Supplement Review

Multiple roles for tumor necrosis factor-α and lymphotoxin α/β in immunity and autoimmunity

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Chapter summary

Tumor necrosis factor (TNF)-α and lymphotoxin (LT) α/β play multiple roles in the development and function of the immune system. This article focuses on three important aspects of the effects of these cytokines on the immune response and on autoimmunity. In several experimental systems (Jurkat T cells, murine T-cell hybridomas), TNF-α appears to cause a downregulation of signaling through the TCR, revealed by changes in calcium flux, activation of p21, p23 and ZAP70, and a decrease in nuclear activation of NF-κB. Previous and present results suggest that TNF-α interferes in some manner with signaling through the TCR, at a locus yet to be delineated. Transgenic expression of LTβR-Fc in nonobese diabetic (NOD) transgenic mice results in prevention of type 1 diabetes in NOD mice as long as the level of expression of the fusion protein (under the control of the cytomegalovirus promoter) remains above a level of 2–3 μg/ml. Once the expression levels of the fusion protein have dropped below this critical level, the diabetic process resumes and the animals become diabetic at 40–50 weeks of age, whereas nontransgenic littermates develop diabetes by 25–30 weeks of age. The paradoxical effects of neonatal TNF-α administration in NOD mice in increasing incidence of and hastening onset of type 1 diabetes, while neonatal anti-TNF administration completely prevents all signs of islet cell autoimmunity, are due partly to the low levels of $CD4^+CD25^+$ T cells in NOD mice. These low levels are reduced by a further 50% on neonatal administration of nontoxic levels of TNF-α. In contrast, neonatal administration of anti-TNF-α results in a dramatic increase in the levels of $CD4^+CD25^+$ regulatory T cells, to levels beyond those seen in wild-type untreated NOD mice. TNF-α and LTα/β thus have pleomorphic regulatory effects on the development and expression of autoimmunity.

Keywords: autoimmunity, immunity, lymphotoxin α/β, tumor necrosis factor alpha

Introduction and historical background

The cytokines TNF-α and LTα/β and their receptors play key roles in the development of the immune system and in immune regulation, inflammation, and autoimmunity. Manipulation of these cytokines in their receptors has revealed numerous aspects of their function in both health and disease, particularly in autoimmune diseases. Recent basic studies and corresponding clinical trials have revealed a major role for TNF-α in the pathogenesis of rheumatoid arthritis (RA), with a dramatic response in two-thirds of the patients to TNF blockade, either with a monoclonal antibody or with a soluble TNF receptor. Similarly, recent findings have shown that blockade of LTα/β by soluble LTβ receptor can suppress the normal immune response and interfere with the development of autoimmune diabetes in the NOD mouse.

A glossary of specialist terms used in this chapter appears at the end of the text section.

Table 1

Diseases in which tumor necrosis factor (TNF) blockade causes exacerbation

Disease	Intervention	Result	Mechanism	References
1. Multiple sclerosis	Anti-TNF, soluble TNFR	Increase in CNS lesions and disease activity	? T-cell activation	[1,2]
2. Experimental allergic encephalomyelitis (EAE)	TNF-α null mutation exacerbation of EAE	Failure of usual regression of T-cell reactivity; prolonged	? T-cell activation	[3]
3a. Murine 'lupus' in (NZB×NZW)F1 mice	TNF administration (adult)	3–4 month delay in disease onset	? Inhibition of T-cell activation	[4]
3b. Murine 'lupus' in (NZB×NZW)F1 mice	Anti-TNF administration (adult)	Earlier disease onset with increased severity	? T-cell activation	[5]
3c. Murine 'lupus' in (NZB×NZW)F1 mice	Heterozygous TNF null mutant	Earlier disease onset with increased severity	? T-cell activation	[6]
3d. Murine 'lupus' in (NZB×NZW)F1 mice	Anti-IL-10 administration (adult)	Delayed onset and decreased severity	Increase in endogenous TNF, leading to decreased T-cell activation	[5]
4a. Type 1 diabetes mellitus in (NOD) mice	TNF i.p. in adult mice	Delayed onset, decreased incidence of diabetes	? Inhibition of T-cell activation	[7]
4b. Type 1 diabetes mellitus in (NOD) mice	Anti-TNF in adult mice	Variable, earlier onset with increased incidence	? T-cell activation	[8]

CNS, central nervous system; i.p., intraperitoneally; NOD, nonobese diabetic; TNFR, TNF receptor.

This review will focus primarily on the effects of TNF and TNF blockade, and of LTα/β and a blockade of this cytokine with soluble LTβ receptor in several autoimmune diseases, both in spontaneous models in the mouse and in patients with autoimmune disease.

Effects of TNF-α and TNF-α blockade on T-cell function in autoimmunity

There is a growing body of evidence that blockade of TNF action in patients and experimental animals increases disease activity and severity in some, but not all, T-cell-dependent autoimmune diseases. Diseases in which blockade of TNF action causes exacerbation or prolongation of pre-existing autoimmune diseases, or the appearance of new signs of autoimmunity, are presented in Table 1.

Diseases such as multiple sclerosis, experimental allergic encephalomyelitis, and type 1 diabetes (T1DM) are all T-cell mediated as well as T-cell dependent. Murine 'lupus' in the (NZB × NZW)F1 strain is antibody mediated, but clearly T-cell dependent for the development of the pathogenic IgG autoantibodies. The findings presented in Table 1 have been confirmed in all cases by at least two separate studies, and in most cases by several studies. Many possible mechanisms for these results have been excluded in one or more of the diseases listed. These excluded mechanisms include alterations in $CD4^+/CD8^+$ ratios, alterations in Th1/Th2 ratios, alterations in levels of expression of Fas and Fas ligand, and effects on levels of expression of IL-12, IL-4, etc. [8,9].

