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The TNF Family in T cell Differentiation and Function – Unanswered Questions and Future Directions

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Abstract

Proteins in the TNF/TNFR superfamily are recognized as major regulators of the activity of conventional CD4 and CD8 T cells, and also of regulatory T cells (Treg). Stimulatory molecules such as OX40, CD27, GITR, DR3, CD30, 4-1BB, TACI, and TNFR2 can promote division and survival in T cells, enhance effector activity including cytokine production, and drive the generation of T cell memory. They also display the capacity to block the development of inducible Treg cells or inhibit suppressive activity in Treg cells. Additionally, molecules such as Fas, TNFR1, and TRAILR promote apoptotic death in T cells and generally limit T cell activity. Although our knowledge of these proteins is quite good at this point in time, there are still many unknowns regarding their function, their expression patterns, and the involvement of these different molecules at various stages of the T cell response that occurs in autoimmunity, cancer, infectious disease, and during vaccination. Importantly, it is still unresolved how similar or dissimilar each of these receptors are to one another, the extent to which cooperation occurs between family members, and whether alternate TNF-TNFR interactions induce qualitatively different cellular responses. All of the molecules are attractive targets for immunotherapy of human disease, but it is not yet clear how to differentiate between them and make an informed decision as to whether any one protein may be the preferred focus of clinical development for a given specific disease indication. This review will highlight unanswered questions related to these molecules and the biology of T cells, and describe possible future directions for research in this area. Expanding our knowledge of how the TNF/TNFR family control T cells will undoubtedly help fulfill the promise of these molecules for providing efficacious clinical therapy of immune system disease.

Keywords

T cells; OX40; CD27; 4-1BB; GITR; HVEM; TACI; TNFR2; DR3; CD30; Fas; TNFR1; TRAILR

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1. Introduction

A protective or pathogenic immune response often relies on the ability of either CD4 or CD8 T cells, or both subsets, to accumulate in high numbers as effector cells and to gain strong functional activity (often displayed as Th1/Th2/Th17 or CTL phenotypes). This equally applies to responses against foreign antigens expressed by infectious agents and to responses against self-antigens in autoimmune disease or in cancer. The initial T cell response occurs over 3–7 days in a primary response and more rapidly in recall responses. In some cases, CD4 or CD8 T cells may also differentiate into regulatory cells (inducible or iTreg) that afford tolerance and protect against damaging self-reactivity, as well as inadvertently hindering anti-tumor and anti-pathogen immunity. Additionally, CD4 T cells differentiate into follicular helpers (Tfh) to promote B cell immunity and antibody production within a slightly longer period, generally 5–15 days. Collectively, this is ample time for T cells to have multiple interactions with a number of different antigen-presenting cells (APC), including B cells, as well as potential contact with other T cells, other lymphoid cells, and non-lymphoid cells such as stromal cells, epithelial cells and endothelial cells. Each individual encounter with a T cell will engage many cell surface protein receptor-ligand pairs that direct, modulate, and control the activity of that T cell, and as such these receptor-ligand interactions can be viewed as the critical checkpoints for development and persistence of T cell immunity.

The receptors on T cells can include classical costimulatory molecules like CD28, and cytokine receptors such as IL-2R, that can promote clonal expansion; other cytokine receptors like IL-4R, IL-6R, IL-12R, TGF- β R, and IL-23R that promote alternate T cell subsets to develop including iTreg cells; and adhesion molecules and chemokine receptors that direct migration and trafficking between lymphoid tissue and peripheral tissue. Importantly, accumulating evidence over the past 10–15 years has highlighted the contributions of many molecules in the TNF and TNFR superfamilies to different aspects of the response of T cells [1–5].

The signals from engagement of TNF family receptors and ligands have often been generalized as controlling survival versus death in T cells, and this certainly encompasses a large part of their activity. TNFR family interactions can dramatically modulate the frequency of antigen-reactive T cells that accumulate at various stages of the immune response, either because the TNFR family members signal to induce anti-apoptotic proteins or proteins promoting cell division, or because they signal to induce pro-apoptotic molecules. However, other regulatory events such as enhancing cytokine secretion or chemokine receptor expression may be just as important.

