A Common Epitope on Human Tumor Necrosis Factor Alpha and the Autoantigen 'S-antigen/arrestin' induces TNF-α Production

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A common epitope on S-antigen (arrestin), a potent autoantigen inducing experimental autoimmune uveoretinitis (EAU), and on human tumor necrosis factor α (hTNFα) was revealed using two monoclonal antibodies to S-antigen which inhibit EAU induction. The minimal common sequence for monoclonal antibody recognition is GVxLxD in the S-antigen/hTNFα amino acid sequences. Peptides containing this sequence motif exhibited monocyte activating capacity similar to the autocrine stimulatory capacity of hTNFα itself. In the S-antigen this activity was located from residue 40 to 50, corresponding to the peptide PVDGVVLVDPE (epitope S2). In hTNFα, the monocyte activating capacity correlated to residue 31 to 53, corresponding to the peptide RRANALLANGVELRDNQLVVPSE (peptide RRAN). The identified regions define common functional structures in the autoantigen and in the hTNFα molecule. The data suggest a regulatory function of this particular structure in TNFα expression and in autoimmunity.

Introduction

S-antigen, a soluble protein abundant in retinal photoreceptors, is a potent autoantigen which induces experimental autoimmune uveoretinitis (EAU) and pinealitis

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(EAP) [1, 2]. EAU is a model system for human uveoretinitis because the animal disease resembles certain inflammatory conditions in the human eye and sensitization to S-antigen is frequently detected in patients suffering from these conditions. S-antigen is identical to the 48 kDa protein of rod outer segments, named arrestin [3]. Its physiological functions in the visual transduction cascade include light- and ATP-induced binding to phosphorylated rhodopsin, quenching the activation of cGMP-dependent phosphodiesterase [4], and a possible activity as an internal Ca^{2+} buffer in photoreceptors [5]. The complete amino acid sequence (404 amino acid residues) of bovine retinal S-antigen was determined in 1987 [2]. Sequence analysis has since been expanded to retina and pineal gland derived S-antigens from different mammals [2, 6, 7]. An S-antigen-like molecule was isolated from bovine brain. This protein is associated with a regulatory function in the β-adrenergic receptor transduction system similar to the retinal S-antigen in the photoreceptor transduction system, i.e., desensitization of G-protein coupled receptors. This protein was named β-arrestin and exhibits 59% sequence identity with bovine retinal S-antigen [8]. Two S-antigen-like molecules have also been identified in *Drosophila* [9–12]. Monoclonal antibodies (mAbs) that recognize different epitopes of retinal S-antigen also identified S-antigen-like proteins in the cytosol of avian and fish nucleated erythrocytes [13], in extracts from several bovine organs (M. Mirshahi *et al.*, in preparation) and even in plants [14]. The participation of S-antigen in the replication machinery has recently been described [15].

The mAbs S2D2 and S6H8 recognize a phylogenetically conserved antigenic determinant (epitope S2). Injection of these mAbs, especially mAb S2D2, inhibits the subsequent induction of EAU by S-antigen immunization in rats [16, 17]. The suppressive effect of these mAbs on EAU suggests an idiotypic regulatory mechanism and/or immunomodulatory function of the epitope S2, especially concerning inflammatory processes. This assumption prompted us to investigate two different questions: (1) a possible role of the S-antigen and epitope S2 in the induction of cytokines involved in inflammatory processes, i.e., human tumor necrosis factor alpha (hTNFα) [18] and (2) a direct influence of the EAU suppressive mAbs on the TNFα-mediated pleiotropic effects, for example the autocrine-stimulated hTNFα gene expression and secretion [19].

Materials and methods

Reagents

S-antigen was isolated from bovine retinas and purified in two chromatographic steps as described [20]. Recombinant human (rh) TNFα was supplied by Knoll/ BASF AG, Ludwigshafen, Germany. The specific activity of the material was 8.74×10^6 U/mg protein as measured in the biological tumor cell (L929) cytotoxicity assay in the absence of actinomycin D [21].

S-antigen specific peptides and hTNFα specific peptides were synthesized in a continuous flow instrument constructed and operated as described earlier [22]. Peptide chain assembly was performed by the solid phase method on a 1% crosslinked polystyrene support using Fmoc-aa and *in situ* activation by BOP [23]. The synthetic peptides were purified by reverse phase HPLC.

