraction buffer were sonified for 5 s to achieve breakage of the DNA with a cell disruptor with microtip (B15; Branson Sonic Power Co., Danbury, CT), and the RNA was prepared by CsCl gradient centrifugation. Total RNA of $6-8 \times 10^6$ cells was fractionated by formaldehyde/agarose gel electrophoresis and blotted to Hybond-N membranes (Amersham, Braunschweig, FRG). As specific probe for TNF- α , a genomic 625-bp XhoI/HindIII fragment coding for exon 4/3' untranslated region was used. The 600-bp PvuII/PstI TNF- β -specific probe was prepared of the corresponding TNF- β region. All DNA probes were labeled and hybridized as described above. The Northern blots were completely stripped off of the probe by incubation for 1-2 h at 65°C in 0.1% Denhart's, 5 mM Tris-HCl, and 2 mM EDTA, and rehybridized after blank exposure.

Results

The Polymorphic NcoI Recognition Sequence Is Located in the First Intron of the TNF- β Gene. We analyzed genomic DNA from a panel of 70 healthy unrelated individuals for the presence of a polymorphic NcoI restriction site. Hybridization

with a genomic 2.4-kb EcoRI TNF- β probe (see Fig. 1) detected a 5.3-kb band in addition to the 11- and 5.7-kb NcoI fragments hybridizing to the 2.75-kb EcoRI TNF- α probe. This result indicated that the polymorphic NcoI recognition sequence is located within the TNF- β gene. Inspection of the published sequences for the human TNF- α and TNF- β genes showed the presence of one NcoI restriction site in the 3' untranslated region of TNF- α (34, 35) and another one in the first intron of TNF- β (34), resulting in the 5.7-kb TNF NcoI fragment, detected on the Southern blots. To confirm the location of the polymorphic NcoI site in TNF- β , we constructed a NcoI restriction map of the TNF region (Fig. 2). Cosmid cah5, containing the 11-kb NcoI TNF region fragment, was isolated from a genomic cosmid library derived from the homozygous B cell line CAH (HLA-A3, -Bw47, -Cw6, -DR7), homozygous for the TNF 11-kb NcoI fragment (see Fig. 1). The restriction map shows that the NcoI site in the first intron of the TNF- β gene is missing in DNA containing the 11-kb NcoI TNF allele. We wondered whether the two TNF- β alleles (the 5.7/5.3-kb NcoI fragments are indicative for the allele further on TNFB*1, and the 11-kb NcoI fragment corresponds with the presence of the TNFB*2 allele) only differ by the polymorphic NcoI restriction site or whether additional mutations are present within the coding region or in regulatory sequences. We therefore determined 2 kb of DNA sequence in the 5' gene region spanning the polymorphic NcoI site. By sequence comparison to the TNFB*1 sequence (34), another base substitution is present in intron 1, besides the G to A mutation in the NcoI recognition sequence, and a third base exchange results in an amino acid substitution (amino acid 26: AAC = Asn to ACC = Thr for TNFB*2) (Fig. 3, boxes). To define additional allelic differences, we performed both Southern blot analysis and/or PCR amplification of 16 TNFB*1 and 16 TNFB*2 homozygous alleles, respectively, using the restriction enzyme EcoRI (see Fig. 2), as a previously reported polymorphism in the 3' untranslated region was described with a frequency of 6% (25). No homozygous 2.5-kb EcoRI sub-

