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Lymphotoxin and TNF: How it all began- A tribute to the travelers

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Abstract

The journey from the discoveries of lymphotoxin (LT) and tumor necrosis factor (TNF) to the present day age of cytokine inhibitors as therapeutics has been an exciting one with many participants and highs and lows; the saga is compared to that in “The Wizard of Oz”. This communication summarizes the contributions of key players in the discovery of the cytokines and their receptors, the changes in nomenclature, and the discovery of the LT family’s crucial role in secondary and tertiary lymphoid organs. The remarkable advances in therapeutics are detailed as are remaining problems. Finally, special tribute is paid to two pioneers in the field who have recently passed away: Byron H. Waksman and Lloyd Old.

Keywords

The Wizard of Oz; Lymphotoxin; Tumor necrosis factor; Secondary lymphoid organs; Tertiary lymphoid organs; Therapeutic inhibitors of LT and TNF; Byron H. Waksman; Lloyd Old

1. Introduction

1.1. Purpose

Our knowledge of the lymphotoxin (LT)/tumor necrosis factor (TNF) family has been gained over the course of many years. I was asked to provide some insight into the early days of the field as one who has been involved for a long time. “The Wizard of Oz” by Frank Baum [1] is a popular book and movie about Dorothy from Kansas and her friends who encounter many obstacles and much excitement as they travel in search of their hearts’ desires, to be fulfilled by the great and powerful Oz in the Emerald City. Here I provide a somewhat biased account of the adventures of a group of travelers who journey along the “yellow brick road” and unlock the mysteries of LT and TNF from the discoveries of the molecules and receptors, to understanding their beneficial and harmful functions, to

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developing therapeutics that have transformed treatment of some autoimmune diseases. Special attention will be given to two pioneers: Byron H. Waksman and Lloyd Old, who were key movers in the LT/TNF field.

1.2. Description of the LT/TNF family

The immediate LT/TNF family consists of three tightly liked genes within the major histocompatibility complex [2]: TNFα, LTα, and LTβ. TNF is produced as a membrane bound molecule that is clipped by the TNF converting enzyme (TACE) to be released as a homotrimer to bind to one of two receptors, TNFR1 or TNFR2. LTα is released as a homotrimer and also binds to the two TNF receptors, hence explaining its similar activities to TNF. LTα3 also binds to an additional receptor, the herpes virus entry mediator (HVEM) as does LIGHT, which is not a member of the immediate LT/TNF immediate gene family. LTα is crucial for the transport of LTβ to the cell surface [3], resulting in the expression of the cell surface the LTα1β2 complex that binds to the LTβR. A recent report indicates that the LTα1β2 complex can be released via a metalloproteinase [4]. The interactions of ligands and receptors are depicted in Figure 1. Distinctions between the ligands include their regulation and cells or origin. A wide range of cells produces TNFα; this includes macrophages after stimulation by Toll-like receptors and CD4 and CD8 T cells after interaction with antigen. A more limited range of cells, including CD4 and CD8 T cells, B cells [5], and notably, lymphoid tissue inducer (LTi) cells [6], produces LTα and LTα1β2.

2. Discovery

2.1. Lymphotoxin

The 1960s saw the description of a secreted cytotoxic material produced by lymphocytes after stimulation by mitogen [7] or interaction with a specific antigen [8, 9]. Granger and his colleagues named this factor lymphotoxin [10]. (In fact, it is likely that these culture supernatants also contained TNFα). Aggarwal's purification of human LT from a lymphoblastoid cell line [11] provided information for its cloning in 1984 by Patrick Gray [12]; murine LT was cloned in 1987 [13, 14]. Werner Lesslauer's group's resolution of the crystal structure of secreted LTα3 with TNFRI [15] led the way to an understanding of the interaction of the many ligands of the extended TNF family with their receptors. Along the way, LT's name was somewhat arbitrarily changed to TNFβ [16]. The published rationale for this confusing change in nomenclature was that the same in vitro assay (killing L929 cells) was used to evaluate these molecules [16]. Later it was apparent that they were duplicated genes. The change in nomenclature was protested [17], but to no avail until the discovery of LTβ and the LTαβ complex by Browning and Ware [18] and the exciting realization that its biologic activity in lymphoid organ development) differed from that of TNFα [19] (see below). This resulted in renaming TNFβ back to LT (but now LTα!) and the demise of the name TNFβ. This back and forth has continued to engender confusion and frustration for students of this field for many years!