Monoclonal antibodies S2D2 (IgG2b) and S6H8 (IgG2a) to S-antigen [24, 25] were purified from ascites fluid by affinity chromatography on S-antigen-bound Sepharose 4B (S6H8) or on Protein A-Sepharose 4B (S2D2) [16]. The mAb 7D1 (IgG1) against hTNFα and the mAbs ALB9 (IgG1), PM1 (IgG2b) and ALB2 (IgG2a), specific for human leukocyte differentiation antigens (isotype controls) [26, 27], were purified from ascites fluid by affinity chromatography on Protein A-Sepharose 4B.

Western-blot analysis

Recombinant hTNFα (3 µg/lane) was electrophoresed on a SDS-12.5% poly-acrylamide gel and transferred to nitrocellulose membrane by electroblotting [28]. Non-specific binding sites were blocked with 3% BSA in PBS. Incubation with the purified mAbs (5 µg/ml in PBS containing 1% BSA) was followed by incubation with peroxidase-labelled rabbit anti-mouse Ig antibodies (Sigma, Deisenhofen, Germany), and with diaminobenzidine-H_2O_2 in sodium acetate buffer (0.1 M, pH 4.9) according to the manufacturer's recommendations.

PEPSCAN-analysis

Sixty-eight heptapeptides, overlapping by six amino acids (aa), from bovine retinal S-antigen (N-terminal residues 1–74) and 75 heptapeptides, overlapping by five aa, from the complete mature hTNFα molecule were synthesized on polyethylene rods, essentially following the strategy described by Geysen [29]. The synthesis, however, used Fmoc (9-fluorenylmethyloxycarbonyl) chemistry and BOP activation. The polyethylene rods derivatized with β-alanine were purchased from Cambridge Research Biochemicals (Cambridge, UK). PEPSCAN-ELISA tests and stripping of the rods were carried out as described by Geysen [29]. Specific mAb binding was detected using horseradish peroxidase (HRP)-conjugated Protein A (Bio-Rad Laboratories, Richmond, VA, USA), 1:2,000 diluted. The enzymatic activity was revealed with the HRP substrate, 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid $2NH_4$-salt (ABTS) (Serva, Heidelberg, Germany) in 0.1 M Na_2HPO_4, 0.1 M citric acid pH 4.0, containing 0.02% H_2O_2. The reaction was stopped by removal of the rods after 20 min. ELISA absorbance was measured at 405 nm.

Cell preparation

Human peripheral mononuclear leukocytes (PMNL) were prepared from hepari-nized blood samples by Ficoll-Paque (Pharmacia, Freiburg, Germany) density-gradient centrifugation [30].

Northern-blot analysis

Total cellular RNA was isolated after a 2 h incubation of 2×10^6/ml human PMNL with protein or synthetic peptide preparations. The controls were cultured either with or without 10 µg/ml Staphylococcus aureus (Pansorbin, Calbiochem, Frankfurt, Germany). The RNA was analysed on a Northern blot [19] which was sequentially

hybridized with ^{32}P-labelled probes generated by random priming of cDNA inserts. The cDNAs were (a) a 750 bp EcoRI fragment of the coding region of hTNFα cDNA, (b) a 530 bp Bam HI-NdeI IL-1β cDNA fragment (isolated from p11, supplied by U. Gubler, Hoffmann La Roche, Nutley, NJ, USA) and (c) a 560 bp SalI-EcoRI-β actin cDNA fragment.

Determination of hTNFα production

Human PMNL ($2 \times 10^6/0.5$ ml) were cultured in RPMI 1640 (GIBCO) with 10% heat-inactivated fetal calf serum (GIBCO) for 20 h with protein or synthetic peptide preparations. The controls were cultured either with or without 10 μg/ml *Staphylococcus aureus* (Pansorbin, Calbiochem). Cell-free supernatants were harvested and stored at $-20°C$ until they were tested for hTNFα activity. The hTNFα activity was determined either by ELISA specific for hTNFα [31] or by the tumor cell (L929) cytotoxicity assay in the presence of actinomycin D [21].