Several lines of evidence suggest that TNF levels inversely affect T-cell responsiveness and TCR signal transduction [4,8–14]. These studies have documented a decrease in T-cell proliferation, cytokine production, and calcium flux in normal T cells and TCR transgenic T cells, following chronic exposure to TNF in both *in vitro* and *in vivo* studies. More recently, Cope and coworkers [15] have demonstrated a decrease in phosphorylation of CD3zeta p21, p23, and ZAP 70 in T-cell hybridomas cultured *in vitro* in the presence of nontoxic levels of TNF-α.

In our own laboratory (Munson *et al*., manuscript in preparation), in collaboration with the laboratory of Dr Arthur Weiss, we have found that chronic 5-day exposure of Jurkat T cells to nontoxic levels of TNF results in a 90% reduction in TCR-mediated nuclear activation of NF-κB (as detected by a reporter construct encoding a NF-κB binding site coupled to the luciferase gene) after TCR stimulation. These results suggest that the immunostimulatory effects of TNF blockade in many autoimmune diseases (Table 1) may be due to release of T cells from endogenous TNF-α-mediated inhibition. If so, this would imply that chronic TNF-α exposure in some manner down-regulates signal transduction mediated by the TCR.

Because TNF has such pleotropic effects, and because tumor necrosis factor receptor 1 (55 kb) (TNFR1) and tumor necrosis factor receptor 2 (75 kb) (TNFR2) are so widely expressed, it is possible, and indeed probable, that other not mutually exclusive mechanisms may also contribute to the effects presented in Table 1.

Table 2

Diseases in which tumor necrosis factor (TNF) blockade is therapeutic

Disease	Intervention	Result	Mechanism	Reference
1a. Rheumatoid arthritis	Anti-TNF, soluble TNFR	65% of patients have a dramatic decrease in disease activity	Blockade of TNF-induced inflammatory response (? decreased macrophage activation)	[16]
1b. Rheumatoid arthritis	Anti-TNF, soluble TNFR	Up to 15% of patients develop α-dsDNA antibodies. 0.2% develop mild SLE	? T-cell activation	[16]
1c. Rheumatoid arthritis	Anti-TNF, soluble TNFR	A few patients develop CNS findings suggestive of MS	? T-cell activation	[17]
2. Crohn's disease	Anti-TNF, soluble TNFR	Dramatic decrease in disease activity in up to 80% of patients	? Decreased monocyte/macrophage activation	[18]
3. Psoriasis	Anti-TNF	Dramatic clearing of skin lesions, decrease in associated arthritis	Blockade of TNF-induced inflammation	[19]

CNS, central nervous system; MS, multiple sclerosis; SLE, systemic lupus erythematosus; TNFR, TNF receptor.

Diseases in which TNF blockade is therapeutic

Diseases in which blockade of TNF action has been shown to be therapeutic are presented in Table 2. Three diseases (RA, Crohn's disease, and psoriasis) form an interesting group that contrasts sharply with the diseases in which TNF blockade causes exacerbation (Table 1). RA is thought by many to be a T-cell-mediated disease but, unlike the diseases in which TNF blockade causes exacerbation (Table 1), anti-TNF therapy results in a dramatic decrease in symptoms, and in some cases a near complete remission, although the disease recurs relatively promptly after cessation of anti-TNF therapy.

Much less is known or conjectured about the pathogenesis of Crohn's disease and psoriasis. With respect to Crohn's disease, recent evidence indicates that one of the principal predisposing genetic factors is a series of mutations in the NOD 2 gene, a regulator of NF-κB, a master regulator of genes involved in inflammation [20,21]. These genes are expressed in monocytes and macrophages, and are thought to be a part of the innate immune response. The prominence of macrophage-produced cytokines (IL-1, TNF-α, and IL-6) in RA and the prominence of a gene expressed in monocytes and active in the innate immune system suggest that those diseases in which TNF blockade is therapeutic may primarily be the result of overproduction of TNF and related cytokines by macrophages and monocytes. This is perhaps initially triggered by activated T cells, but the major mediator of inflammation is the macrophage rather than the T cell.

Some of the side effects of anti-TNF therapy in RA (development of anti-dsDNA antibodies, development of overt systemic lupus erythematosus, and central nervous system lesions suggestive of multiple sclerosis [Table 2]) are known manifestations of the diseases presented in Table 1, in which blockade of TNF action leads to exacerbation.

Effects of neonatal administration of TNF and anti-TNF on T1DM in the NOD mouse

Table 3 presents the effects of neonatal administration of nontoxic doses of TNF-α to newborn NOD mice, which results in a striking increase in incidence and a much earlier onset of diabetes. Additionally, administration of anti-TNF in doses beginning at 20 μg/g body weight and rising to 100 μg every other day for the first 21 days after birth results in complete and prolonged (1 year) absence of both diabetes and almost all signs of islet cell autoimmunity [10]. Recent evidence has shown that NOD mice treated in the neonatal period with TNF have a further sharp decrease in their already low levels of $CD4^+CD25^+$ regulatory T cells. Conversely, anti-TNF treatment in the neonatal period results in a dramatic *increase* in these $CD4^+CD25^+$ regulatory T cells. Preliminary studies suggest that the increase in $CD4^+CD25^+$ T cells alone is sufficient to explain the complete prevention of T1DM, since regular transfer of small numbers of these T cells to young NOD mice prevents the development of T1DM.