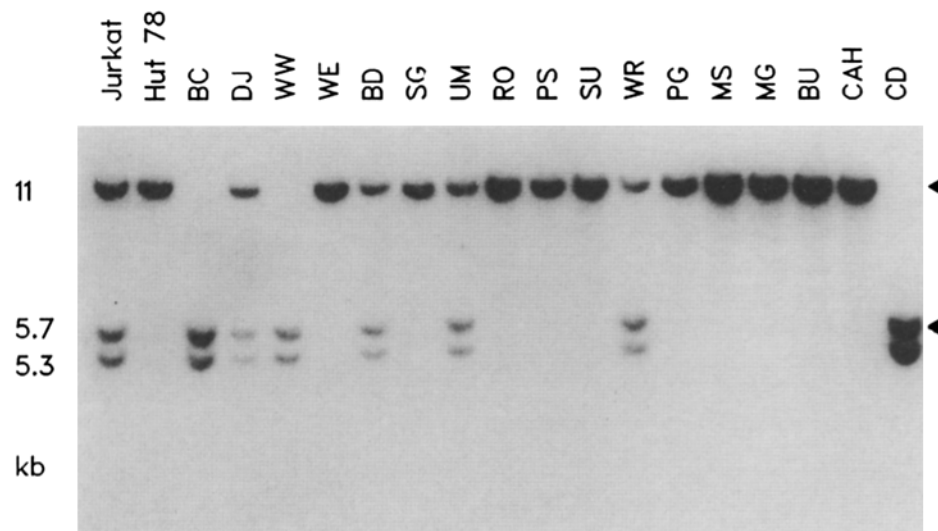


Figure 1. Genomic Southern blot analysis of the NcoI polymorphism in the TNF locus. Genomic DNA isolated of various cell lines (Jurkat, Hut 78, CAH) and of PBL of 16 individuals was digested with NcoI, and 10 μ g of each digest was separated on a 0.7% agarose gel, transferred to nylon membrane, and sequentially hybridized to the 2.75-kb EcoRI fragment of the TNF- α gene and the 2.4-kb TNF- β EcoRI fragment (see Fig. 2). Closed arrows on the right indicate the NcoI bands detected with the TNF- α probe, whereas the TNF- β probe hybridized to the 5.3-, 5.7-, and 11-kb NcoI fragments. Molecular weights in kilobases on the left are given in the sizes as obtained from the isolated cah5 cosmid.

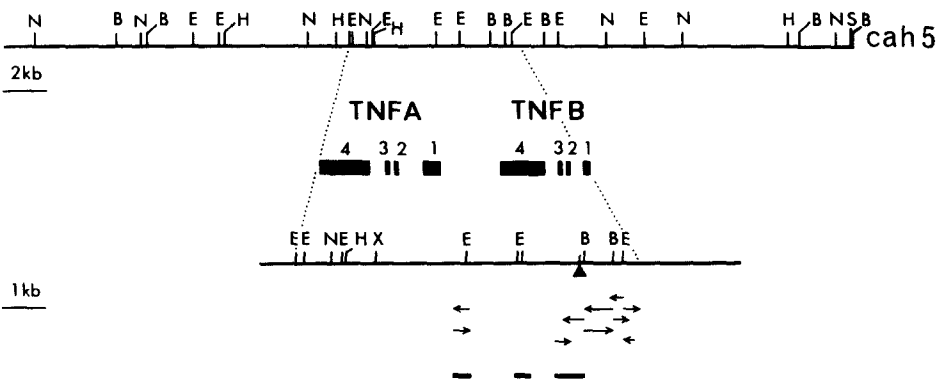


Figure 2. Structure of the TNF locus isolated in cosmid cah5. The restriction map of cosmid cah5 in its centromer to telomer orientation in the HLA complex is shown on the top line, and an extended map of the TNF locus is presented below. The exons of the TNF- α (TNFA) and the TNF- β (TNFB) genes are indicated as closed boxes with the numbering above. Arrows underneath mark the orientation and the extent of the DNA sequencing. The bars at the bottom show the fragments that have been PCR amplified from genomic DNA. The following restriction sites are indicated: B, BamHI; E, EcoRI; H, HindIII; N, NcoI; S, Sall (X, XhoI, only shown for the extended TNF locus). The filled triangle marks the polymorphic NcoI site in intron 1, which is absent in the TNF- β gene of the cah5 allele (TNFB*2).

type was found, only one heterozygous individual (HLA-A2, -Bw60/-w62, -Cw3, -DR3/4) out of 32 with the 2.4-kb EcoRI fragments was detected. The further use of the B144 and 2.9-kb Sall/HindIII DNA probes (described in Materials and Methods), both in close vicinity to the TNF locus, did not detect any RFLP in the DNA of 40 individuals investigated.

The Polymorphic NcoI Site Is Linked with an Amino Acid Substitution at Position 26. Several TNF- β sequences (protein, cDNA, and genomic) have been published that either code for AAC = Asn (10, 34, 36) or contain in exchange ACC = Thr at amino acid position 26 (9, 35, 36). We wondered whether the NcoI RFLP is linked with the presence of AAC at amino acid position 26. We therefore amplified 740 bp of genomic DNA from exon 1 to intron 3 encompassing the polymorphic NcoI site and the variant triplet coding for amino acid 26. Amplified genomic DNA of nine individuals and one amplificate of a gorilla, each homozygous for the 11-kb band, and five human DNAs homozygous for the 5.7/5.3-kb NcoI fragments, were directly sequenced. In all cases, the CCATGG (NcoI) sequence and the additional substitution G at position 368 correlated with the triplet AAC, and CCATGA with C at position 368 and ACC (Fig. 3, boxes). Furthermore, heterozygous DNA as amplification/sequencing control showed both bases. This result demonstrates the existence of two TNF- β protein variants and that the NcoI RFLP is indicative for this polymorphism in the TNF- β protein. Thus, two allelic forms for the TNF- β gene are found at the protein level and four alleles can be detected at the DNA level (NcoI and EcoRI RFLP). Further sequence comparison with the genomic DNA sequence reported for the TNFB*1 allele (34), and the partial sequence described by Nedospasov et al. (35) derived from a TNFB*2 allele, resulted in two additional nucleotides, not found in our cah5 sequence (Fig. 3, arrows), besides the three allelic nucleotide changes discussed above (Fig. 3, boxes). The TaqI restriction site, reported by both authors following the EcoRI