The stimulatory or survival-inducing TNFR family members that have been characterized as expressed by T cells, and shown to promote T cell clonal expansion or pro-inflammatory activities in T cells, are OX40 (CD134), 4-1BB (CD137), CD27, GITR (CD357), CD30, HVEM (CD270), DR3, TACI (CD267), and TNFR2 (CD120b). In contrast, inhibitory or death-inducing molecules in the TNFR superfamily that can be found on T cells are Fas (CD95), TRAILR1 and TRAILR2 (DR4/CD261, DR5/CD262), and TNFR1 (CD120a), and these can restrict T cell accumulation. Furthermore, many TNF family ligands can also be

expressed on T cells, like CD40L (CD154), LIGHT (CD258), LT $\alpha\beta$, OX40L (CD252), 4-1BBL, and CD70 (CD27L). The most often cited concept regarding why these latter molecules are on T cells is that they primarily act as ligands to induce functional activity via their cognate receptors expressed on cell-types that T cells encounter, including professional APC, neighboring T cells, and other lymphoid or non-lymphoid cells. This is exemplified by CD40L and LT $\alpha\beta$ that can promote division and survival in APC through CD40 and LT β R, respectively. Molecules like CD40L and LT $\alpha\beta$ additionally can function in feedback regulation, whereby signals through their receptors on APC or other cell-types leads to the upregulation of more stimulatory TNF family ligands such as OX40L, 4-1BBL, and CD70, hence indirectly allowing amplification of the T cell response.

Other TNF family ligands are produced as soluble molecules by T cells and essentially act like inflammatory cytokines. Their activity again can be manifest through promoting responses in non-T cells, or they can simply function in paracrine or autocrine fashion on T cells. For example, LIGHT produced by T cells in membrane form or as a soluble molecule can promote survival signals in other T cells through binding its receptor, HVEM, or it can promote cytokine production by binding HVEM on cells such as eosinophils or epithelial cells. Most notably TNF, FasL, and TRAIL are also produced as membrane or soluble molecules, and function as cytotoxic effector proteins when made by CD8 T cells and Th1 cells. These molecules are particularly important for controlling viral replication as well as other regulatory events in immune responses.

Given the quite extensive literature at this point in time, the question is then do we know all we need to know about TNF/TNFR molecules and their control of, or participation in, T cell responses? The answer is an emphatic no. This review will not attempt to catalogue what we do understand about individual TNF/TNFR members, as this has been done before [1–15], but rather will try to highlight areas where our knowledge is poor or lacking, and where we might benefit from more basic and applied experimentation. There has been fantastic success with the TNF blockers in autoimmune disease, and biologics that neutralize BAFF and RANKL (also in the TNF family), or kill CD30-positive tumor cells, are now additionally on the market [4], but none of these are principally thought to be directed against T cell activity. Biologics to neutralize several TNF family molecules (LIGHT, OX40L, CD40) are in clinical trials for T cell-mediated inflammatory disease and agonists to several TNFR members (4-1BB, OX40, CD27, CD40, GITR) are being tested to augment T cell immunity in cancer, with others family members also being considered as possible therapeutic targets [4]. However, arguably, we do not yet know how best to target the TNF/TNFR superfamily to modulate T cell-driven disease, and we do not understand which TNF superfamily interaction or individual molecule would be the preferable target for any particular disease indication. These are primary goals for the research community, but before we can easily address them, fundamental questions in T cell biology still need to be answered.

2. Control of T cell clonal expansion and accumulation

The initial response of a naïve T cell is often separated into three phases, namely expansion, contraction, and memory generation. As mentioned above, signals through a large number of

TNFR family molecules (OX40, 4-1BB, CD27, GITR, CD30, HVEM, DR3, TACI, TNFR2) have been shown to promote clonal expansion and the accumulation of high numbers of antigen-specific effector-type T cells in mouse models ranging from basic immunization schemes with nominal antigen to responses against viruses to models of autoimmune disease. Often this has also been accompanied by a parallel effect on the frequency of memory T cells that develop.

Given that such a large number of molecules display what appears to be a similar activity in the expansion phase of the T cell response, a primary question is whether this then means that the accumulation of T cells (effector and memory) in *any* immune response is *always* driven by multiple TNFR interactions, and if so are *all* the aforementioned receptors relevant or only select ones? The short answer is, we do not know. Being able to address this in responses against viruses, autoantigens, and tumor-associated antigens, is likely going to be key to our ability to effectively design therapeutic strategies in the years to come to either positively or negatively target these molecules. Certainly, one can find literature within the same apparent basic or disease model showing the importance and activity of many of these different TNFR molecules [3, 5], but in most cases the reports do not originate from the same laboratory and often the experimental protocols differ in small but potentially significant degrees precluding straightforward conclusions. There are some studies particularly in viral systems where several TNFR molecules have been studied side-by-side (e.g. [16, 17]), but these are relatively rare at present. Therefore, while implied, we do not actually have direct proof that the T cell response in every situation is being driven by two, or three, or multiple, TNFR interactions. More importantly, it is difficult to predict which molecules might be the primary drivers of any given T cell response, and it is likely that this will be highly variable and the nature of the TNFR interactions that are critical will not be the same in all T cell responses. Thus, there is still a need for many more studies of TNFR molecules and their relative contributions to the initial T cell response and the generation of populations of effector T cells in alternate inflammatory situations.