Results and discussion

Epitope mapping on the S-antigen

Monoclonal antibodies S2D2 and S6H8 to S-antigen can inhibit the EAU induced by S-antigen immunization [16, 17]. We identified the epitope recognized by these inhibitory mAbs using the PEPSCAN method [29]. The epitope S2 was localized to residues 40 to 50 of S-antigen, corresponding to the sequence PVDGVVLVDPE (Figure 1a). This sequence is conserved in retina and pineal gland S-antigens from various mammals and in bovine β-arrestin, and is immunochemically detectable in other vertebrates, invertebrates [25] and even in plants [14]. The epitope S2 is situated far from the presently known disease-inducing [2, 32–38] and T-cell stimulating [34–36] sites. The possible action of epitope S2 in immunoregulation is further implied by the finding that the humoral immune response against this epitope is related to disease inducibility in genetically distinct rat strains: indeed, the antibody response to S2 is low in susceptible Lewis rats, high in refractory Brown Norway (BN) and Long Evans strains, and intermediate in susceptible Lewis × BN F1 hybrids [39].

Biological function of epitope S2

These observations prompted us to test a possible direct effect of epitope S2 on the production of inflammatory cytokines such as TNFα [18]. For this approach, adherent human PMNL were employed to study the conditions that regulate TNFα synthesis *in vitro*. S-antigen and peptide S2 (EPVDGVVLVDPE) were found to induce hTNFα mRNA (Figure 3a) and IL-1β mRNA expression (data not shown). In addition, detectable amounts of released hTNFα protein were measured in a hTNFα specific ELISA (Table 1) and in the TNFα bioassay (data not shown). These results suggest that the EAU suppressive mAbs inhibit TNFα induction and/or influence one of the TNFα mediated pleiotropic effects. However, under *in vitro* conditions, we could not demonstrate any effect of the mAbs S2D2 and S6H8 either
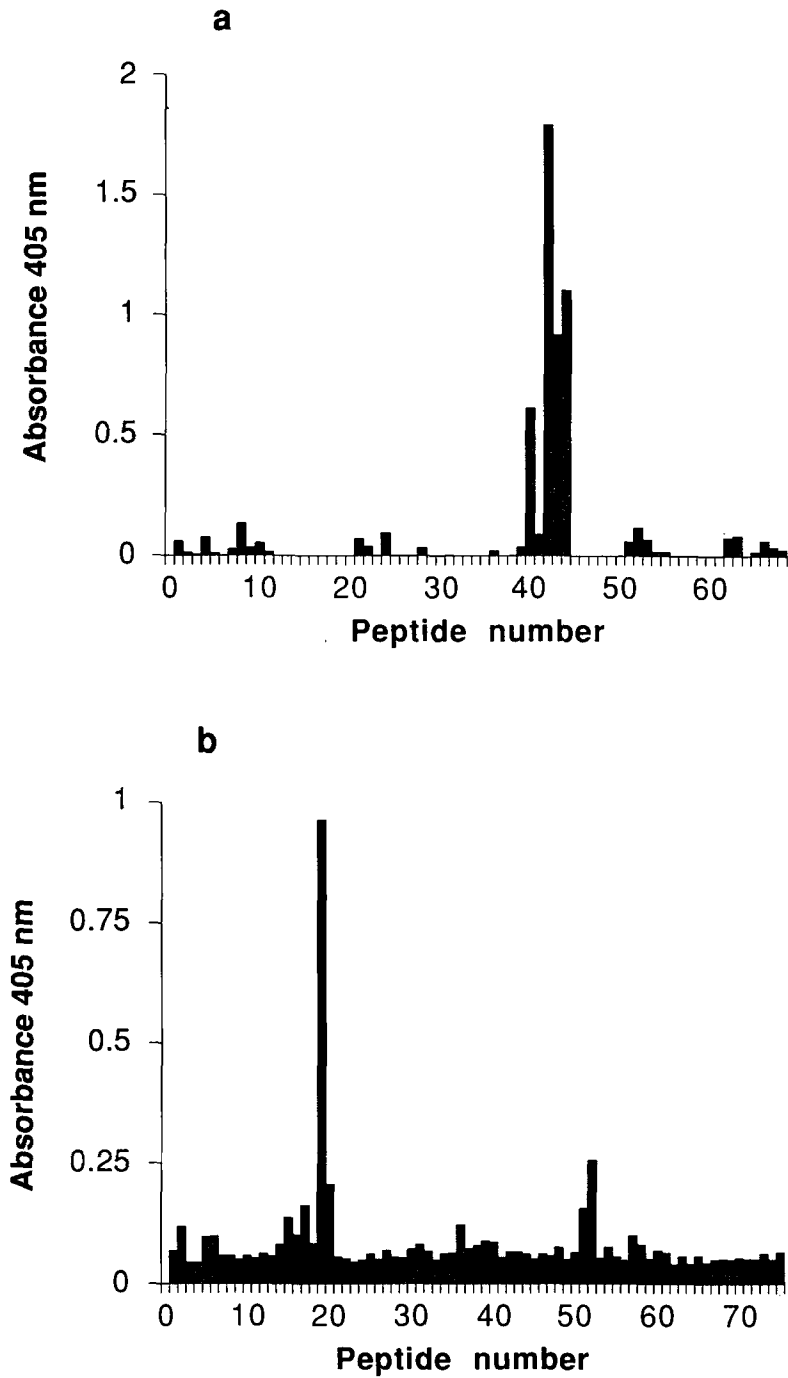
a