5' site (deletion of C in position -808; Fig. 3, arrow) is not present in the cah5 TNFB*2 allele. We analyzed DNA of 38 unrelated individuals for the presence of this 5' flanking TaqI site by genomic Southern blot analysis and hybridization with the 2.4-kb EcoRI TNF- β gene fragment and the 5' 700-bp BamHI probe. We only detected the TaqI fragments corresponding to the cah5 TNFB*2 allele (data not shown). Only one substitution has not been redetermined for both TNFB alleles. Interestingly, this A instead of the G at -626 is within a putative TPA-responsive DNA element (TRE)¹ sequence in the cah5 TNFB*2 allele, with high affinity for the group of inducible nuclear factors AP-1/c-fos/jun, and probably would change the binding affinity.

Analysis of Putative Regulatory Elements of the TNF- β Gene. We analyzed the TNFB and TNFA promoter regions for the presence of known nuclear factor binding motifs. In Fig. 3, we marked the DNA sequences that either have been shown to bind transcription factors, or that have been shown in other studies to be of high affinity, some of which are conserved in the TNF- β genes between man and mouse (37, 38). The TNFB-kB motif located at -95 bp, which binds NF-kB with high affinity (Messer, G., manuscript submitted for publication), and the TATA box as well as a SP-1 factor binding sequence in between, are exactly conserved in sequence and position in mouse and man. A potential NF-kB binding site is present at position -1030 bp, which is located ~935 bp upstream of the high affinity -95-bp NF-kB motif. By gel shift competition assays (data not shown), we could demonstrate that this sequence binds NF-kB with 10-fold lower affinity than the -95 kb element, but with higher affinity than the kB binding site found functional in the IL-2 promoter (39). The significance of the -1,030-kb NF-kB binding site is not clear yet, as this sequence is contained within the Alu repeat, and it has not been demonstrated so far that single

¹ Abbreviation used in this paper: TRE, TPA-responsive DNA element.

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-1220
(t)15gagacagagcttgcctgctccccaggctggaatacagtggtcgcgacttgactcactgcagcctccgcctccagggtcaataatctccagcctcagcctcccgagtagct
                                NF-κB
gggactgcagatgcccaccagcagcctggctaattttgattattatagagatgagtttcacatgttgccagcgtggtctcaaactcctgacctcaagtaatccgccaccctcag
-970
actccc aaagtgccaggattacaggtgtgagccactgcaccaggtcggaacaat ttaaataatgtattggctctgcaaatgcagcttcagaacaagtccttagctgtccccacc
← Alu
accctaagtaccacccttaagcctaccctatggaattctgaaactcctttgtagaaaactttggaaggtgctgcccacattgatcctggaatgtgtttatctgggttatataa
-730
atctgttctgtggaagccacctgaagtcaggaagagatggagggtcctctcaggagtgagatgagacctcatcacttgactgtccagcatcctctgagttcaggggaccataaaa
↑                                     TRE
tctcttccaaactaggacactttcaagagtgaagggggatccattaatatcttcacctggacaagaggcaaacaccagaatgtccccgatgaaggggatataataatggacctcttga
-490
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-250
ctgaccaccctcctggcctccagccacagattcccctgaccgactccctttccagaactcagtcgctgaaccccgagcctgtggttctctctagccctcagccttctctgcct
TRE           NF-κB           SP-1
tgcctgaaacacagcagatcttctaagccctgagggttccccgggccccagccccgacctagaaccgctcctgctgcccagctgcacctgccccttctctatataggcctgag
-10       +1           SP-1
cgtccgggcccCAGGGGCTCCGCACAGCAGGTGAGGCTCTCTGCCCATCTCCTTGGGCTGCCGCTCGTGCCTTTGGACTACGGCCAGCAGTGTCTGCCCTCTGCTGGCCCTCG
GTCCCTCTGCACCTGCTGCTGGATCCCCGGCCTGCTGGCCCTGGGCTTGtgggttggtttggtttctctctgctctcctcctccatctctcagctcattgtctctgtcac
+231
acattctctgtttctgcatgagctccctctgttcccctctctctctctctctctcctctgctcacctggggttctctgactgcatctgtcccctctctctgctgctctctctc
g           SP-1           SP-1           met thr pro
gggggctcggggggtctctcctccagggcgagggtctgctctccgcccgtgcccggctcccgctcactgtctctctctctctctctcttctctgcagGTTCTCCCC ATG ACA CCA
pro glu arg leu phe leu pro arg val cys gly thr thr leu his leu leu leu leu gly leu leu leu val leu leu pro gly ala gln
CCT GAA CGT CTC TTC CTC CCA AGG GTG TGT GGC ACC ACC CTA CAC CTC CTC CTT CTG GGG CTG CTG CTG GTT CTG CTG CCT GGG GCC CAG
+558
gtgaggcagcaggagaatggggctgctggggtggctcagccaaaccttgagccctagagccccctcaactctgtttctcccctag          gly Leu Pro Gly Val Gly Leu Thr
GGG CTC CCT GGT GTT GGC CTC ACA
Pro Ser Ala Ala Gln Thr Ala Arg Gln His Pro Lys Met His Leu Ala His Ser Thr ACC Leu Lys Pro Ala Ala His Leu Ile g
CCT TCA GCT GCC CAG ACT GCC CGT CAG CAC CCC AAG ATG CAT CTT GCC CAC AGC ACC CTC AAA CCT GCT GCT CAC CTC ATT G gtaaacatcc
          +792
acctgacctcccagatgtccccaccagctct