Many years ago [1] it was proposed that TNFR molecules are likely to act in a temporal manner on T cells, one after another (kinetic-use), allowing the response to be sustained in the short-term and long-term, and ensuring memory develops. For example, CD40L can be induced rapidly on T cells following antigen recognition and ligate CD40 on APC such as dendritic cells or macrophages. CD40 signals in turn can induce molecules like OX40L and CD70 that would then ligate OX40 and CD27 on the T cells, implying in some scenarios CD40 activity may precede the activity of OX40 and CD27. Along the same lines, certain TNFR molecules like CD27, DR3, TNFR2, HVEM, and GITR are constitutively expressed on most CD4 and/or CD8 T cells, whereas others such as OX40, 4-1BB, and CD30 are induced after antigen encounter, with their appearance sometimes occurring several days after the start of the T cell response. Moreover, some constitutively-expressed molecules can also be downregulated or upregulated after T cells are activated, additionally suggesting temporal activity will happen to an extent during the development of the majority of T cell responses. Perhaps most importantly, many of the ligands of these receptors are not immediately available and need to be induced via antigen recognition or by innate stimuli, which further substantiates the kinetic-use models. However, this then complicates the

simple hypothesis that the expression of the receptors on T cells can teach us all we need to know about the timing of action of the TNFR family. As well as acting temporally, another idea is that a number of TNFR molecules are also engaged and functional at the same time (concurrent-use). This could occur if, for example, a T cell expresses 2, 3, or 4, of these proteins as it is responding, and the APC with which the T cell interacts expresses all of the respective ligands. Temporal and concurrent activities are not of course mutually exclusive. Thus, who comes first, who comes second, who comes third, etc, is not at all clear, even in the most basic of model systems. Simply put, we would benefit from much more information on how TNFR molecules integrate together, and the relative importance of each individual receptor with respect to the others in controlling the accumulation of effector and memory T cell populations.

We are severely lacking in our understanding of the expression characteristics of most of the TNFR molecules in vivo. Data in animal models of disease are useful, but most importantly data from human samples during the course of immune disease may be essential. This similarly applies to the expression characteristics of the TNF ligands. As stated above, most TNF family molecules are not found constitutively and they have been hard to analyze because they are often weakly and transiently expressed on the surface of cells, either due to rapid cycling and endocytosis, cleavage from the cell membrane, or simply tight regulation. Therefore our knowledge of the concurrent or temporal expression of those TNF ligands that may drive T cell accumulation (TL1A, OX40L, 4-1BBL, CD30L, CD70, LIGHT, TNF) is almost non-existent in vivo at the present time. This begs the question of how much does their expression vary from one inflammatory or autoimmune disease to another, and from one stage of disease to another? Also, where are these ligands? During the initiation of a T cell response, it is logical that the TNF ligands, if expressed, will be on professional APC (DC, macrophages), but as the response progresses over time into the effector stages, B cells may be one primary source in follicles or germinal centers. Alternatively, perhaps non-lymphoid cells in the disease target tissue are most relevant at later stages of the T cell response. Isolated instances of the expression of some of these ligands have been reported in human or mouse tissues such as heart muscle or vascular endothelium, but it is not clear how widespread is their distribution, and again importantly how often are the various TNF family ligands co-expressed and are there patterns of co-expression that would dictate functional importance. For example, in diabetes or asthma, the most important expression to characterize may be that in the pancreas and lung respectively, and knowledge of TNF family ligand expression on peripheral blood cells, or lymph node and spleen cells, may be irrelevant or in some cases misleading. It then cannot be stressed enough that more, and varied, studies of mouse tissues and human biopsy material are needed to allow a better appreciation for the ability of T cells, and other lymphoid or non-lymphoid cells, to bear and use alternate TNFR and TNF family molecules.

Lastly, within the scope of this discussion, is the question of redundancy. Even if a TNF family receptor is expressed on a T cell and its ligand is available to that T cell, does this mean the interaction is active in contributing to the accumulation and persistence of T cell populations? It is possible that an interaction can occur but that it has no major physiological effect, which is the true definition of redundancy. In this regard, there are a few negative reports in the literature where no role for a TNFR family molecule has been found during