b

Figure 1. Immunoreactivity profile of (a) 68 heptapeptides, overlapping by six amino acids, from bovine retinal S-antigen (N-terminal residue 1–74) and (b) of 75 heptapeptides, overlapping by five amino acids, from the complete mature hTNFα molecule. The S-antigen/hTNFα common epitopes were revealed with mAb S6H8 (1 µg/ml).

Table 1. *hTNFα release, stimulated by protein or peptides*

		TNFα (ng/ml)
Not stimulated		<1.0
S-antigen		5.4
Peptide S2	EPVDGVVLVDPE	3.6
hTNFα peptides		
Residues 31–42	RRANALLANGVE	2.5
Residues 37–48	LANGVELRDNQL	1.9
Residues 43–54	LRDNQLVVPSEG	1.7
Residues 49–60	VVPSEGLYLIYS	<1.0
Residues 79–90	TISRIAVSYQTK	<1.0
Residues 25–36	QLQWLNRRANAL	<1.0
Residues 31–53	RRANALLANGVELRDNQLVVPSE	20.4

TNFα release was determined in the supernatant of human PMNL cultures after 20 h incubation with 10 μg/ml of the indicated preparations. TNFα concentrations were determined in the hTNFα specific ELISA.
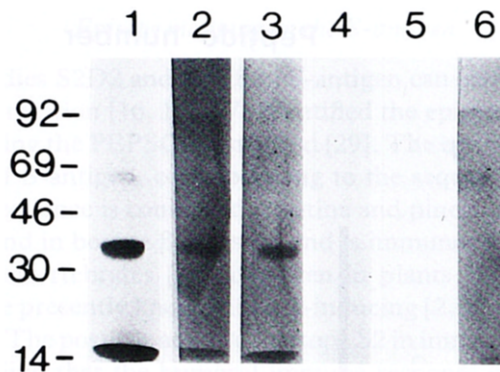

Figure 2. Western blot analysis of recombinant hTNFα using mAbs 7D1 against hTNFα (lane 1), S2D2 (lane 2) and S6H8 (lane 3) against S-antigen and control mAbs ALB9 (lane 4), PM1 (lane 5) and ALB2 (lane 6) with unrelated specificities. Numbers on the left indicate molecular weight markers.

on the induction of monocyte activation by TNFα itself for TNFα biosynthesis or on the cytotoxic effects of TNFα in the bioassay (data not shown).

Epitope mapping on TNFα

By Western blot analysis, we found that mAbs S2D2 and S6H8 specifically recognize hTNFα in both its monomeric and dimeric forms (Figure 2). A PEPSCAN analysis of hTNFα was therefore performed using mAbs that recognize epitope S2 of S-antigen. The analysis revealed a cross-reactive epitope in the hTNFα molecule at residues 39 to 45, i.e., peptide NGVELRD (Figure 1b). The common motif in the sequences 40–50 of S-antigen (PVDGVVLVDPE) and 39–45 of hTNFα (NGVELRD) is GVxLxD. Examination of the protein sequence data bank

Table 2. *Vertebrate proteins containing the GVxLxD motif (from protein sequence data banks SwissProt and NBRF)*