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Figure 3. The 5' sequence of the human TNF- β gene (TNFB*2), derived from *cah5*. The nucleotide sequence of the exons is given in capital letters to distinguish it from 5' flanking and intronic nucleotides numbered in relation to the transcription start site (+1) (9, 10). The protein coding DNA sequence is presented in triplets with the amino acids displayed above and the sequence of the mature TNF- β polypeptide in capital letters. The amino acid substitution in the TNFB*1 sequence is shown below the variant triplet. The three nucleotide changes in the TNFB*1 allele, including the resulting NcoI recognition sequence and the variant amino acid at position 26, are boxed. The NF- κ B binding sites, the TATA box, and the two sequences used as oligonucleotides for PCR amplification are underlined. The 3' border of the Alu repetitive element in the 5' flanking region is marked. Nucleotides in which the published TNF- β genomic sequences (34, 35) differ from the *cah5* sequence, but which have been confirmed by restriction enzyme digestion or PCR amplification and direct sequencing of several TNFB*1 and TNFB*2 alleles to be identical to the cloned *cah5* gene, are indicated by an arrow. One difference in position -626 was not checked in our amplification/sequencing protocol and is shown as such below the *cah5* nucleotides. DNA sequence motifs for the interaction with transcription factors are highlighted by stippling. Only those sites are shown that either have been shown by us (NF- κ B sites) or in other systems to bind the corresponding nuclear factors with high affinity, or that are conserved in sequence and location in the TNF- β gene of the mouse (5' AP-1, NF- κ B site -95 bp, SP1 elements -65 and +410 bp, and the TATA box) (37, 38).

NF- κ B sites separated by such a distance can strongly cooperate in stimulation of transcription (40). A putative AP-2 recognition motif overlapping the 5' half-site of the -95-bp TNFB- κ B site is also found in the mouse sequence (38; EMBL/GenBank corrected version, accession number Y00467). Furthermore, two putative TRE sequences in position -626 and -126 are positioned upstream of the -95-bp NF- κ B site and were reported to be high affinity motifs of the *H-ras* gene and the murine IL-2 gene for TPA inducibility (41, 42). The -126 TRE site has the same sequence consensus and is in the ap- proximal position as the TRE found to confer IL-1 inducibility in the IL-2 promoter (43). Interestingly, the prepositioned intron 1 of the TNF- β gene, spliced out of the 5' untranslated region, also contains two additional TRE binding sites, one TRE site is a homologue of the *c-fos*/*jun* respon-

sive element of the *ras* oncogene and a reverse homologue to the murine IL-2 gene -150 TRE_p element (41, 42). Both motifs have been shown to act alone as strong inducible elements for TPA induction. Here they might cooperate synergistically due to their head-to-head orientation with three-base spacing as an imperfect palindromic motif for two AP-1 molecules, shown to increase the affinity up to 60-fold (44). The TRE elements of the TNF- β gene described above all seem to bind the AP-1, *jun*, *c-fos* heterodimer transcription factor family, and do not agree with the character of the related CRE/*c*-AMP-responsive element (45). No CRE binding motif in TNF- β was found, whereas one has been reported for the TNF- α promoter in position -105 bp (46). The first intron also has a strong binding potential, conserved in mouse, primates, and man, for the constitutively expressed transcrip-

-687
 CAGCATTATG AGTCTCCGGG TCAGAATGAA AGAAGAGGGC CTGCCCCAGT GGGGTCTGTG AATTC^{△△△}CCCGGG
 -597
 GGTGATTCA CTCCCGGGG CTGTCCCAGG CTTGTCCCTG CTACCCGCAC CCAGCCTTTC CTGAGGCCTC
 AAGCCTGCCA CCAAGCCCCC AGCTCCTTCT CCCCGCAGGG CCCAAACACA GGCCTCAGGA CTCAACACAG
 -444
 CTTTTCCCTC CAACCCCGTT TTCTCTCCCT CAAGGACTCA GCTTTCTGAA GCCCCTCCA GTTCTAGTTC
 TATCTTTTTC CTGCATCCTG TCTGGAAGTT AGAAGGAAA