2.2. Tumor necrosis factor

TNF was discovered by Lloyd Old's group as a factor in serum in response to endotoxin that caused necrosis when directly injected into tumors [20]. As noted above, this somewhat

cumbersome assay was replaced by in vitro cytotoxicity against L929 cells. LPS-stimulated macrophages were a major source of TNF (later called TNFα). The Genentech group cloned the gene for murine [21] and human TNF [22]. Beutler and Cerami isolated a factor from a macrophage cell line that affected adipocytes in culture, which they called cachectin [23]. A sequence comparison determined that this was TNF. The observation that TNF was produced under septic conditions and that it might contribute to wasting led to rethinking about its role and trepidation concerning its use as an anti-tumor agent.

2.3. TNF and LTβ Receptors

A material that inhibited TNF was isolated from human urine by David Wallach in 1989 [24]. This was determined to be a TNF receptor. The groups of Loetscher and Lesslauer [25, 26] cloned the p55 (TNFRI) and p75 (TNRFII) receptors and it was revealed that both receptors bind TNFα and TNFβ (LTα). The gene for LTβR was cloned by the Immunex group of Smith and Goodwin [27] and found to bind both the LTα1β2 complex and LIGHT. The cloning of the receptors and ligands resulted in an explosion of knowledge concerning the signaling pathways of the immediate LT/TNF family and also those members of the extended TNF family.

2.4. Which cytokine is more important? Fashions come and go

Several years of research following the original descriptions of LT and TNF revealed important information about their cellular source of origin, mechanism of cytotoxicity through DNA fragmentation [28, 29], and signaling through the classical and alternative NFκB pathways. However, the original dream that TNFα and TNFβ (LT) would be useful as anti-tumor agents was not realized, as it was apparent that TNFα was a mediator in sepsis. The LT field lagged behind that TNF field for several reasons. Although recombinant human LT was available, murine LT proved difficult to prepare and thus signaling studies were not undertaken.. Furthermore, the most widely used monoclonal antibody to mouse TNF appeared to also neutralize LT [30] and for many years there was no antibody specific for murine LTα. TNF's implication in sepsis suggested that its inhibition might have clinical benefit; LT is not produced by macrophages after LPS and its inhibition was thus not an appropriate target for sepsis. Although both LT and TNF are clearly pro-inflammatory [31, 32] with effects on chemokine induction and changes in endothelial cells [33, 34], many researchers concluded that LT was a weaker, less important member of the family, and it languished in semi-obscurity with its new name, TNFβ. The generation of the LT and TNF transgenic and knock out mice and the discovery of LTβ resulted in LT enjoying resurgence in popularity as a subject of study and potential clinical relevance.

3. Roles in Secondary and Tertiary Lymphoid Organs

3.1. LT is crucial for secondary lymphoid organ development

In order to determine whether there were biologically significant differences between LT and TNF, and whether either molecule could induce Type 1 diabetes, mice transgenic for LTα or TNF under the control of the rat insulin promoter (RIP) were produced [31]. Both mice exhibit florid infiltrates in the islets of Langerhans that were later realized, at least in the RIPLTα mouse, to resemble lymphoid organs [35] (see below). Although the