the response of a T cell or in a T cell-driven disease (e.g. [18, 19]), but whether these instances reflect true redundancy has not been adequately evaluated. Nevertheless, negative data is also very useful in being able to answer the questions posed above, and to understand how much cooperation (temporal or concurrent) does occur between alternate molecules in driving T cell priming and how the use of individual molecules may vary from response to response or between inflammatory diseases. Therefore, linking expression to activity is also vital. This can be done in vitro with human cells but is difficult in human disease without clinical trial, although it is easily studied in mouse models. One simple measure of the potential activity, and possible time of activity, of TNF ligands that can be performed in both mice and humans is to assess the amount and kinetics of the soluble versions of these molecules in serum or other biological fluids. There are a growing number of such studies in the human literature, however they are limited in not only the number of patients analyzed for any given disease, but perhaps as importantly limited in longitudinal analyses that would possibly lead the timing and nature of any future therapeutic strategies. Furthermore, in this area, there is still the question of what the soluble version of a TNF family ligand represents, and whether they are simply inflammatory cytokines or neutralizing decoy molecules, and whether they are useful biomarkers for disease activity and/or reflect their participation in the response. Regardless, an expanded knowledge of the expression and function of TNF molecules both from animal models and from human samples is likely to be an essential piece of the puzzle for understanding when alternate TNF/TNFR molecules participate in driving clonal expansion and accumulation of T cells and how inflammatory responses differ from disease to disease. This could also be essential for therapy of disease and for making an informed decision on which interaction may be the one to target (positively or negatively) for any particular stage of a given disease.

3. Development of Subpopulations of Effector and Memory T cells

Another aspect of TNF family biology that relates to T cell responses is whether different TNF/TNFR interactions selectively expand, or drive the development of, alternate subsets of T cells. Here, we can firstly think of this simply in terms of CD4 versus CD8 T cells. For example, some literature suggests 4-1BB and CD27 are more important for driving accumulation of CD8 T cells compared to CD4 T cells, and other literature that OX40 is more important for CD4 T cell responses. Understanding to a much greater extent this type of preferential activity particularly in infectious disease and autoimmune disease is of great value. A major factor that might dictate the involvement in favoring a particular T cell subset is again whether the molecule is expressed on the T cell being studied and whether its ligand is available. As discussed above, this may strongly vary depending on the overall inflammatory milieu, and it is also highly likely that there are suppressive influences (from cytokines like IL-10 or inhibitory molecules like PD-1 or CTLA-4) that will strongly dictate whether a particular TNF ligand or receptor is available to different T cell subsets. We have little in-depth knowledge of any type of alternate regulation of TNF molecules on varying subsets of T cells, again stressing how essential it is to understand when and where these molecules are expressed and what positively or negatively modulates their expression.

Within the same framework, an important feature of a T cell response is the decision to diverge into a subset within a lineage. For example, CD4 T cells can become follicular

helper cells (Tfh), or CD4 and CD8 T cells can become terminal effectors (sometimes referred to as short-lived effector cells, SLEC, which could be Th1/Tc1, Th2/Tc2, or Th17/Tc17) or they can become effectors that will persist as a population to ultimately form the memory pool (sometimes referred to as memory precursor effector cells, MPEC). Other variations on this theme are subsets of memory T cells [20] and there is certainly heterogeneity in memory T cell populations. Two primary subsets, effector memory (Tem) and central memory (Tcm) T cells have been characterized, although this may be overly simplistic. Tcm have been described to home to secondary lymphoid tissues, expand greatly upon encounter with recall antigen, and contribute to long-term protection, whereas Tem may primarily circulate through extra-lymphoid tissues and exert rapid effector function without the need to expand in numbers. Alternatively, other investigators have highlighted the propensity of subpopulations of T cells to stay in peripheral tissues [21]. These tissue-resident memory cells, Trm, may be a subset of Tem cells, or distinct, but the ability to accumulate in a particular tissue is of obvious importance, whether the T cell is reactive against epitopes expressed by pathogens that primarily infect or localize in one tissue, or is specific for a self-antigen with restricted location.

Do TNF/TNFR interactions then contribute to lineage or subset decisions during T cell responses and is this restricted to particular molecules or a general feature of many molecules? For example, in one study in mice lacking OX40 there was a selective impairment in the generation of CD4 Tem cells but essentially no effect on the development of Tcm cells [22]. Studies of humans deficient in TNFR molecules may help understand this type of T cell regulation as well, although these are likely to be limited. The first patient with an OX40 deficiency was recently described and found to have reduced proportions of CD4 Tem cells and poor CD4 T cell recall reactivity to several antigens, whereas other subsets like Tfh cells and CD8 T cell subsets were relatively normal in peripheral blood, at the least providing some correlation with the mouse data [23]. This suggests there can indeed be selective or differential activity in the TNFR family in contributing to T cell priming, but again a major question is whether this type of effect will translate across variable immune responses in infectious or inflammatory disease and will apply to multiple TNFR molecules. There are not many studies to date that have addressed this kind of regulation and certainly not comparing one TNFR molecule to another. Again, we could greatly benefit from more insight into this important area of T cell biology.