Figure 4. Partial 5' TNF- α promoter region determined from cosmid cah5. The 312-bp fragment of the TNF- α promoter region (-687 to -370), corresponding to nucleotides 3411-3723 of the TNF region published by Nedospasov et al. (35), was amplified from genomic DNA using the following oligonucleotide pair (5' primer: CAGCATTATGAGTCTCCGGGTC; 3' primer: CTA^{△△△}ACTTCCAGACAG-GATGC) and sequenced directly by priming with the same oligomers. Triangles indicate nucleotides missing in the TNF- α promoter sequence reported by Nedwin et al. (34). The arrow indicates a C that is present in the other sequences (34, 35), and the dot marks the position where Nedwin et al. (34) reported an A.

tion factor SP1, and the SP1 motif close to the 3' splice site of intron 1 has the highest binding activity reported for SP1 (47).

Analysis of the TNF- α 5' Promoter Region Linked to the NcoI RFLP. As the NcoI RFLP in intron 1 of the TNF- β gene might also be linked to variations in the proximate, downstream localized TNFA, we compared the genomic sequences of the TNF- α gene that have been published together with the corresponding TNFB*1 (34) and TNFB*2 (35) alleles, though homozygosity in respect of the NcoI RFLP of the

DNA used for library construction cannot be presumed. No difference has been reported for the amino acid sequence of the genomic TNF- α clones, but intriguingly, a cluster of 10 nucleotide differences is found between the two sequences (Fig. 4) (35) within a 160-bp long G/C-rich region of the TNF- α 5' flank. The corresponding region in the mouse TNF- α gene was shown to regulate the stimulation of TNF- α production by LPS (48, 49). Although we did not detect any differences in TNF- α production upon stimulation of PBMC with PHA between individuals varying in the TNF-NcoI-

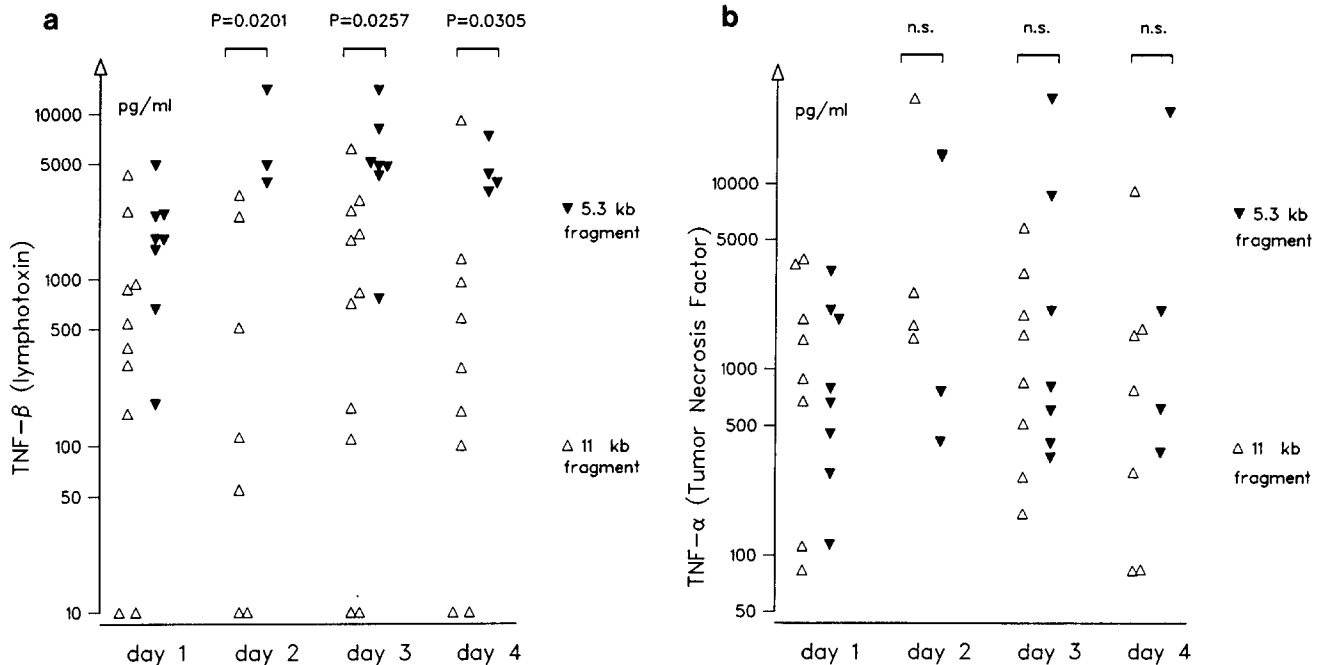


Figure 5. TNF protein production after PHA stimulation of PBMC of individuals matched for the NcoI polymorphism of the TNF- β gene. Comparison of donors homozygously typed for the TNFB*1 (5.3-kb fragment; filled arrowheads) and TNFB*2 (11-kb fragment; open arrowheads) alleles in regard to TNF protein production ([a] TNF- β and [b] TNF- α production) upon PHA stimulation of PBMC. Each symbol represents one individual, tested two to three times. Due to paucity of cells, not every subject could be measured at every point. In the stimulation of 2×10^6 cells with $2 \mu\text{g/}$ PHA, supernatants were harvested after 1, 2, 3, and 4 d. The protein levels were assayed by a TNF- β - or TNF- α -specific ELISA and are shown in picograms per milliliter. *p* values were determined by the Mann Whitney test.