morphological appearance of the infiltrates differs slightly in the RIPLT and RIPTNF mice [31], this alone did not reveal for a major difference in the biologic activity of the molecules. Neither mouse line developed diabetes unless a co-stimulator molecule unless the β cells also made a co-stimulator molecule [36]. Even though there was little difference when the transgenic mice were compared, the analysis of the knock out mice revealed dramatic differences in biologic activity. LTα knock out mice have major defects in SLOs with no lymph nodes, no Peyer's patches, highly disorganized spleens [37, 38], and defective nasal associated lymphoid tissue [39]. Mice deficient in LTβ have a similar, but slightly less drastic phenotype in that they retain mesenteric, cervical and sacral lymph nodes [19, 40] indicating that the LTαβ complex plays a major role in secondary lymphoid organs, with some role for LTα3 alone. Additional data indicating that LTα has unique activities as LTα3 in addition to its contribution to the LTα1β2 complex derives from the observation that LTα3 from innate lymphoid cells regulates IgA in the gut by regulating homing of T cells, and that this occurs through TNFRI and TNFRII [41]. There is also an alteration in the gut microbiome. These events occur independently of LTβ, which even though it also regulates IgA production, it does so in a T cell-independent manner. Mice deficient in TNF exhibit a much less severe SLO phenotype when compared to mice deficient in LTα or LTβ [42]. There are reductions in marginal zone macrophages, but the lymphoid organs are all present.

LT regulates lymphoid organs in ontogeny by its production by lymphoid tissue inducer cells (LTi cells, also called ILC3 cells) acting on stromal lymphoid tissue organizer cells (LTo) [6, 43] by means of their induction of lymphoid chemokines [44] and endothelial adhesion molecules [45–48] during development. In the adult, LT maintains lymphoid organs through its production by T cells, B cells, and DCs.

3.2. LT induces tertiary lymphoid organs

TLOs, or more accurately, tertiary or ectopic lymphoid tissues, are accumulations of cells that arise in non-lymphoid organs during chronic immune stimulation in autoimmunity, graft rejection, atherosclerosis, microbial infection, and some tumors [47, 49, 50]. These tissues have many characteristics of SLOs including T and B cell compartmentalization, lymphoid chemokines, antigen presenting cells, conduits, high endothelial venules and lymphatic vessels [51] and appear to act as sites of local antigen presentation. Mice transgenic for LTα under the control of the rat insulin promoter (RIPLTα mice) exhibit such infiltrates [31, 32, 35], as do mice transgenic for both RIPLTα and RIPLTβ, but not RIPLTβ alone [48]. The most obvious difference between the infiltrates in the pancreata of these two types of mice has to do with the nature of the HEVs. Those in RIPLTα infiltrates express MAdCAM-1, but their peripheral node addressin (PNAd) is only expressed abluminally, whereas those in the double transgenics express PNAd luminally and abluminally [48]. These differences are due to differences in expression of GlyCAM-1 and HecGlcNac6st2 (also termed HEC6ST, gene name *chst4*) [46, 48]. The LTαβ complex is crucial for these genes whose expression is necessary for luminal and abluminal PNAd [52] characteristic of a mature HEV [6] that can attract L-selectin^{+} naïve and memory cells to populate LNs and TLOs. This in turn allows presentation of antigen at the local site-beneficial in infection, but detrimental in autoimmunity where it can give rise to determinant spreading and disease exacerbation.

3.3. Exploiting information from SLOs and TLOs to develop mice for in vivo imaging

We were struck by the presence of HEVs and LVs in TLOs that appeared to be very similar to those in SLOs and resolved to determine if their functions and regulation were actually the same. In these ongoing experiments we are studying their regulation and function and have developed mice that have green fluorescent HEVs and red fluorescent LVs. This was accomplished by means of the pCLASPER recombineering technique [53] to isolate regulatory elements of Hec6St in the case of HEVs [54, 55] or Prox-1 in the case of lymphatic vessels [56] to drive reporter genes. In the case of the Hec6st reporter mice, the expression of both the endogenous gene and the transgene are inhibited by treatment LTβR-Ig, an inhibitor of LTαβ signaling [54]. The transgene is regulated identically to the endogenous gene in development and is expressed in HEVs in TLOs [54]. These data indicate that regulation of the HEVs by LTαβ is similar in TLOs and SLOs. Lymph nodes of mice with green fluorescent HEVs have been imaged in vivo [53, 57], demonstrating that it is possible to image events in real time in TLOs and determine if and how HEVs in that context act as portals for naïve cells to exacerbate autoimmunity or defend against tumors.

