The discovery of TNF-α: a historical perspective

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Abstract. Tumor necrosis factor (TNF), also known as TNF-alpha (TNF-α), is a pleiotropic pro-inflammatory cytokine that exerts multiple biologic effects. The research journey into the role of TNF has been a roller-coaster ride filled with ups and downs and it is still ongoing. At its discovery, huge expectations were laid upon this cytokine going so far that the cloning of human TNF, which had been achieved and published in 1984, was examined in the general press around the globe, and its therapeutic potential in cancer treatment was welcomed as the opening of a new era in cancer treatment. Nevertheless, the first clinical studies did not yield the expected results and it seemed for a moment that the glory of TNF was ending. But the discovery of the pro-inflammatory effect of TNF changed the course of the research journey of this cytokine. TNF is a highly pleiotropic cytokine and accordingly, it plays a pivotal role in many physiological functions, and is involved in a wide variety of pathological conditions. TNF has become a major target in chronic inflammatory diseases, and its neutralization have delivered the first insights into the development of a novel category of pharmaceuticals, the "biological drugs".

Key words: tumor necrosis factor, cytokine, cancer treatment, TNF-α

Introduction

The story of the discovery of TNF dates back more than 100 years and it is closely related to the history of anticancer treatment. William B. Coley, a surgeon from New York, was the first to use the endotoxin-induced antitumor activity in the treatment of sarcoma patients by local injection of streptococcal broth cultures (1). Over the course of the following years, as scientific research progressed, interest in Coley's mixed toxins faded away but never disappeared completely.

In 1968, Kolb and Granger, researchers from the University of California, reported a cytotoxic factor produced by lymphocytes and named it lymphotoxin (LT). The authors described some of the physical and chemical characteristics of LT, a cytotoxic factor released by human lymphocytes in vitro after stimulation with phytohemagglutinin (PHA). They observed that LT is a heat-sensitive, trypsin-resistant molecule exhibiting properties characteristic of a protein having a molecular weight of approximately 85,000 and a pH stability around 7 (2). Credit for this discovery was shared with Ruddle and Waksman from Yale University, who reported the same activity (3).

In 1975 the research article entitled "An endotoxin-induced serum factor that causes necrosis of tumors" was published by Carswell and colleagues in Proceedings of the National Academy of Sciences of the USA. According to Carswell et al., the cause of tumor necrosis was not bacterial endotoxin itself but rather a substance produced by host cells, most likely macrophages, that led to the death of both mouse

and human tumors. Carswell research group named this cytokine "tumor necrosis factor", now known as TNF-α (4) (Table 1).

Several articles describing the production, purification, and characterization of TNF-α and LT were published in the early 1980s. LT and TNF factors were both described based on their ability to kill L-929 mouse fibrosarcoma cells (5-7). Based on its capacity to engage the same cell surface receptor as TNF-α, it was discovered that LT (or TNF- β, as it is now commonly called) is another cytokine with cytotoxic actions similar to TNF-α and that it is structurally linked to TNF (8). Over the following15 years, papers emerged describing a large family of related molecules with contradictory roles in cell death, cell survival and organogenesis (9). In 1984 and 1985 human and mouse genes of TNF-α and LT were cloned (10-13) (Table 1).

Later on, the whole superfamily of 19 ligands related to TNF and 29 receptors was revealed. TNF superfamily ligands include TNF, the lymphotoxins (CD27L, CD30L and CD40L), FASL, APO2L/TRAIL, LIGHT, RANKL, APRIL and BLyS/BAFF. They are homotrimer type 2 transmembrane proteins that bind one or more receptors from the TNFR superfamily. TNF receptor (TNFR) superfamily members are Type 1 transmembrane proteins. Members of this family include TNFR1 and TNFR2, LT-βR, CD40, NGFR, OX40, FAS, CD27, CD30 and BAFFR (9, 14, 15). However, within this superfamily, TNF-α was recognized as a distinctively dominant intercellular communicating molecule with crucial roles in the innate and adaptive immune response.

Members of the TNF superfamily mediate a broad range of processes in development and physiologic functions in the organism, such as inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, and morphogenesis. Most of TNF molecules are produced by immune cells, which are involved in in host defense mediating an inflammatory response against infectious agents and malignancies (16). Besides the immune system, TNF proteins play a pivotal role in cardiovascular, neurologic, pulmonary, and metabolic diseases and in numerous autoimmune diseases. As a matter of fact, due to their involvement in a variety of pathological conditions, TNF superfamily members have become active targets for drug development in the last 20 years.