RFLP, we tested the possibility that TNF- α alleles might differ significantly in their promoter sequence by PCR amplification and direct sequencing of the TNF- α 5' region of five individuals each homozygous for either TNFB*1 or TNFB*2, and in addition, we determined the sequence of the corresponding segment of clone cah5 (see Fig. 4). Sequences of the amplified fragments run in parallel did not detect any differences between the 10 different amplified TNF- α promoter regions (including two A1/B8 haplotypes), which were identical to the sequence obtained from the cah5 TNF- α fragment.

The TNFB*1 Allele Leads to a Higher TNF- β Response upon PHA Stimulation of T Lymphocytes. To investigate both NcoI-defined alleles for functional differences in TNF- β and TNF- α gene expression, PBMC from healthy individuals homozygous for the 11-kb NcoI fragment ($n = 11$) or the 5.3-kb NcoI fragment ($n = 9$) were stimulated with mitogen (PHA) to activate TNF production. TNF- α and TNF- β protein levels were determined by ELISA on days 1, 2, 3, and 4. Due to paucity of cells, TNF- α and - β could not be measured on all days for each individual. Despite great interindividual variation in TNF- β production, mean TNF- β levels were higher in TNFB*1 homozygous subjects on all days (Fig. 5 a). These

differences were statistically significant on day 2 (TNFB*1, 7.6 ± 3.0 ng/ml; TNFB*2, 0.5 ± 0.4 ng/ml; $p=0.021$), on day 3 (TNFB*1, 5.2 ± 1.6 ng/ml; TNFB*2, 1.5 ± 0.6 ng/ml; $p = 0.0257$), and on day 4 (TNFB*1, 4.7 ± 0.9 ng/ml; TNFB*2, 1.4 ± 1.0 ng/ml; $p = 0.0306$), when analyzed by the Mann Whitney test. The TNF- β responses were a stable feature of the individuals, as repeating the experiments after varying time intervals resulted in the same phenotype. High responders always showed an elevated TNF- β production and low responders gave a reduced TNF- β response. In both groups of homozygous individuals, TNF- β titer in culture supernatants upon PHA stimulation correlate with the level of TNF- β transcripts of the concomitantly performed TNF- β mRNA analysis (Fig. 6, a and b). The TNF- β mRNA signals were stronger in the TNFB*1 group. In contrast to TNF- β , no statistically significant difference in TNF- α protein (Fig. 5 b) or mRNA levels between the two groups was assessed (data not shown).

Discussion

The TNF NcoI polymorphism, previously thought to be present within the TNF- α gene, has been analyzed by sev-