As well as our fundamental understanding of the activity of the TNF family, this knowledge may also be crucial to our ability to manipulate immune disease, both in autoimmune/inflammatory conditions and with vaccination for infectious disease and cancer. There are many questions on this subject that have yet to be addressed. Does blocking a particular TNFR interaction selectively impair only a subpopulation of T cells (e.g. Tcm cells, Tem cells, SLEC) and if so is this an advantage or disadvantage in terms of altering disease progression? If select TNFR molecules control the generation of Trm or their persistence, are these more attractive for clinical targeting? In cancer or infectious disease, can agonists to TNFR molecules skew the T cell response down a particular pathway, and if so is this good or bad? Let's say, if stimulating a TNFR primarily promotes SLEC or MPEC, this may be at the detriment of Tfh cells, resulting in a good protective CD4 or CD8 T cell response but an impaired antibody response, as seen recently with an agonist administered during

LCMV infection [24]. This would be acceptable and perhaps preferable in tumor therapy, but might be undesirable in infectious disease depending on how important antibody responses are for pathogen control. Other unanswered questions are whether certain TNFR molecules also control tissue homing of T cells? We have little or no knowledge of this, but it would likely depend on the ability of individual molecules to promote (or possibly suppress) expression of alternate chemokine receptors on T cells. Again, this is important as it might dictate which TNF/TNFR molecule is the preferred target for clinical intervention (with antagonist or agonist therapy), dependent on the primary tissue that is central to the specific immune disease in question.

4. Clonal contraction, and Persistence and Reactivation of Memory T cells

As well as TNFR molecules driving the earlier phases of T cell responses to promote division and survival, lineage commitment, memory generation, and homing, these molecules also may influence T cell responses at a later time after the peak of the effector response. Generally, T cells undergo contraction in numbers at the population level, during which many die and only a proportion survive becoming memory cells. Whether TNFR family members play a strong and universal role in controlling the extent of contraction is not entirely clear. Apoptotic signals from Fas, TNFR1, or the TRAILR, have often been quoted as being responsible for promoting death of effector T cell populations, but in reality this has not been investigated in many situations to allow any definitive statements on the importance of these molecules to this stage of most T cell responses. Similar to the hypotheses on temporal or concurrent activity of the stimulatory molecules in promoting T cell accumulation, it is not well understood if these death receptors also act in sequence or together, or are selective for subsets of T cells (e.g. SLEC, Tfh cells). There is a reasonable literature on how a deficiency in the death receptors or their ligands impacts CD8 T cell activity during viral infections [15], but many questions exist with regard to when and why these molecules are active or not required, and also their use or activity on CD4 T cells. For example, if one blocks a single death receptor, or all of these death receptor interactions concomitantly, several days into an immune response, does this promote a greater number of memory T cells to develop? If so, is there selectivity in the type of memory cell affected, such as enhancing Tcm over Tem, or primarily controlling the generation of Trm over lymphoid-organ homing memory cells?

Whether TNFR family interactions are major contributors to the persistence of memory T cell populations is additionally not clear. Given that many of the TNF family receptors or their ligands that control T cell responses are inducible and only transiently expressed, and that memory T cells are largely resting cells existing in a non-inflammatory environment and only turn over slowly in response to IL-7 and IL-15, it is logical to suggest that the TNFR family will not be of great importance for memory persistence in the absence of antigenic stimulation. However, our knowledge here is also poor. The TNFR molecules described to stimulate T cells have primarily been classified as costimulatory receptors, meaning that their primary function is to quantitatively or qualitatively augment signaling initiated when antigen triggers the T cell receptor (TCR). However, studies of 4-1BB and OX40 showed that these molecules can exert a survival activity in CD4 T cells and CD8 T cells that is completely independent of antigen recognition and TCR signaling, in addition to

being able to enhance TCR-dependent activities such as cytokine production [25–27]. This indirectly implies that memory T cells might indeed be maintained in part by TNFR interactions. Studies of 4-1BB and OX40 have suggested these molecules can be induced in the absence of antigen on memory CD8 and CD4 T cells by IL-15 and IL-7, respectively [25, 28], and therefore there is the potential that these molecules may in certain circumstances contribute to the survival of memory T cells. 4-1BBL is expressed in the bone marrow on most stem cells [29] where many memory CD8 T cells migrate, and it was shown to contribute to the persistence of these cells in one study [30]. Similarly, lymphoid tissue inducer (LTi) cells in some settings may regulate the persistence of memory CD4 T cells as they express both OX40L and CD30L [31]. Collectively, this suggests the possibility that any stimulatory TNFR molecule that can be expressed or induced on memory T cells, including 4-1BB, OX40, and CD30, could provide survival signals to these cells if they are ligated regardless of the presence or absence of antigen. Therefore, another largely unanswered question is whether there are niches in the body where subpopulations of memory T cells will reside or circulate through, where expression of many, or alternate, TNFR molecules and their ligands coincide, and if so is this a major contributing factor to the longevity of T cell memory in the absence of antigenic stimulation?