ProxTom mice with their red (tdTomato) fluorescent lymphatic vessels have also been successfully imaged in vivo [56]. Previous studies of sections of lymph nodes revealed remarkable plasticity of lymphatic vessels [58, 59] with robust lymphangiogenesis that occurs at early times after immunization and gradually resolves [59]. Interestingly, these early lymphatic vessels are defective in their ability to transport DCs [59] due to defects in lymphatic contraction [60]. We have demonstrated such lymphangiogenesis after immunization by in vivo imaging of lymph nodes of ProxTom mice [57].

4. Therapeutics

4.1. TNFα inhibitors

Once it became apparent that TNF would not be an effective anti-tumor agent because of its unfortunate activity that mimicked septic shock, attempts were made to develop reagents that could inhibit sepsis. Robert Schreiber and colleagues developed an anti-mouse TNF antibody that also appeared to have anti LT activity that was effective against sepsis in mice, but only if administered **before** LPS. Vilçek and colleagues developed a monoclonal mouse human chimeric monoclonal antibody, cA2 [61], which neutralized cachexia in mice transgenic for human TNF [62]. An alternative approach is to use a truncated portion of the p55 TNFRI in an Fc fusion protein. Originally called Lenercept, this is also protective against sepsis in mice. Later, Etanercept (EMBREL®) was developed using a similar strategy; in this case, the material is a truncated version of the p75 (TNFRII)-Fc fusion protein. Completely humanized versions of the receptor fusion proteins have also been developed (summarized in [63]).

Early attempts to inhibit TNF in situations other than sepsis included murine models of cerebral malaria and multiple sclerosis (MS). Georges Grau, Pierre Vassalli, and colleagues demonstrated that rabbit anti-TNF antibody protected mice against cerebral malaria even if administered 4 days **after** exposure to *Plasmodium berghei.* Unfortunately, this is not effective in humans suffering from malaria [64]. My group in collaboration with that of Bob

Clark used the Schreiber monoclonal anti-TNF antibody in to inhibit transfer of experimental autoimmune encephalomyelitis (EAE) [65] and later with G. Jeanette Thorbecke to inhibit relapsing EAE [66]. These results suggested that inhibition of TNF might be efficacious in human MS. Unfortunately, Lenercept protein was ineffective in a clinical trial of relapsing-remitting MS and in fact led to exacerbation of the disease in some individuals. The field carried on with the hope that inhibition of TNF might be effective in other autoimmune diseases. Mark Feldmann, Fionula Brennan, and Tini Maini were struck by the high levels of TNF in the joints of RA patients [67] and Feldmann and Maini conducted the first successful anti-TNF randomized trial against RA using cA2 (Infliximab) [68]. The anti-TNF therapies have revolutionized the treatment for RA, psoriasis, and inflammatory bowel disease.

Lenercept and etanercept inhibit both TNFα and LTα, thus expanding their range beyond the anti-TNFα antibodies. It has recently been reported that etanercept is effective at reducing both TNFα and LTα in the synovium of RA patients, particularly those who are high clinical responders [69]. Infliximab, the anti-TNF antibody, is less effective at reducing LTα levels. These observations are consistent with a direct effect of the TNF receptor blockers against both TNF and LT rather than a secondary reduction due to reduction in LT-producing cells infiltrating the joint. Whatever the mechanism, the data suggest another look at combined therapies is warranted.