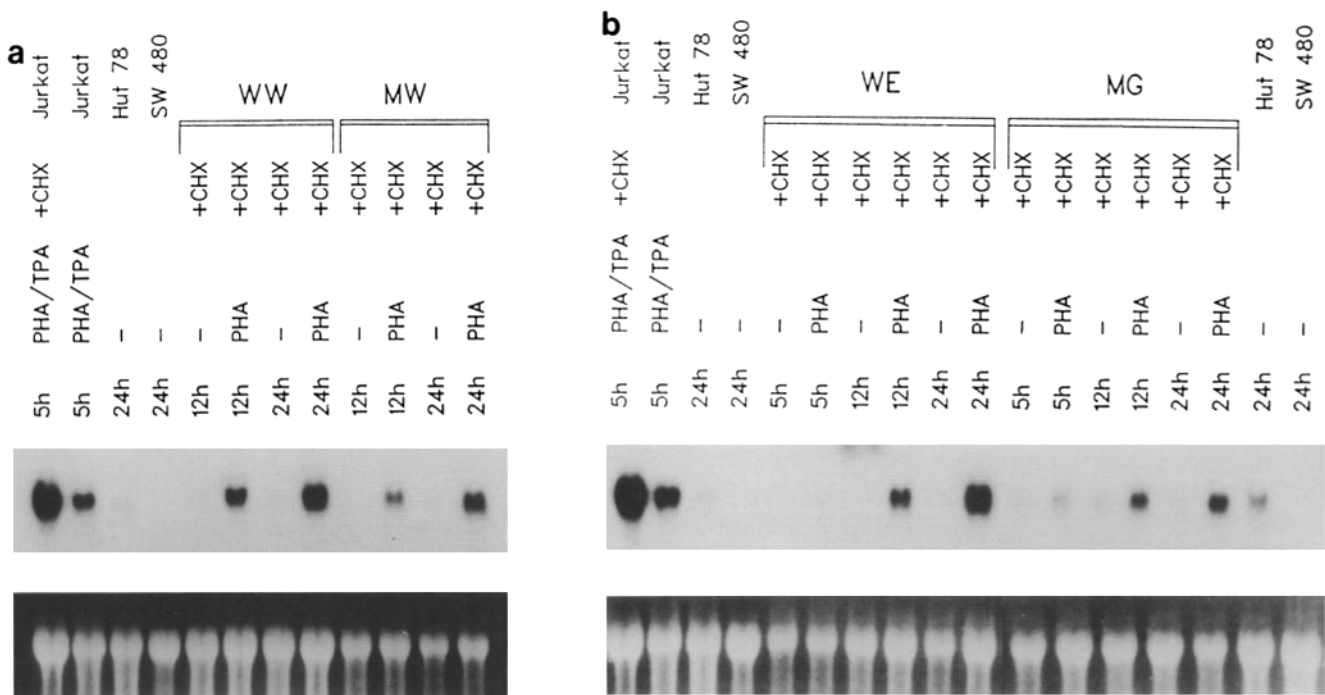


Figure 6. Northern blot analysis of RNA isolated from control cell lines and PBMC upon stimulation. Total RNA was isolated from the cell lines SW480 and Hut 78 and Jurkat cells after stimulation with PHA/TPA. To amplify the TNF- β mRNA level, cycloheximide (CHX) ($10 \mu\text{g/ml}$) was added to some cultures 3 h before harvesting (compare 5-h stimulation of Jurkat cells with and without CHX). CHX treatment only increased the TNF- α and TNF- β mRNA signals, but did not change the relative character of the inducibility. Hut 78 expresses TNF- β constitutively and SW 480 was included as negative control. The result of the hybridization with the TNF- β probe is shown above the ethidium bromide staining of the 28S ribosomal RNA included as control for the amount of RNA loaded on the gels. (a) PBMC of two individuals homozygous for the 5.3/5.7-kb TNF- β NcoI fragments (8×10^6 of W.W. and 6×10^6 of M.W.) were stimulated with PHA, and RNA was analyzed after the intervals indicated. The weaker signals in the stimulation of PBMC of M.W. is due to less RNA applied on the gel (see ethidium bromide staining of the 28S RNA). (b) 8×10^6 PBMC of individuals W.E. and M.G. (homozygous for the 11-kb TNF- β NcoI fragment) were stimulated and mRNA analysis was performed as described. As W.E. and M.G. stimulations were tested on a separate gel, the positive (Hut 78) and the negative (SW 480) controls are shown again on the right side to demonstrate that this hybridization was exposed longer than the Jurkat/Hut 78/SW 480 control on the left and the Northern blot in a (see a stronger TNF- β signal in the Hut 78 lane on the left).

eral groups. Its less frequent allele, as defined below (TNFB*1, $p = 0.06-0.1$), was shown to be strongly associated with the HLA-A1, -B8, -DR3 haplotype (22-24), well known for its close association with various autoimmune diseases such as SLE, IDDM, celiac disease, myasthenia gravis, and rheumatoid arthritis (50). In this report, we show that the NcoI RFLP reported for TNF- α is instead located in the first intron of the TNF- β gene. Moreover, the described substitution is linked with two additional exchanges in TNFB, one present in the first intron and the other leading to an amino acid substitution at position 26. Thus, we can define two TNF- β alleles: TNFB*1 containing the NcoI restriction site and AAC at position 26, and TNFB*2 for the gene lacking the NcoI restriction site and coding for ACC.

The functional determinants of the TNF- α and TNF- β molecules have not been well characterized so far. It was shown that the COOH terminus is essential for cytotoxic activity (3). TNF- α , and presumably TNF- β , are described to exert their biological functions as trimers (51), as recently confirmed by the three-dimensional structure of TNF- α existing as a trimeric molecule ordered in a β -barrel jelly-roll configuration (52). Since TNF- β has several activities in addition to its cytotoxicity against sensitive target cells, the biological significance of the single amino acid substitution cannot be assessed, unless it has been tested in a variety of functional assays.

We asked whether the defined alleles might also influence the production of TNF- α and TNF- β by activated PBMC isolated from donors homozygous for the TNFB*1 and TNFB*2 haplotypes. Here, we demonstrate a fivefold higher secretion of TNF- β by PBMC of TNFB*1 homozygous donors, while no significant differences in TNF- α titers were found (although the previously published genomic sequences would predict significant difference in the inducibility of TNF- α due to variation in the 5' sequence [34, 35]). The direct genomic sequencing data reported here revealed no differences in the promoter region of the TNF- α gene (see Fig. 4), and are concordant with TNF- α protein analysis.