An additional very important facet of a T cell response is the ability of memory subpopulations to exhibit recall activity when antigen is encountered again. A general concept quoted in the literature is that memory T cells react easier and faster than naïve T cells, and certainly some subsets like Tem cells do exert immediate effector function without the need for further differentiation. However, there is reasonable, albeit not extensive, evidence that, in addition to signals provided by recall antigen through the TCR, memory T cells still do require other signals for their maximal response. These second signals may contribute to clonal expansion of Tcm cells, or full effector activity of Tem, Tcm, or Trm cells, or may modulate homing capacity. TNFR molecule interactions are likely to play crucial roles in reactivation of memory T cells in many situations, and indeed a number of studies have found reduced secondary expansion of memory populations in mice where OX40, 4-1BB, CD27, or HVEM interactions were neutralized (e.g. [16, 32, 33]). However, again, an in depth understanding of which TNFR molecules might contribute, and to what aspect of the recall response, and when they may contribute, is severely lacking. These are fundamental questions relevant to therapy of autoimmune and inflammatory disease and to booster or repeat vaccination strategies.

Within the same framework is the question of whether T cells differentiate, either under conditions of chronic or serial recall antigen encounter over time or in particular inflammatory environments, into a memory state where they become reliant (focused) on only select ligand-receptor interactions for their response. For example, it has been known for many years that CD27, which is normally constitutively expressed on all T cells, can be irreversibly lost on subsets of memory T cells, particularly CD8 T cells. Often this has coincided with loss of the Ig superfamily costimulatory molecule CD28, and been associated with chronic stimulation. Why are CD27 and CD28 lost? Does this reflect a poorly responsive state in the T cell because it negates these major stimulatory pathways, or do these T cells now rely more on other molecules, and particularly other members of the TNFR family, for their response, i.e. do CD27- or CD28-negative memory T cells retain the

ability to express molecules like OX40, CD30, 4-1BB, or GITR? There is a moderate amount of data suggesting this might be the case, such as a report of human CD28-negative T cells that found they could divide and make cytokines and cytotoxic molecules when stimulated by 4-1BBL [34], but a greater understanding of this awaits many more studies. Similarly, are there subsets of memory T cells that develop that lose the ability to express molecules like HVEM, OX40, 4-1BB, GITR, TNFR2, or DR3, but retain expression of very select TNFR family molecules? Knowledge of this is likely to be tremendously important when considering which molecules to target in inflammatory disease. If a persistent or chronic inflammatory condition is driven by memory T cells that are only responding to one or two TNF family ligands, this will strongly dictate which molecules would be targets for effective immunotherapy.

5. Modulation of Regulatory T cell Activity

There is little doubt that regulatory T cells (Treg) are a major part of T cell biology and their accumulation and/or activity will impact the accumulation and activity of effector and memory T cells. Two primary types of CD4⁺ Treg cells have been described, thymic-derived or natural Treg (nTreg), and inducible or adaptive Treg (iTreg) that differentiate from naïve and perhaps memory T cells [35], and CD8 T cells can also exert Treg activity.

A number of studies of OX40, 4-1BB, TNFR2, and GITR have demonstrated that their signals can block naïve T cells from differentiating into Foxp3⁺ iTreg cells, and in some cases IL-10⁺ iTreg cells, which will further contribute to the ability of these molecules to promote the clonal expansion and development of effector and memory T cell populations. Whether the other TNFR members that are thought primary drivers of T cell immunity (CD27, CD30, DR3, HVEM, and in some cases TACI) display this activity is not yet clear. Nevertheless, a complication in our understanding of the impact of TNFR molecules on regulatory T cell populations is the fact that most if not all of the molecules that are expressed on naïve/effector/memory populations are also expressed (constitutively or inducibly) on nTreg cells and already differentiated iTreg cells. A simple concept, fitting in with the overall function of the stimulatory TNFR members being to promote T cell priming, is that any direct activity of these molecules on the aforementioned Treg cells would involve inhibiting their suppressive function. Indeed, some studies of molecules like OX40 and GITR have reported this, although a major unanswered question is how do intrinsically stimulatory molecules block suppression and what is the exact suppressive activity that is targeted? However, studies of OX40, 4-1BB, TNFR2, GITR, and including DR3, have also found that ligation of these molecules can promote survival and/or expansion of these Treg cells, analogous to the activities they display on effector T cells. This may not be surprising assuming similar stimulatory signaling pathways can be engaged in both effector T cells and Treg cells. Therefore, what, if anything, dictates which activity (blockade of suppressive function, or promotion of expansion) will predominate in a Treg cell?

Under steady state conditions, there is little evidence at present that TNFR molecules are major regulators of the expansion, persistence, or suppressive activity, of nTreg cells or already differentiated iTreg cells, although this needs to be investigated in more depth.