While this effect on regulatory T cells may be the primary explanation for the effect of neonatal TNF and anti-TNF on T1DM, there is another possibility. It has been postulated [9] that, by decreasing TCR signaling, neonatal TNF exposure results in a decrease in thymic T-cell-negative selection, particularly of autoreactive T cells. Correspondingly, neonatal exposure to anti-TNF, by increasing signaling through the TCR, may result in an increase in thymic T-cell-negative selection of autoreactive T cells, thus preventing diabetes.

The second model of T1DM presented in Table 3 is that induced in C57BL/6 mice by introduction of a transgene encoding the rat insulin promoter coupled to the TNF-α coding sequence. These mice develop early severe insulitis,

Table 3

Effect of neonatal tumor necrosis factor (TNF) and anti-TNF therapy on type 1 diabetes (T1DM) models

Model	Intervention	Result	Mechanism	References
1a. T1DM in NOD mice	TNF, 1–2 μg i.p., q.o.d for 21 days from birth	Increased incidence and earlier onset of diabetes ? Decrease in thymic T-cell-negative selection	? Further decrease in $CD4^+CD25^+$ regulatory T cells	[9,10] (A Wu and HO McDevitt, unpublished observations)
1b. T1DM in NOD mice	Anti-TNF, 20–100 μg i.p., q.o.d. for 21 days from birth	Complete, prolonged (1 year) absence of diabetes and islet cell autoimmunity ? Increase in thymic T-cell-negative selection	Dramatic increase in $CD4^+CD25^+$ regulatory T cells	[9,10] (A Wu and HO McDevitt, unpublished observations)
2. T1DM in C57BL/6 mice expressing RIP-TNF	TNF overexpressed in β cells	Severe insulitis, but diabetes never occurs (unless transgenic RIP-B7.1 is introduced)	RIP-TNF appears to have induced a Th2 shift in islet-reactive T cells	[11,12]

i.p., intraperitoneally; NOD, nonobese diabetic; q.o.d., every other day; RIP, rat insulin promoter; Th2, T helper cell type 2.

due to the overexpression of TNF in the β-islet cells. Despite this severe continuing insulitis, however, these transgenic animals never develop diabetes, and can be induced to do so only on introduction of a second transgene, rat insulin promoter-B7.1 [11,12].

Analysis of this model indicates that there is an increase in the number of macrophages and dendritic cells attracted to the islets by the local expression of TNF-α. Despite this, the T cells in the islets of these mice appear to be 'tolerant' to islet cell autoantigens, and the T cells have undergone a Th2 shift in the β-islet cell reactive T-cell population. While this model appears to be distinct from that in the wild-type NOD mouse, it is noteworthy that the severe prolonged insulitis in these animals does not result in the development of islet cell destruction, but does result in a form of T-cell tolerance; presumably due to some type of downregulation or alteration in T-cell function, an effect similar to the effects described in Table 1 (part 3a).

Potential mechanisms

Among the potential mechanisms by which blockade of TNF action might increase or activate autoimmunity (Table 1), an inverse effect of TNF levels on signal transduction through the TCR and/or a direct effect of TNF levels on T-cell apoptosis in the periphery mediated by TNFR2 are the most prominent (Table 4).

There are several reasons for the first of these two prominent potential mechanisms. First, it is difficult to envision how TNF blockade could activate macrophage function, antigen presentation or any other of the inflammatory functions of TNF-α. Second, several lines of evidence (see earlier) indicate that *in vitro* or *in vivo* exposure to TNF is capable of decreasing the T-cell response as measured by T-cell proliferation, cytokine production, and calcium flux. Third, preliminary studies by Cope and coworkers [15] and Munson *et al*. (manuscript in preparation) have shown that chronic exposure to TNF is capable of decreasing the activation of several of the proximal proteins in the TCR signaling pathway, and is also capable of decreasing TCR-mediated activation of NF-κB. The latter effect is important, since signaling by TNF through one arm of the TNF receptor signaling pathway and signaling by the TCR can both result in activation of NF-κB.

The possibility that TNF acts by increasing T-cell programmed death via TNFR2 must also be considered, since both mechanisms could be operative. The functions of TNFR1 are well characterized and include the induction of programmed cell death via the caspase-8 pathway, as well as the activation of a large array of molecules involved in the inflammatory response, primarily through the activation of NF-κB. The functions of TNFR2 are less well characterized.

Studies in R1, R2 or double-receptor-deficient mice have shown that TNFR1 is responsible for a number of host defense and inflammatory responses [22]. TNFR2-deficient mice have dramatically increased serum levels of TNF in response to endotoxin, and they show exacerbated inflammation in several inflammatory models [22]. This suggests that a primary role of TNFR2 is to suppress or regulate TNF-mediated inflammatory responses. The TNFR1 receptor is the high-affinity receptor for soluble TNF [23], but it is expressed at much lower levels on T cells and peritoneal exudate macrophages [22]. This difference in expression levels makes evaluation of the phenotype of receptor-deficient mice complex. Thus, in TNFR2-deficient mice, experimental allergic encephalomyelitis is a much more severe and acute disease [24]. It