Previously, significant interindividual variations in the level of TNF- α induction have been described in normal healthy individuals, which were interpreted as inherited interindividual differences (53, 54). In addition, high and low responders were observed with regard to TNF- α production in males, but no linkage to the NcoI RFLP was found (54), a result in agreement with our data on the TNF- α response. Interestingly, a correlation was found between TNF- α production and MHC class II genotype, showing a reduced level for HLA-DR2 (54, 55) and DQw1-positive donors but higher TNF- α titers in individuals with DR3 and DR4 (54). The group of donors analyzed here was selected with regard to the NcoI RFLP, thus, a correlation with HLA-DR haplotype cannot be evaluated. Previous measurements of TNF- β production *in vitro* did not detect a correlation of high or low response patterns to a particular HLA class I or class II genotype, a result to be expected from our findings, as the subjects of previous studies had not been matched for the NcoI RFLP. Thus far, a reproducibly significant differ-

ence in the induction of TNF- β production was only found in association with the NcoI RFLP. Due to the distribution of the two TNFB alleles, only few individuals homozygous for the TNFB*1 allele could have been included in the other experiments. We did not observe significant differences in TNF inducibility with regard to the sex in the panel of individuals tested, a result supported by a previous investigation of a large collective of healthy donors not matched for the TNFB genotype.

The TNF protein production is regulated by very complex mechanisms (56-58). We could show that the difference of the TNFB alleles in the inducibility of TNF- β is presumably regulated at the level of transcription and is not due to variability in post-translational regulation, or to increased affinity of the anti TNF- β antibody used in the ELISA to the TNFB*1 product. The high responders showed an increased level of TNF- β mRNA already after 4 h of stimulation, whereas no differences were found for the amount of TNF- α mRNA when comparing the activation results of PBMC for both TNFB alleles. The result did not change when we added cycloheximide to the cultures 3 h before harvesting. The application of the protein synthesis inhibitor only resulted in a dramatic increase of the TNF- α and TNF- β hybridization signals, but did not affect the relative level of TNF inducibility. Thus, the increased amount of TNF- β mRNA in stimulated PBMC of individuals homozygous for the TNFB*1 allele cannot be explained by different mRNA stability but should be due to higher transcriptional activity. Moreover, the two TNFB alleles do not differ in the 3' untranslated region and in the sequences known to influence mRNA stability (34, 35).

To find out the transcriptional mechanisms, mediating TNF- β induction, we inspected the 5' region for sequence patterns conserved with regard to cell type specificity and similarity in man and mouse. So far, very little is known about the regulatory mechanisms that control transcription of TNFB. The characterization of one NF- κ B/KBF1 binding sequence motif in a promoter position of the TNF- β gene as a mediator in T cells that is conserved in mouse and man (Messer, G., manuscript submitted for publication), led us to search for further protein binding patterns within the two TNF- β alleles. We identified several nuclear factor binding motifs that should be relevant in the regulation of TNFB transcription and that are also present in other genes specifically expressed in T cells (see Fig. 3). None of the allelic base pair substitutions is present in a known transcription factor recognition sequence, although the first intron contains such sequences. Thus, the allelic differences leading to an increased level of TNF- β mRNA for the TNFB*1 allele upon stimulation might point to novel regulatory sequence elements.

The TNFB*1 allele is strongly linked to the HLA-A1/-B8 (8.1) haplotype (22-24), and the altered production of TNF- β and the variant polypeptide structure might contribute to disease susceptibility of this haplotype. In animal models, agents that regulate TNF production or activity (3) or application of TNF- α (20) can inhibit advancement of pathogenesis of the diseases. The role of TNF- β in the development

of chronic autoimmune disease could be in the local stimulation of class II molecules in costimulation with IFN- γ , as proposed for IDDM, of human pancreatic islet cells in vitro (59). It may also inhibit the function of β cells or contribute to the cell destruction in this disease. An aberrant local production of cytokines was proposed for the nonobese diabetic mice model, and it was proposed that a TNF- α and granzyme A production of intra-islet infiltrating lymphocytes might possibly play a role in the tissue damage (60).

It is conceivable that an allelically varying TNF- β response of activated T lymphocytes might contribute to the slow and self protruding inflammatory mechanisms of local autoimmune reactions, even, for example, as a cofactor in a multifactorial etiology. Thus, the finding that the two NcoI-defined TNFB alleles differ in structure and in the level of TNF- β inducibility might be relevant for MHC-associated predispositions for autoimmune diseases.

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Note added in proof: After submission of this manuscript, G. C. Webb and D. D. Chaplin (61) also localized the NcoI RFLP in the first intron of the TNF- β gene.

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