Rather, most of the current evidence in this area comes from manipulating the TNFR molecules, particularly in experiments with agonist reagents. For example, striking studies with agonist antibodies of 4-1BB have shown they can suppress disease in many models of autoimmunity and inflammation, primarily by promoting a type of CD8⁺ Treg cell, although CD4⁺ Treg cells have also been implicated in these activities [36, 37]. Similarly, studies of agonist antibodies of DR3 and OX40 in certain model systems have also resulted in suppression of T cell-driven inflammation, again through promoting the expansion of nTreg or iTreg cells [38, 39]. The questions then are: how often do TNFR molecules positively promote survival or expansion of mature Treg cells, especially under inflammatory conditions; which molecules together with 4-1BB, OX40, and DR3 exhibit these activities, and importantly when are they manifest; and what is the consequence to the Treg and effector T cell balance when neutralizing or agonist TNFR reagents are administered in alternate inflammatory situations? Depending on the conclusions, this could have a great impact on the therapeutic activity of TNFR-directed biologics. For instance, when blocking molecules such as OX40, DR3, GITR, CD30, and TNFR2 with the aim of reducing effector and memory T cell accumulation in autoimmune disease or allergic disease, will nTreg cells or pre-existing iTreg cells also be negatively affected? If Treg cell accumulation and activity are reduced this might prevent a state of tolerance from being gained, perhaps resulting in transiently suppressed disease because of fewer effector/memory T cells, but no long-lasting effect as there will be no regulation stopping the reemergence of pathogenic effector/memory populations. Similarly, if agonist reagents are used for cancer immunotherapy or as vaccine adjuvants, will their efficiency in promoting the generation of effector or memory T cells against tumor-associated and pathogen-derived antigens be limited or negated by also promoting more Treg cells to accumulate? These are fundamental issues that need to be addressed in multiple situations to understand the action of each individual TNFR molecule, and ultimately to understand how best to target TNFR molecules for immunotherapy and which may be the preferable molecule to target in any given disease scenario.

6. Signal Transduction in T cells

Lastly, is the more basic issue of how TNF family receptors signal in T cells. In many respects, we will not fully understand how TNFR molecules control T cell activity until we know how they recruit intracellular adapters and kinases and positively or negatively control gene transcription. Our knowledge of how the death receptors (Fas, TRAILR, TNFR1) form signaling complexes and exert their activity through FADD/TRADD-driven caspase pathways is very good, including how TNFR1 can switch to being an NF- κ B-inducing pro-inflammatory molecule [40–42] - although arguably more could be done with T cells as much of this information comes from studies of cells such as fibroblasts. But a fundamental question that remains in this area is whether these death receptors integrate their signaling pathways in T cells. If so, do several molecules assemble supra-signaling complexes and is this essential for promoting T cell apoptosis? Further, does the signaling activity of each death receptor vary depending on the nature of the T cell? For example, by definition SLEC should be highly susceptible to death signals whereas MPEC should not undergo apoptosis even if death receptors are ligated. There was some attention paid to this quite a number of years ago with studies of “activation induced cell death” in T cells which were largely

focused on Fas [40], but it is not clear if we have a really strong understanding of what does and does not make a T cell receptive to undergoing apoptosis from the death receptors and how this is controlled intracellularly. Additionally, no significant information exists on whether there is direct or indirect intracellular cross-talk with other inhibitory receptors on T cells such as CTLA-4 and PD-1, and if a stimulatory TNFR molecule is engaged at the same time as a death receptor, what determines the functional outcome in T cells and how does this translate into signal complex formation?

Similar considerations apply to the stimulatory receptors and how they function in T cells. Although there is a reasonable literature on the intracellular pathways targeted through 4-1BB and OX40 [8, 43], and much of this has been done on either T cell hybridomas or primary T cells, our understanding of their signaling capacity is still actually limited. Moreover, the literature on signal transduction through CD27, GITR, DR3, HVEM, CD30, TACI, and even TNFR2 is almost non-existent in T cells. Each molecule can recruit a range of TRAF (TNFR-associated factor) adaptors, and activate one or both of the NF- κ B pathways (canonical and non-canonical), but which TRAFs are recruited in T cells to each individual molecule is largely unknown, nor whether there are quantitative differences in the ability of these molecules to promote canonical or non-canonical NF- κ B signaling. There is a strong literature on the importance of IKK α / β / γ -dependent canonical NF- κ B to enhancing division, survival, and cytokine production in T cells, and supportive data that a good proportion of the effects of the stimulatory TNFR molecules on T cells may be attributed to this. However, we do not fully understand the significance of activating NIK-IKK α -dependent non-canonical NF- κ B in T cells. This includes whether this pathway is differentially activated by stimulatory TNFR molecules, or whether it controls only very specific functions such as IL-9 production that was recently reported [44], or whether it acts similarly to the canonical pathway in controlling clonal expansion or survival of T cells [45].