Table 4

Mechanisms

Intervention	Potential mechanisms
1. Adult TNF therapy (delays type 1 diabetes, prevents β-cell destruction, and delays glomerulonephritis in B/WF1 mice)	1. A decrease in TCR signal transduction and effector T-cell function mediated through TNFR1 2. An increase in T-cell apoptosis mediated by TNFR2 3. Very little effect has been found on $CD4^+CD25^+$ regulatory T cells in adult mice
2. Adult anti-TNF therapy (variably hastens type 1 diabetes, increases B/W F1 nephritis, and increases late EAE)	1. An increase in TCR signal transduction and effector T-cell function through TNFR1 2. A decrease in T-cell apoptosis through TNFR2 3. Very little effect has been found on $CD4^+CD25^+$ regulatory T cell numbers by anti-TNF
3. Neonatal TNF therapy (increases diabetes incidence and hastens onset in NOD mice)	1. Further decreases $CD4^+CD25^+$ regulatory T cells, possibly via TNFR2-mediated T-cell apoptosis 2. Possible activation of macrophages and dendritic cells, increasing insulitis 3. A decrease in TCR signal transduction via TNFR1, permitting potentially autoreactive T cells to escape negative selection, emigrate to the periphery and cause diabetes
4. Neonatal anti-TNF therapy (completely prevents type 1 diabetes in NOD mice)	1. Dramatically increases $CD4^+CD25^+$ regulatory T cells, possibly by blocking TNFR2-mediated T-cell apoptosis 2. Possibly decreases macrophage and dendritic cell activation so that regulatory T cells can function effectively 3. Possibly increases TCR signal transduction so that autoreactive T cells are negatively selected in the thymus and/or in the periphery

EAE, experimental allergic encephalomyelitis; NOD, nonobese diabetic; TCR, T-cell receptor; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1 (55 kb); TNFR2, tumor necrosis factor receptor 2 (75 kb).

is not clear whether this is due to the lack of a downregulatory influence of TNFR2 or to the increased levels of TNF that are released in inflammatory responses because of the lack of the R2 receptor [25–31].

The TNF and TCR signaling pathways

With respect to the specific effects of TNF on signal transduction through the TCR, it should be noted that Cope and coworkers have reported that chronic TNF exposure of T-cell hybridomas results in a downregulation of activation of CD3zeta p21 and 23 and ZAP 70 [15]. It is not clear how TNF exposure leads to a decrease in the activation of these proximal TCR signal transduction proteins. However that decreased activation is achieved, it would be expected to lead to a decrease in activation of PLC-γ and PKC, and a corresponding decrease in the activation pathway from CD28 to NF-κB.

As noted earlier, Munson *et al*. (manuscript in preparation) have shown in Jurkat T cells that chronic exposure to low levels of TNF-α leads to a striking reduction in TCR-induced NF-κB activation after a 5-day period of incubation. TNF exposure may act through both pathways to result in a decrease in NF-κB activation. This would be expected to result in a decrease in T-cell activation and T-cell response. Conversely, exposure to anti-TNF and blockade of all endogenous TNF should prevent the normal endogenous TNF effects on NF-κB activation. In both these latter observations [15] (Munson *et al*., manuscript in preparation), the pathways and signaling proteins that are affected by chronic exposure to TNF are unknown, and they are extremely difficult to identify because of the complexity of both signaling pathways.

Actual and potential interactions between cell receptor signaling pathways (receptor cross-talk): interaction between TNF-α and the insulin receptor

Insulin resistance is an important metabolic abnormality often associated with stress, infections, cancer and obesity, and is especially prominent in non-insulin-dependent diabetes. Increased production of TNF-α is frequently observed in the first three of these conditions.

In obesity, it has been observed that adipocytes produce low levels of TNF-α that increase in obesity [32]. In 1994, Spiegelman and associates found that TNF-α inhibits signaling from the insulin receptor [33] and that this was associated with reduced tyrosine kinase activity of the insulin receptor [34]. Subsequently, Spiegelman's group showed that this reduced signaling through the insulin

receptor was due to the induction of serine phosphorylation of insulin receptor substrate-1 (IRS-1). Serine phosphorylation converts IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity [35]. (In 1997, Hotamisligil and coworkers also demonstrated that TNF contributes to obesity by increasing the release of leptin from adipocytes [36].)

The effects of TNF on signaling through the insulin receptor were verified earlier this year when White and coworkers [37] showed that TNF-α, insulin, and insulin growth factor-1 all act to serine phosphorylate IRS-1 on serine 307. Serine 307 phosphorylation of IRS-1 by TNF-α requires the action of MEK (also activated in the TNF signaling pathway), while serine 307 phosphorylation by insulin and insulin growth factor-1 requires association of JNK-1 with IRS-1, and the activation of the phosphatidylinositol 3-kinases.

This is an excellent example of receptor cross-talk. Insulin, insulin growth factor-1 and TNF-α all stimulate inhibitory phosphorylation of serine 307 on IRS-1 but do so through the use of different kinase pathways, all intersecting at IRS-1 and serine 307. A similar type of interaction may explain many other receptor cross-talk phenomena, possibly including the effect of TNF on downregulation of signaling through the TCR.

Interactions between TNF and IL-10

As noted by Ishida *et al*. [5], chronic administration of anti-IL-10 to adult (3–4 months old) (NZB × NZW)F1 female mice (which would be expected to remove the inhibitory effects of IL-10 and to lead to an exacerbation of disease) paradoxically results in a delay in the onset of glomerulonephritis. This paradoxical result is almost entirely due to the failure of IL-10 to downregulate TNF-α production by macrophages and T cells, since simultaneous administration of anti-TNF with anti-IL-10 results in no effect on disease in this model. Thus, endogenous TNF, unopposed by IL-10, exerts the same delaying and preventive effect that is seen with administration of TNF in these mice [4,5]. (Anti-TNF administration alone caused a much earlier onset of fatal nephritis in these mice [5].)

It is clear from the observations already cited [4,5] that TNF-α, IFN-γ, and other inflammatory cytokines have an intimate reciprocal relationship with IL-10 [38]. This is another example of receptor cross-talk, in this case 'cross-inhibition'. When this inhibition is released, macrophages are then capable of increasing their production of inflammatory cytokines such as TNF.