4.2. LT inhibitors

4.2.1. LTβR-Ig—An LTβR-Ig fusion protein developed by Browning and colleagues [70] inhibits signaling of both LTα1β2 and LIGHT. It prevents development of most lymph nodes when administered to pregnant mice [71] with particularly striking results on blocking HEV maintenance through effects on GlyCAM-1 and Hec6ST [45, 59]. This reagent, has been effectively used in several mouse models of autoimmunity, including collagen arthritis [70] and salivary and lacrimal gland inflammation in the NOD mouse model of Sjögren's syndrome [72, 73]. Because so many chronic autoimmune diseases exhibit TLO characteristics, and because LTα1β2 is so crucial for HEV development and maintenance, it was thought that an inhibitor of this pathway might be efficacious in treatment of autoimmune diseases. However, the original promise of Baminercept, the material administered to humans [74], was not realized as it failed to meet its endpoint in a phase II trial in RA. Nevertheless, based on the success in treatment of salivary and lacrimal gland inflammation in mice, a Phase II trial is currently underway aimed at human Sjögren's syndrome (http://clinicaltrials.gov/ct2/show/study/NCT01552681).

4.2.2. Anti-LTα antibody—Jane Grogan's group has developed a humanized anti-LTα monoclonal antibody, designated MLTA3698A or Pateclizumab that reacts with both LTα3 and LTα1β2 [75]. The existence of a dual recognition molecule suggests that an approach may be useful that goes beyond inhibiting just one aspect of the LT family. Encouraging results reported in a phase I clinical trial in RA patients [76] provide even greater optimism for a multipronged approach.

4.3. Summary and future directions

Much work remains with regard to inhibition of the LT/TNF pathways in therapeutics. Why are some RA patients resistant to anti-TNF therapy? Perhaps the armamentarium could be increased to include reagents that target all three members of the LT/TNF family. How do we minimize the side effects that include reactivation of latent tuberculosis? How do we target TNF and LT at the local site while sparing the beneficial effects of these factors? Caution is warranted to prevent drastic effects on SLOs, given the crucial role of LT in their induction and maintenance.

In some cases chronic inflammation is beneficial. Breast cancer is a striking example where there exists a positive correlation of beneficial outcomes (long term survival, fewer metastases and deaths) with TLOs in the tumor, particularly if the density of HEVs is high [49]. Presumably, the TLO acts as a site for priming of naïve cells and thus induces resistance to the tumor. Thus, the future may include therapeutics that actually encourage the development of HEVs at the site of a tumor to allow generation of a local defense.

5. A Tribute to Two Pioneers

5.1. Introduction

In the majority of this communication, I have paid tribute to many of our fellow travelers. Here, for special notice, are two of the early champions of the field who are known for so much more than a single discovery and who have died since the last TNF Congress.

5.2. Byron H. Waksman (1918–2012)

Byron Waksman's early studies were on the role of the thymus in delayed type hypersensitivity in rats [77–80] and he can be considered a discoverer of the functions of that hitherto mysterious organ. He revealed the role of the thymus in tolerance by injecting soluble protein antigens into the thymus and demonstrating selective lack of reactivity to those antigens [81]. These experiments were precursors to our understanding of the exquisite control of self-antigen expression by Aire in the thymus [43]. He was a student of many models of autoimmunity including EAE and RA. His interest in understanding mechanisms of inflammation was crucial in the discovery of LT (called cytotoxic factor) with me [9] and IL-1 (called lymphocyte activating factor) with I gal Gery [82]. For many years Dr. Waksman was Chair of the Microbiology Department at Yale University School of Medicine. He joined the National Multiple Sclerosis Society as Director of Research and Medicine and served as President of the Waksman Foundation for Microbiology established by his father, Selman Waksman, the Nobel Prize winner for the discovery of streptomycin. In his later years, well into his 90s, Byron Waksman continued his involvement at New York University and Harvard University, attending lab meetings and giving seminars. Byron Waksman was above all a scientific communicator. He founded a program for scientific journalism at the Marine Biological Laboratory at Woods Hole and the European Initiative for the Communication of Science at the Max Planck Institute in Munich, Germany. In summary, Byron Waksman made crucial scientific contributions and was always aware of the broader clinical and societal implications of his work.