Retinal S-antigen (bovine, human, mouse and rat)
Pineal gland S-antigen (rat)
β-arrestin (bovine)
Tumor necrosis factor α (human and bovine)
Coagulation factor VIII precursor (human)
Complement components C4 and C9 (human and mouse)
Integrin beta-4 subunit precursor (human)
Interferon-induced 17 kDa protein, interferon-induced 15 kDa protein (human)
Cholesteryl ester transfer protein precursor (human)
Acetyl-CoA acetyltransferase (EC.2.3.1.9.)
Liver glucose transporter protein (human and mouse)
Lysozyme C (rat and mouse)
Lysozyme M (mouse)
cAMP-dependent 3',5'-cyclic phosphodiesterase 1-4 (rat)
Complement component C4-related sex-limited protein (mouse)
Prolactin precursor
Hemoglobin I and III (chicken)
Band 3 anion transport protein (chicken)
Cytochrome P450 IVB1 (rabbit)

(SwissProt and NBRF) identified the GVxLxD motif in other vertebrate proteins as well as in S-antigen and TNFα (Table 2).

Biological function of TNFα peptides

The finding of an hTNFα region that cross-reacts with the S-antigen mAbs suggested that this antigenic determinant might be associated with the autocrine stimulatory potential of hTNFα [21]. Indeed, the hTNFα-dodecapeptides, RRANALLANGVE (residues 31 to 42), LANGVELRDNQL (37 to 48) and LRDNQLVVPSEG (43 to 54), covering the area of the epitope common with S-antigen were able to activate human PMNLs, whereas a series of other hTNFα-derived dodecapeptides, including peptides positioned directly adjacent to the region of homology, did not induce TNFα mRNA (Figure 3b and c) and IL-1β mRNA expression (data not shown). In addition to hTNFα mRNA expression, we studied (as for S-antigen and peptide S2) the influence of these three dodecapeptides on the induction of biologically active hTNFα protein. Small but measurable amounts of hTNFα protein were released after stimulation of PMNL with the hTNFα peptides (Table 1). Essentially identical results were obtained in the TNFα bioassay (data not shown). A synthetic peptide covering the hTNFα sequence from residue 31 to 53, (RRANALLANGVELRDNQLVVPSE) (peptide RRAN), and including the complete region of homology, showed an efficiency on hTNFα mRNA expression comparable with that of rhTNFα itself (Figure 3d). The failure of the other hTNFα dodecapeptides to induce TNFα expression indicated that contaminating endotoxin levels in the protein and synthetic peptide preparations were not responsible for the observed human PMNL activation. S-antigen-, peptide S2- or
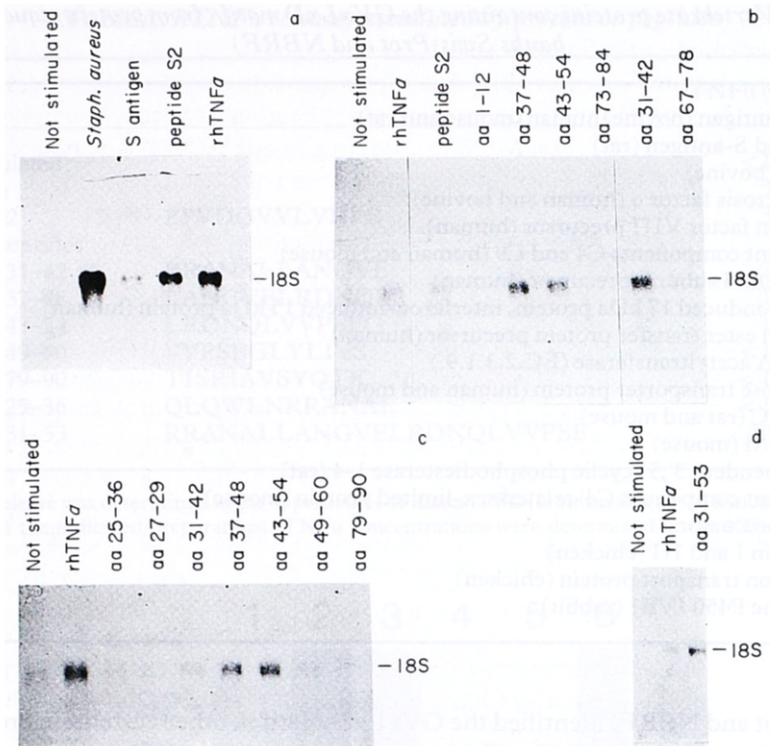
Figure 3. Induction of TNFα mRNA expression in cultures of the adherent fraction of human peripheral mononuclear cells. Monocytes were either not stimulated, or stimulated with one of the following preparations: (a) *Staphylococcus aureus* (10 µg/ml), S-antigen (2 µg/ml), peptide S2 (EPVDGVVLVDPE) (2 µg/ml), hTNFα (1 µg/ml); (b) 1 µg/ml of hTNFα, peptide S2, hTNFα specific peptides aa 1 to 12 (VRSSSRTPSDKP), 37 to 48 (LANGVELRDNQL), 43 to 54 (LRDNQLVVPSEG), 73 to 84 (HVLLTHTISRIA), 31 to 42 (RRANALLANGVE), 67 to 78 (QGCPSTHVLLTH); (c) 1 µg/ml hTNFα or hTNFα specific peptides aa 25 to 36 (QLQWLNRRANAL), 27 to 38 (QWLNRRANALLA), 31 to 42 (RRANALLANGVE), 37 to 48 (LANGVELRDNQL), 43 to 54 (LRDNQLVVPSEG), 49 to 60 (VVPSEGLYLIYS), 79 to 90 (TISRIAVSYQTK); (d) 1 µg/ml of hTNFα, or hTNFα peptide RRAN, aa 31 to 53 (RRANALLANGVELRDNQLVVPSE).