Interactions between TNF and the TCR

Developing evidence [9,15] (Munson *et al*., manuscript in preparation) has shown that chronic TNF exposure downregulates components of both the TNF and the TCR signaling pathways. This evidence indicates that TNF has an effect both at the level of NF-κB expression, a very distal part of activation through both the TNF and TCR, and at the very proximal locus of activation of the CD3 components of the TCR.

Effect of TNF-α on Jurkat TCR activation

Jurkat T cells were cultured in the presence or absence of human recombinant TNF (10 ng/ml) for 1–6 days. Cells were then transfected with various transcription factor binding site–luciferase reporter constructs, incubated overnight, and washed and stimulated with phorbol 12-myristate 13-acetate and ionomycin or with anti-CD3 alone or in combination with anti-CD28, in the absence of TNF. Cells were cotransfected with a plasmid encoding a truncated CD25 molecule that could be used for normalizing the transfection efficiency by staining for anti-CD25 and performing FACS analysis.

TNF treatment for 3–5 days resulted in an 86% decrease in NF-κB-binding to a class I NF-κB binding site–luciferase reporter construct following stimulation with phorbol 12-myristate 13-acetate and ionomycin, and resulted in an 83% decrease following stimulation with anti-CD3 + anti-CD28 after normalization for transfection efficiency. Likewise, TNF treatment resulted in similar decreases in AP-1 binding to an AP-1 luciferase reporter, as well as decreases in luciferase production from a dual NF–AT–AP-1 composite reporter. Phosphoblots revealed very little difference in phosphorylation.

The findings cited in previous sections indicate that exposure to TNF-α affects both proximal and distal parts of the TCR signal transduction pathways. To obtain a survey of changes in expression (either up or down) in components of both the TNF and TCR signaling pathways, experiments currently underway will utilize gene expression profiling as the first step in assessing the effect of chronic TNF-α exposure on TCR signal transduction. These experiments will utilize the 'lymphochip' originally used by Alizadeh *et al*. [39] to analyze gene expression in large B-cell lymphoma and, more recently, in Jurkat T cells under a variety of stimulation conditions. These experiments are currently in progress and should permit a more comprehensive assessment of changes in gene expression in both of these receptor signaling pathways. An understanding of precisely how TNF exposure indirectly regulates TCR signal transduction may lead to the development of methods for compensating for the effects of TNF blockade in those autoimmune diseases where this is an appropriate therapeutic measure.

The role of LTα/β and LTβ receptor in autoimmunity

Administration of the soluble extracellular domain of LTα/β coupled to the Fc fragment of IgG as a fusion protein (LTβR-Fc) to NOD mice, either by injection [40] or by

transgenic expression transcribed from the cytomegalovirus (CMV) promoter [41], prevents T1DM in NOD mice. The mechanisms of this prevention and the possible undesirable side effects of these interventions are currently unknown. There are three principal, nonexclusive mechanisms for the effects of LTβ receptor blockade.

The first mechanism is prevention of MAdCAM-1 expression, causing defective T-cell, B-cell, and dendritic-cell homing and localization to peripheral lymphoid organs. Second is the interruption of the positive feedback circuit between LTαβ/LTβ receptor, B lymphocyte chemoattractant (BLC) and BLR-1 (and SLC and ELC), resulting in improper localization of T cells, B cells, and dendritic cells within the spleen and the lymph nodes, thus causing altered or defective T cell, B cell, and antigen-presenting cell (APC) interactions [40,41]. Finally, since soluble LTβ receptor binds both LTα/β and LIGHT (whose cognate receptor is the herpes virus entry mediator [HVEM]), the immunoregulatory effects of the soluble LTβR-Fc may be mediated by blockade of the interaction between LIGHT, LTα/β, and the LTβ receptor.

There is abundant evidence (reviewed in [42–44]) describing the critical role of LTα/β and the LTβ receptor in the development of the immune system. This ligand–receptor pair, as well several other ligand–receptor pairs within the TNF-α superfamily, and a number of transcription factors, chemokines and chemokine receptors have all been shown to be required for proper lymph-node genesis [42–50]. Many of these gene products operate at the very earliest stages of lymph-node formation, as well as at later stages in this process. These effects are beyond the scope of the present discussion, which will focus on the effects of the lack of the LTβ receptor on immune system function, primarily in autoimmunity.

Inhibition of LTαβ signaling through the LTβ receptor in adult mice, by the use of a blocking monoclonal antibody against LTβ receptor or a soluble LTβR-Fc fusion protein, induces marked changes in mice receiving this treatment [40,41,51–54]. In the spleen, discreet B-cell follicles are markedly reduced or absent, follicular dendritic cell (FDC) clusters are lacking, and the marginal zone shows radical changes, with absence of staining with MOMA-1 (a marker for marginal metallophilic macrophages) and reduced staining for ER-TR9 on marginal zone macrophages. In addition, the normal staining for MAdCAM-1 in the marginal zone is absent in treated mice. The normal boundary between the B-cell and T-cell zones in the white pulp of the spleen is disrupted, and the population of ER-TR7+ reticular fibroblasts normally seen around the outside of the white pulp in the marginal zone is absent. Furthermore, germinal centers did not form in these mice following immunization with sheep red blood cells [55].

A single injection of LTβR-Fc was sufficient to eliminate MAdCAM-1 expression 1 week later [40]. Some of the other changes noted earlier required several injections of the fusion protein. Several injections of the LTβR-Fc fusion protein resulted in a progressive decrease in the level of sheep red blood cell-specific IgG1, IgG2a, and IgM responses [40].