5.3. Lloyd Old (1933–2011)

Lloyd Old, considered by some to be the “father of cancer immunology” grew up in San Francisco where he aspired to be a classical violinist. He pursued that dream in Paris but returned to the United States where he pursued his interests in biology and medicine at the University of California at Berkley and the University of California at San Francisco where he graduated in 3 years at the top of his class. He did postdoctoral work with Baruj Benacerraf at Memorial Sloan Kettering where he remained for the rest of his career. His life’s work was devoted to answering three questions: 1) is there an immune reaction to cancer? 2) if so, what are the targets? 3) how can you stimulate that immunity? Dr. Old’s more than 800 publications included the discovery of TNF; the identification of the TL antigens, later called Ly1,2, and 3, eventually called CD4 and CD8; and the identification if the cancer testis antigens- NY-ESO-1.

Lloyd Old was tremendously influenced by the work of William Coley, a surgeon who injected bacterial lysates into cancer patients and in some cases showed remarkable reduction in tumor burdens. We now know that this material called “Coley’s Toxins” likely included substances such as LPS and other activators of Toll-like receptors and induced cytokines such as IL-1 and TNF. Lloyd Old took his fascination with Coley’s toxins along with Helen Coley Nauts, Dr. Coley’s daughter, to the establishment of the Cancer Research Institute (CRI) an organization that has provided crucial support in the form of postdoctoral fellowships and research grants for individuals in the TNF field. Dr. Old was instrumental in the Cancer Vaccine Collaborative, a joint program between the CRI and the Ludwig Institute for Cancer Research. This group is a network of world wide clinical trials and immune monitoring. In all these endeavors Lloyd Old laid the foundation and in fact provided answers to his three questions.

6. The Yellow Brick Road from Coley’s Toxins to therapeutidcs

In this communication, I have presented a brief history of the LT/TNF field with high and low points along the way. These are summarized in Figure 2. I leave it to the reader to decide who embodies the characteristics of the Good Witch Glinda, who could be the Wicked Witch of the North, and who are the most likely embodiments of Dorothy, the Tin Woodman, the Cowardly Lion, and the Scarecrow. In all seriousness, the field has brought out the best in the travelers who have persisted in the face of discouragement and changes in research trends and have shown a remarkably cooperative spirit as they move the field to its present prominence and level of accomplishment. We may not have yet reached the Emerald City, but we are well on our way.