In the first instance, the use of TNF-α seemed promising in the cancer treatment but was soon abolished due to severe toxicity. On the other hand, systemic inhibition of TNF-α was shown to have remarkable therapeutic effects in the treatment of several autoimmune diseases (17). Contemporary, it was shown that continuous use of systemic anti-TNF/anti-LTα biologics could increase the risk of cancer (18). In this review, we summarize findings regarding the discovery of TNF-α and its role in cancer and discuss some unresolved disputes.

The discovery of TNF-α

The notion of the phenomenon that certain cancer patients who developed concurrent bacterial infections sometimes experience concomitant remissions of their malignant disease was established as far as 1700s. In 1774, a Parisian physician Dupré de Lisle injected pus into the leg of a patient with advanced breast cancer and observed that as the leg infection got worse the regression of cancer was more pronounced (19). During the 19th century, Busch and Fehleisen noticed regression of breast cancer and lymphoma after accidental erysipelas infections by erysipelas (a superficial, streptococcal infection of the skin) (20). In 1868, Busch was the first who intentionally inoculated a cancer patient with erysipelas and reported decline in the spreading of the malignancy (21). Fehleisen repeated this treatment experiment in 1882 and identified Streptococcus pyogenes as the causative agent of erysipelas (22) (Table 1). These first attempts remained at the level of mere observations until the experiments by William B. Coley, an innovative surgeon from New York. By examination of a series of medical reports, Coley observed that the concomitant erysipelas infection favored remission of sarcoma. His first experiment was performed in 1891 and consisted of local injection of streptococcal broth cultures in a cachectic patient with inoperable sarcoma of the neck and tonsil, at 3-to-4-day intervals (23) (Table 1). Coley noted only slight local reactions that lasted 24-48 hours, but the tumor slightly diminished in size, with general improvement of patient conditions.

Table 1. Timeline of discovery of the TNF-α and its role in the cancer

Year	Discovery	Reference
1774	Dupré de Lisle injected pus into the leg of a patient with advanced breast cancer and observed that as the leg infection got worse the cancer regressed more	19
1868	Intentionally inoculated a cancer patient with erysipelas, and reported decrease of the malignancy	21
1882	Repeated previous experiment in 1882 and ultimately identified *Streptococcus pyogenes* as the causative agent of erysipelas	22
1892	William Coley treats his first sarcoma patient with erysipelas	23
1896	Coley's mixed toxins used clinically for the first time	25
1931	Bacterial extracts shown to cause tumor necrosis in a guinea pig model of sarcoma	30
1944	Endotoxin is hypothesized as the active principle of tumor necrosis serum	31
1952	Endotoxin alone does not kill tumor cells in vitro	33
1962	Transfer of tumor necrotic activity in serum of endotoxin- treated animals	32
1968	Discovery of LT	2,3
1975	TNF discovered	4
1984-1985	Human and mouse TNF genes cloned	10, 12
1984-1988	Local treatment with recombinant TNF causes tumor necrosis in a range of mouse models	10, 46, 52, 48
1984-present	Identification and characterization of other members of the TNF and TNF receptor families	9, 15
1985	TNF and cachectin are identical	87
1985	TNF-α, in synergism with IFN-γ, activates neutrophils in vitro	85
1986	TNF-α exerts mitogenic effect on both mouse and human untransformed fibroblasts	82
1987	First clinical trials of TNF in advanced cancer	93, 94, 95
1987	TNF produced by cancer cell lines	66
1987	Angiogenic activity of TNF reported	96, 74
1989	Chromatographic purification of a binding protein, now known to be TNFR1 (also known as TNFRSF1A)	39
1990	Purification of a soluble form of the TNFR2 (also known as TNFRSF1B)	40
1989-1990	TNF-α mRNA and protein could be detected in malignant and stromal cells in human cancer biopsies	69, 70
1989–1993	TNF may increase cancer growth and spread	67, 75, 97
1989	TNF-α in combination with IL-1 activates endothelial cells, leading to the significant change in the tumor vasculature	56
1990	Cloning of TNFr1	98, 99
1990	Cloning of TNFr2	100, 101
1992	TNF, IFN-γ and mild hyperthermia treatment using isolated limb perfusion causes tumor necrosis in patients with sarcoma and melanoma	102
1994	First report of clinical activity of TNF antagonists in rheumatoid arthritis	103
1996	First TNF-knockout mouse	76
1999	TNF-knockout mice are resistant to skin carcinogenesis	77