Similar changes in splenic architecture, B-cell and T-cell zone abnormalities, and absence of staining for MAdCAM-1 were also seen in BALB/c mice expressing a transgenic LTβR-Fc fusion protein under the control of the CMV promoter [55]. In this transgenic model, the CMV promoter does not become activated until days 2–3 after birth, by which time the development and population of lymph nodes has occurred [55].

The LTβ receptor has two ligands, LTα/β and LIGHT, another member of the TNF superfamily. The binding of LIGHT to a second receptor, the HVEM, functions as a costimulatory ligand–receptor pair that can promote T-cell proliferation and IFNγ production. Until very recently there were no reagents that could effectively block LIGHT activity *in vivo*. However, a very recent publication [56] has utilized a soluble HVEM-Fc receptor molecule to block the action of LIGHT *in vivo*. These studies showed that the HVEM-Fc was capable of downregulating the T-cell response to stimulation with concanavalin A and anti-CD3. Furthermore, multiple injections of HVEM-Fc in 5-week-old to 6-week-old NOD mice (100 μg per injection) was capable of decreasing the development of T1DM in the NOD recipients from 80% at 25 weeks to 25%. It is thus clear that at least part of the effects of the soluble LTβR-Fc are due to blockade of the interaction of LIGHT with HVEM.

The preliminary results presented in this recent study [56] did not demonstrate the extensive morphological changes in the spleen and lymph nodes that are seen with the soluble LTβR-Fc. Clearly, further studies need to be carried out, but it appears that the effects of LTβR-Fc on the morphology in the spleen and lymph nodes, the absence of the marginal zone, and of staining with MOMA-1 and MadCAM-1 antibodies indicate that the effects of blockade through the LTβ receptor may involve LTα/β, LIGHT, and MadCAM-1. The initial results indicate that LTβR-Fc is more effective in preventing diabetes than is HVEM-Fc.

Several studies over the past 4 years [13–15] (Munson *et al*., manuscript in preparation) have revealed that LTαβ/LTβ receptor are critical for the development of natural killer cells, dendritic APCs, and FDCs. Membrane lymphotoxin is required for dendritic cells to infiltrate the lymph nodes, while mature FDC networks require LTβ receptor expression by stromal cells, and LTαβ and TNF

expression by B cells. FDC development is dependent on TNF signaling and on LTβ receptor signaling by B cells (Munson *et al*., manuscript in preparation).

Mebius *et al*. [57] showed that, during fetal lymph node development, the lymph node post-capillary high endothelial venules express MAdCAM-1. This permits the early lymphoid precursor cells that are $\alpha_4\beta_7^+$, CD45$^+$, CD4$^+$ and CD3$^-$ to enter the lymph-node anlage. These cells also express surface LTβ and the chemokine receptor BLR-1 (CXCR5), and are capable of becoming natural killer cells, dendritic APCs, and follicular cells [58–60]. More recently, the mediators by which LTαβ/LTβ receptor signaling attracts B cells, dendritic APCs, and FDC to the developing lymph node follicle have been delineated [61–63]. The picture that emerges from these studies can be briefly summarized as follows.

Membrane-bound LTβ (produced by CD45$^+$, CD4$^+$, CD3$^-$ lymphoid precursors; see earlier) [57,61–63] binds to LTβ receptor on stromal cells, leading to the release of BLC (CCL13). By binding to its receptor on B cells (BLR-1, CXCR5), BCL attracts B cells to the area in the lymph nodes and the spleen where production of BLC is maximal. BLC binding to its receptor leads to B-cell activation and increased expression of LTαβ, which then leads to a further increase in expression of BLC, thus establishing a positive feedback loop. By inducing upregulation of membrane-bound LTαβ, BLC promotes further FDC development. At the same time, by binding to its receptor, LTαβ also stimulates induction of SLC (6 C-kine), a weak B-cell chemoattractant and a strong T-cell chemoattractant. SLC expression is induced in the region immediately adjacent to the B-cell region, creating a T-cell-rich region in the patterns seen in normal lymph nodes and spleen.

LTαβ binding to LTβ receptor also drives the expression of PNAd, MAdCAM-1 and V-CAM on the post-capillary high endothelial venules in the developing lymphoid tissue. SLC expression by endothelial cells and stromal cells, driven by LTαβ/LTβ receptor, also results in the expression of ELC by stromal cells. ELC is a strong T-cell chemoattractant, and the combined action of SLC and ELC, both binding to CCR7 on T cells, results in the well-defined segregation of T-cell and B-cell zones in normal lymphoid tissue. SLC and ELC also bind to CCR7 on dendritic cell precursors, thus attracting these cells to the developing lymphoid architecture.

Chemokines, triggered by the binding of LTαβ to LTβ receptor (and also by TNF-α binding to its receptors), thus establish the normal microarchitecture of the lymph node and the spleen.

From the presented results, it is clear that LTβ receptor blockade by a LTβR-Fc fusion protein will have multiple effects. These include a decrease in B-cell production of LTαβ and BLC, a decrease in expression of PNAd, MAdCAM-1, and VCAM-1, and a decrease in SLC, and to a lesser extent, ELC production by stromal cells in the T-cell zone. These effects explain many of the manifestations seen in LTβ receptor-deficient and LTβ-deficient mice, and in mice expressing a LTβR-Fc fusion protein: the loss of the marginal zone in the spleen; the partial mixing and disruption of normal T-cell/B-cell zone separation in the splenic white pulp and lymph nodes; the decrease in MAdCAM-1 expression; the decrease or absence of primary follicles and FDC clusters; and the diminution in T-cell–B-cell–APC interactions, resulting in isotype-switching defects in specific antibody responses [40,41].