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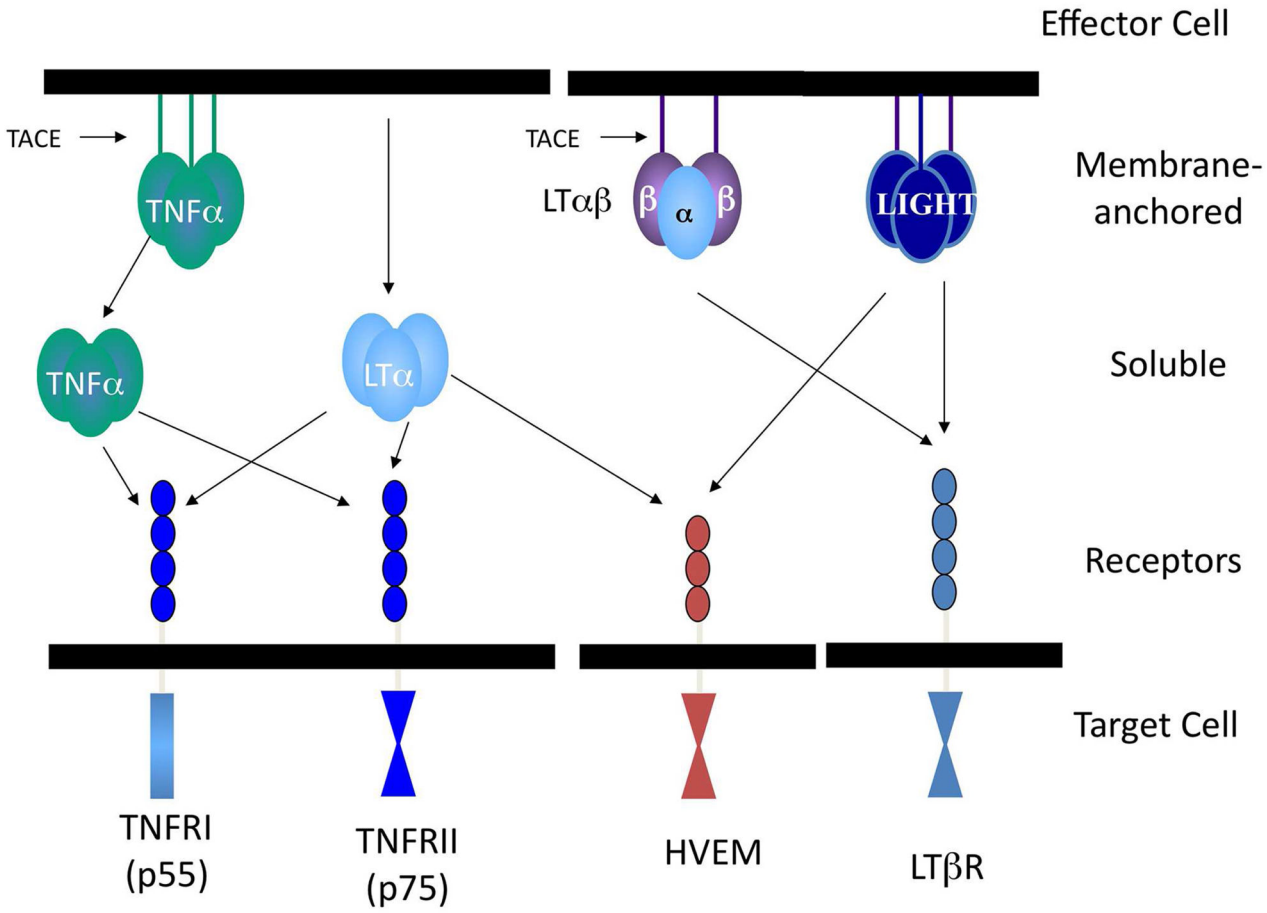

Figure 1.

A schematic depiction of the members of the ligands and receptors of the members of the lymphotoxin (LT)/tumor necrosis factor (TNF) family.

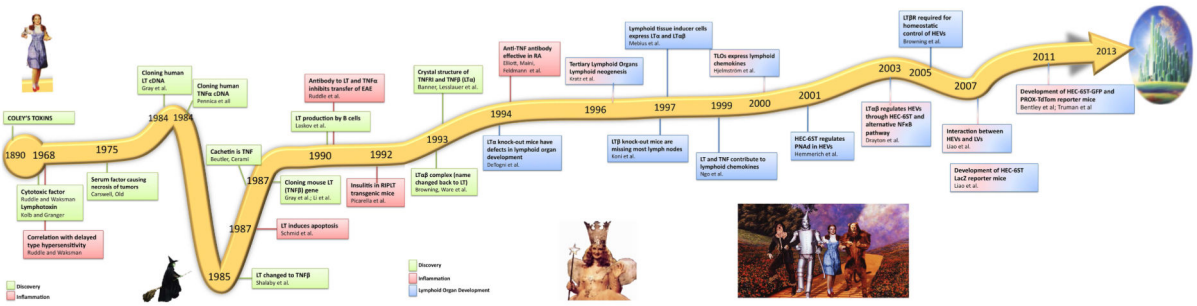

Figure 2.

The time line of the key discoveries in the field of LT and TNF depicted schematically with homage to “The Wizard of Oz” [1]. The insert depict the 4 main characters in the book, Dorothy, the Tin Woodman, the Cowardly Lion, and the Scarecrow.