hTNFα-derived peptides did not interfere in the hTNFα specific ELISA and were not directly cytotoxic to the target cells used in the TNFα bioassay. Even when cross-linking of peptide RRAN was achieved with the mAbs S2D2 and S6H8, no cytotoxic effect was detectable on TNFα sensitive murine or human tumor cells [40]. In addition, S-antigen, peptide S2 and peptide RRAN were not able to stimulate PGE$_2$ production in a mouse fibroblast line (L929) as did hTNFα (data not shown).

These data provide new insight into the mechanism of autocrine-stimulated hTNFα gene expression, defining a functional structure in the hTNFα molecule for PMNL activation. This is also supported by recent data demonstrating fibroblast chemotaxis induced by a hTNFα peptide aa (31–68) [41]. The identified region is highly conserved among TNFα sequences derived from several species [42, 43] and has been predicted to be a potential receptor binding site [44–46]. However, on two

different cell lines we were not able to demonstrate competition of peptide RRAN with radiolabelled rhTNFα for receptor binding.

TNFα production stimulated by S-antigen, peptide, S2, hTNFα itself and the peptide RRAN could play a direct role in local inflammatory processes, for example in ocular autoimmune disease. Modifications of peptide RRAN to produce peptide variants with agonistic or antagonistic activity or the production of chemical inhibitors for this region in the TNFα molecule are future experimental or treatment perspectives. The inhibitory effect of mAbs S2D2 and S6H8 on EAU induction could be explained by their binding to the TNFα-inducing site(s) of the S-antigen and a blockade of the TNFα inducing function. However, a direct neutralizing effect of these mAbs on the monocyte activating capacity of S-antigen, peptide S2 or peptide RRAN in the described TNFα production *in vitro* system was not observed (data not shown). Therefore, in the EAU model, active immunization programmes are underway using peptide S2, peptide RRAN and/or human S-antigen peptides covering peptide S2 region (peptide 4: aa 31–50 and peptide 5: aa 41–60) in order to protect against S-antigen induced EAU and EAP. Preliminary results suggest that suppression of EAU is related to the immune response against the homologous sequence of S-antigen and TNFα (Y. De Kozak, personal communication). EAU and EAP induced by the retinal autoantigens rhodopsin and interstitial retinoid binding protein (IRBP) [1] and experimental autoimmune encephalomyelitis induced by myelin basic protein, could serve as model systems for the putative contribution of TNFα to several human neurological diseases [47–49]. The possibility of direct cytokine induction by autoantigens, bypassing the necessity for specific antigen recognition, by T/B cells deserves investigation in such models of autoimmune diseases.

Acknowledgements

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