The effect of LTβR-Fc on development of T1DM in the NOD mouse

Injection of relatively large doses of LTβR-Fc in NOD mice at 12 weeks of age, when insulitis is already well established in untreated littermates, results in nearly complete reversal of insulitis, and in failure to develop diabetes up to 30 weeks of age [40]. Expression of LTβR-Fc, under the control of the CMV promoter, prevents diabetes in NOD mice during the period in which expression of the fusion protein is greater than 2 μg/ml serum [41]. When expression of the transgene fusion protein drops below this critical level, however, the mice begin to develop diabetes, at approximately 40–50 weeks of age, with an incidence of 40% at the end of 1 year.

Both of these findings show that blockade of the LTαβ/LTβ receptor ligand–receptor system is capable of downregulating the diabetic process and of preventing the development of T1DM. Furthermore, the second study [41] shows that once the level of transgenic fusion protein has fallen below a critical level, the immune system is perfectly capable of reinstituting the diabetic process, leading to overt clinical diabetes.

It should be noted that in NOD LTβR-Fc mice, there was very little diminution in insulitis; the size of lymphocytic infiltrates in the islets and the frequency of insulitis were indistinguishable between transgene-positive and transgene-negative littermates. This result suggests that the ability of lymphocytes to migrate to areas of inflammation is not impaired under this type of treatment, but that the ability to generate a sufficient diabetogenic T-cell response that would result in islet cell destruction is impaired, possibly because of interference with normal T cell–B cell and T cell–APC interactions.

Two major mechanisms for the effects of LTβR-Fc can thus be envisioned to explain the observed effects. First, it is possible that interference with LTαβ binding to its receptor may decrease the expression of adhesins and integrins sufficiently to prevent insulitis and diabetes.

Earlier studies from this laboratory [64] have shown that administration of a monoclonal antibody to the α_4 integrin in young NOD mice is capable of preventing the development of diabetes in these animals. Thus, interference with $\alpha_4\beta_7$ and $\alpha_4\beta_1$ binding to their respective receptors can prevent the development of diabetes. However, the finding that insulitis persists in mice expressing a LTβR-Fc fusion protein would indicate that these lymphocytes are capable of entering inflamed areas.

The second possible mechanism for diabetes prevention may involve the effect of LTαβ/LTβ receptor blockade in interfering with normal separation between T-cell and B-cell zones, normal dendritic cell localization, and faulty interactions of T cells with both B cells and APCs [65]. In the latter case (i.e. in LTβR-Fc transgenic mice), where the degree of interference with this ligand–receptor system is presumably less than that induced by injection of very high doses of the fusion protein, it may be possible to prevent the development of diabetes with doses of the fusion protein that do not seriously interfere with the development of the normal immune response to environmental antigens.

LTα/β or LTβ null mutations, leading to LTβ deficiency, prevent autoimmunity not only in NOD mice [40,41], but also in experimental allergic encephalomyelitis and experimental murine colitis models [66,67]. Near-complete LTαβ blockade (as in [40]) may cause increased susceptibility to infection. Conversely, lesser degrees of blockade (as in [41]) (indicated by the persistence of insulitis) may result in prevention of diabetogenesis for shorter periods, requiring repeated administration, albeit with less severe immune suppressive side effects.

Effect of transgenic expression of a soluble LTβ receptor on diabetes in NOD mice

F9 LTβR-Fc transgenic mice were followed for the development of diabetes by expanding the population to 40–50 female mice, along with nontransgenic littermates. This experiment was carried out in two different founder lines: one expressing high (0.8–30 μg/ml) levels of the transgene protein (line 1610), and one expressing lower (0.38–1.1 μg/ml) levels of the transgene protein (line 201). The 201 line developed diabetes at almost the same rate as nontransgenic littermates (albeit slightly delayed). However, female mice in the 1610 line did not develop diabetes by 30 weeks of age, with the exception of one animal in a group of 26 females. Examination of these animals by histology and immunohistochemistry showed that the 1610 line at 6 and 12 weeks demonstrated similar anatomical abnormalities in the spleen, the lymph node, and Peyer's patches as had been noted in the original LTβR-Fc transgenic mice described earlier.

In the 1610 NOD line (unlike results in BALB/c mice), LTβR-Fc protein expression began at relatively high levels on embryonic day 16.5, reaching 2–30 μg transgene protein/ml serum by neonatal day 7. Over the next 10–12 weeks, however, the fusion protein concentrations fell to an average of 3 μg/ml at 10 weeks of age, and to less than 2 μg/ml at 20 weeks of age [41]. This is an unusual result, since transcription from the CMV promoter normally is expected to occur in many tissues throughout life. In the LTβR-Fc BALB/c mice, levels of LTβR-Fc remain constant up to 40–50 weeks of age.

Beginning at 35–40 weeks of age, female NOD 1610 mice unexpectedly began to develop diabetes, such that almost 50% of them developed diabetes by 65 weeks. Thus, once the LTβR-Fc protein levels fell to 1–2 μg/ml or lower, the diabetic process was capable of resuming. This resumption led to the development of T1DM, albeit 15–30 weeks after the nontransgenic littermate females.

Histologic analysis of these mice at 12 and 17–19 weeks of age showed that they had almost the same degree of insulitis as seen in control littermates (unlike the results described by Fu and coworkers [40]). The anatomical abnormalities in the spleen, which were seen in 4-week-old to 12-week-old mice, were greatly reduced by 30 weeks of age, with the exception of the redevelopment of the marginal zone. At 20 and 30 weeks, the structure of the marginal zone and the expression of MAdCAM-1 and MOMA-1 were still absent.