There is also still much to be learned about how the TRAF molecules integrate TNFR signals in T cells. Many reviews have quoted the range of TRAFs that can bind to each TNFR molecule but most of this literature derives from transient transfection systems. For example, OX40, GITR, 4-1BB, CD30, HVEM, TNFR2, DR3, and CD27 all have the potential to bind TRAF2, but only GITR, CD30, HVEM, 4-1BB, and TNFR2 may bind TRAF1. Other differences have been suggested between these molecules in binding to TRAF3 and TRAF5. However, because the recruitment of these TRAF proteins has largely not been assessed in T cells when the respective receptor is ligated, either by its natural ligand or by agonist reagents, it is not clear if different receptors can truly build unique scaffolds of adaptors or whether most of these molecules when signaling into T cells essentially form similar, if not identical, signaling complexes. Also, again, does this vary depending on the T cell subset (SLEC, MPEC, Tem, Tcm, Trm) and if so why? Another question is how much of the signaling activity and complex formation of these TNFR molecules is also dictated by extrinsic stimuli? A recent study found that TRAF1 was specifically downregulated in T cells responding during chronic viral infections, correlating with loss of responsiveness to signals through 4-1BB. Moreover, exposure to IL-7 was found to restore responsiveness through this receptor by promoting TRAF1 expression [46]. Is this type of activity then widespread, and how much does the availability of TRAF adaptors impact the function of TNFR members on the various T cell subsets? Furthermore,

irrespective of TRAF availability, another largely unexplored area is whether the signaling complexes that are formed by individual TNFR molecules are modulated by signals transmitted through other T cell-expressed receptors such as the TCR itself, or CD28, or cytokine receptors, or the inhibitory ITIM-containing molecules like PD-1? There are limited studies in this regard (e.g. [26]). Much more needs to be learned, particularly in T cells that may have differentiated into alternate states, such as the exhausted-type or anergic-type T cells that are characteristic of chronic viral infections or infiltrate tumors and that may be heavily regulated by inhibitory signals from non-TNF family receptors.

Most of the stimulatory TNFR molecules expressed on T cells have also been shown to be capable of promoting activity in pathways more associated with the TCR, such as via Jun N-terminal kinase (JNK) and the AP1 transcriptional complex. However, the majority of this data is in simplified systems, and again reports in T cells responding to antigen under physiological conditions are not extensive. Another pathway that may be common to some of the TNFR costimulatory molecules involves phosphatidylinositol 3-kinase (PI3K) and the downstream protein kinase B α (Akt/PKB). The importance of these molecules to the costimulatory activity of OX40 in T cells has been documented extensively [8, 26] and evidence suggests that HVEM, TNFR2, 4-1BB, and DR3 also may engage this pathway for regulating T cell activity, including promoting effector T cell expansion and suppressing the generation of iTreg [47]. It is then possible that PI3K/Akt might also be central to many of the stimulatory effects of the TNFR family, but differential contributions to proliferation, survival, differentiation, and other facets, may manifest depending on the stage of the T cell response. At present there is little indication of qualitative differences in signaling between the various stimulatory TNF family receptors in T cells, which would go well with the notion of concurrent and temporal activity of these molecules in driving and sustaining T cell responses that was discussed above, but only expanding our knowledge of how they signal as individual entities, or whether they signal as co-clustered aggregates of multiple receptors, will provide answers to qualitative versus quantitative activity. In effect, much more needs to be learned before we can truly understand the degree of integration or degree of separation in signaling that might exist between each molecule when they are expressed and functional on T cells.

Conclusions

Research into how the TNF/TNFR superfamily controls and modulates T cell responses has progressed well over the past 10 years. Although fundamental knowledge of the immune system is good at any level, our primary goal should be to learn how to manipulate human immune system diseases. Indeed, studies have progressed to the stage that biologics that block or stimulate many of the TNF family receptors or ligands are being widely discussed as candidates for targeting T cell-driven processes, and some biologics have already entered clinical trial. However, undoubtedly we need more work in both basic and applied situations to realize the full contribution of these molecules to T cell immunity and to then realize the potential of biologics against these molecules for modifying human disease. The discussion here has presented a number of areas in T cell biology where our understanding of the TNF family is poor or not complete. Although not comprehensive, the questions posed here will hopefully spur further research in the next 10 years to more fully appreciate the influence of

these proteins and to provide a framework for the most effective manner in which to manipulate them for our benefit.

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Highlights

TNF/TNFR superfamily proteins are major regulators of T cells

OX40, CD27, GITR, DR3, CD30, 4-1BB, TACI, and TNFR2 promote clonal expansion and T cell memory

Fas, TNFR1, and TRAILR promote apoptotic death and limit T cell activity

There are many unknowns regarding their function, expression, and integration in T cell responses

Knowledge of how they control T cells will help fulfill their promise as targets for clinical therapy