Soluble LTβR-Fc fusion protein therefore prevents T1DM in NOD mice, while levels remain above a critical threshold. Of great interest, following the decay of fusion-protein expression, the diabetic process spontaneously restarts and leads to development of overt diabetes in 50% of mice, with a 30-week delay (roughly the time period during which higher levels of the fusion protein were expressed) [41].

The results of this study are significant because they indicate that relatively low levels of a LTβR-Fc fusion protein are capable of inducing LTβ receptor blockade and of preventing the diabetic process in genetically susceptible NOD mice. Furthermore, it is clear from this study that no permanent inhibition of the immune response is induced by expression of the soluble LTβ receptor. This, of course, raises the possibility that the LTβR-Fc fusion protein, given at carefully determined times and doses, might permit prevention of the development of T1DM without significant degrees of suppression of the immune response to foreign antigens.

Effect of TNF-α and its blockade on the development of $CD4^+CD25^+$ regulatory T cells in the NOD mouse

Introduction

$CD4^+CD25^+$ T cells are derived from the thymus and have gained recent attention as mediators of peripheral tolerance

[68–70], as well as potentially having a role in the protection of a transplanted organ from immunological attack [70]. These cells are found in many mouse strains, including the NOD mouse, and in humans. $CD4^+CD25^+$ T cells act essentially as suppressor T cells in the periphery. These cells are anergic and do not respond to stimulation with anti-CD3, they are capable of suppressing the antigen-specific activation of $CD4^+CD25^-$ T cells, they require cell–cell contact for this suppression to be effective, and they may function through the expression of CTLA-4 molecules on these T cells interacting with B7 (on either APCs or on the target T-cell population) [68].

The NOD mouse is a well-defined animal model used to study T1DM. Our laboratory has previously demonstrated that neonatal administration of TNF (first 3 weeks of life) accelerates the onset of and increases the incidence of T1DM in the NOD mouse, while neonatal administration of anti-TNF completely abrogates all manifestations of T1DM for up to 1 year [71]. Neonatal TNF administration also further decreases the number and function of $CD4^+CD25^+$ regulatory T cells. Neonatal administration of anti-TNF, in contrast, increases the number of $CD4^+CD25^+$ T cells and does not alter the ability of these cells to suppress disease in a transfer system. These data suggest that the numbers and function of this regulatory population may be regulated in either a positive or negative manner by their cytokine (specifically, TNF) milieu.

Initial results

Ourselves and other workers [72] have shown that the NOD mouse is numerically deficient in the $CD4^+CD25^+$ T-cell population, suggesting a compromised ability to maintain a state of nonresponsiveness to self-antigens. Direct supplementation of the neonatal NOD mouse with three injections of 2×10^5 $CD4^+CD25^+$ T cells can significantly delay the onset of T1DM in this mouse model. Continued weekly administration of similar small numbers of $CD4^+CD25^+$ T cells results in failure to develop diabetes for as long as this treatment is continued. These data highlight the potential therapeutic value and the potency of these cells, and are compatible with the concept that the anti-TNF-induced increase in $CD4^+CD25^+$ T cells is the basis for the failure of anti-TNF-treated mice to develop diabetes.

Future studies

There is abundant evidence that the thymus of the NOD mouse displays significant anatomical abnormalities that are thought to be related to the T1DM disease process. The precise relationship is unknown. The NOD thymus exhibits giant perivascular spaces with abnormal retention of thymocytes and with premature signs of age-associated breakdown of the thymic epithelium [73–75]. To the extent that the NOD thymic architecture could influence the selection and ultimate trafficking of $CD4^+CD25^+$ T cells, it becomes important to understand the development of $CD4^+CD25^+$ T cells in the thymus. There is little to no information available in this area of study. There is no data available regarding the development of $CD4^+CD25^+$ T cells, and how TNF and/or anti-TNF may affect their development. These studies are currently under way.

Concluding remarks

The studies presented in this review, as well as a number of studies from the literature, make it clear that TNF-α and its receptors (TNFR1 and TNFR2), LTβ receptor and its LTα/β and LIGHT ligands, and the interaction of LIGHT with HVEM are all important regulators of the development and maintenance of structural integrity and function of the T cells in their interactions with other T cells, B cells and APCs in the development of the normal immune response and in the development of autoimmunity. Further understanding of the molecular basis for the downregulation of the immune response produced by pharmacological doses of TNF-α may lead to the development of new therapies to prevent the excessive stimulation of the immune system seen in TNF blockade, as occurs in RA.

Furthermore, a detailed understanding of the effects of soluble LTβ receptor and soluble HVEM receptor on the function of T cells, and the interaction of T cells with other T cells and with APCs in the white pulp of the spleen and the developing follicles in the lymph nodes, may lead to relatively nontoxic methods for downregulating the autoimmune response in diseases such as T1DM, where individuals at risk in the prediabetic stage can be identified and subjected to this kind of blocking therapy.

Glossary of terms

BLC = B lymphocyte chemoattractant; CMV = cytomegalovirus; FDC = follicular dendritic cell; HVEM = herpes virus entry mediator; IRS-1 = insulin receptor substrate-1; LTα/β = lymphotoxin α/β (α2β1, aα1β2); LTβR-Fc = the extra cellular domain of LTα/β coupled to the Fc fragment of IgG as a fusion protein; NOD = nonobese diabetic; T1DM = type 1 diabetes, juvenile onset, insulin-dependent diabetes mellitus, due to insulin deficiency; TNFR1 = tumor necrosis factor receptor 1 (55 kb); TNFR2 = tumor necrosis factor receptor 2 (75 kb).

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