Several months after treatment discontinuation, the tumors began to increase, reaching their former size. Coley attempted one more time with the injection of a new culture, which resulted in a severe attack of erysipelas which was almost fatal for the patient. Interestingly, the tumor of the neck began to break down on the second day of the infection. The infectious attack lasted for 2 weeks after which the large tumor of the neck completely disappeared. The patient regained his usual health and strength and was in good condition

for 8 years, until he experienced a relapse and died of the disease (1). From this first attempt Coley learned several important lessons: (i) erysipelas was not easy to induce; (ii) erysipelas was not easily controllable once induced and could be life-threatening in oncologic patients; (iii) some retardation of tumor growth, which was temporally associated with the injections even when erysipelas did not develop, was present; (iv) during the severe attack of erysipelas, a dramatic regression of the disease was observed. After several attempts resulting in patients' death, Coley began using the heat-killed versions of streptococci, which had only minor therapeutic effects. After coming across a publication in which was demonstrated that the virulence of streptococcal cultures could be enhanced by co-injection of the animals with heat- killed *Serratia* (24), Coley introduced his "vaccine" which consisted of less dangerous filtrates from cultures of heat-killed *Streptococcus pyogenes* and heat killed Gram-negative endotoxin producing *Serratia marcescens* (25). Coley's toxins consisted of an undefined mix of factors extracted from both Gram-positive and Gram-negative bacteria. Most likely, the combination of endotoxins induced a cytokine cascade that eventually led to effective antigen presentation to the immune system and cancer remission. Coley treated over 800 patients and approximately half of them had an impressive clinical improvement (26). From today's point of view, these data are quite controversial since they contain several potential biases: they were uncontrolled and quite frequently not reproducible; their effectiveness, as reviewed 40 years later by William Coley's daughter Helen Coley Nauts, was mostly based on anecdotal evidence.

Nevertheless, in 1934, the American Medical Association wrote that Coley's toxins "may sometimes play a significant role in preventing or retarding malignant recurrence or metastasis" and that "occasionally they may be curative in hopelessly inoperable neoplasms" as in that time frame they were the only known treatment for cancer (27). If the data produced by Coley were to be taken as they are, we could conclude that Coley was able to obtain rapid and extraordinary responses in patients who would represent a major challenge to oncologists even today (28, 29).

As research advances were made in the field of cancer treatment mainly by developing radiotherapy and chemotherapy, interest in Coley's mixed toxins significantly diminished. However, several groups of scientists continued this line of research. In 1931, it was shown that bacterial extracts caused tumor necrosis in a guinea pig model of sarcoma (30), in 1944 isolated lipopolysaccharide (LPS) from bacterial extracts tumor regression in a mouse model of cancer (31). O'Malley et al. were the first to use the term "tumor necrotizing factor" in a study in which they demonstrated that serum from endotoxin-treated animals led to necrosis of the tumor in animals with experimental cancers (32). In 1975,working on a murine model Meth A sarcoma, Carswell et al., discovered that the factor responsible for the "hemorrhagic necrosis" of transplanted tumors in animals was the host cells and not endotoxin itself. More than 20 years before, Algire et al. (33) proved that endotoxin does not kill tumor cells in vitro, speculating that hemorrhagic necrosis could be secondary to endotoxin-induced hypotension, leading to circulatory stasis and tumor ischemia. Carswell group went further and proved that endotoxin forced the host to release a toxic factor for the tumor, offering a more evident justification for endotoxin's indirect impact. Carswell group partially characterized TNF as a glycoprotein with a molecular weight of about 150,000 kDa which migrates with α-globulins (34) (Table 1). The cellular origin of TNF was unknown at the time, but the authors speculated that macrophages may be the source of it since the macrophage-inciting chemicals were required for its demonstration. In support of this hypothesis, the spleen of infected mice enlarged two hours after bacillus Calmette-Guerin (BCG) infection, with massive macrophage hyperplasia and elevated TNF circulating levels (4). One of the most intriguing findings of Carswell et al.'s work was the "provocative" behavior of macrophages which gained specific toxicity against malignant cells after exposure to agents such as BCG, endotoxin, and some protozoa (35, 36). Based on their findings, Carswell et al. hypothesized that because TNF exhibits discriminatory toxicity in vitro for transformed cells, it could potentially mediate the selective cytotoxicity of activated macrophages as well. That was the beginning of characterizing TNF as a strong pro-inflammatory mediator in the immune system, as it will be demonstrated years later. Between 1984 and 1985 the group of Aggarwal

structurally identified two different TNFs and cloned their genes. By using hundreds of liters of conditioned medium collected from the human lymphoblastoid cell line RPMI 1788, they have purified a protein 25 kDa in size, which was initially termed lymphotoxin-α (LT-α) and then renamed TNF-β (6, 7). Aggarwal et al. isolated a second cytotoxic factor with a molecular mass of approximately 17kDa and named it human TNF-α (5) using the same experimental approach (cell lysis assays and antibodies against lymphotoxin) and hundreds of liters of conditioned supernatants of human promyelomonocytic cell line HL-60. Although it is unclear whether the factor discovered by Carswell et al. through functional studies was the same factor examined by Aggarwal's group, it was the latter group that characterized TNF and LT at the molecular level by analyzing the amino acid sequence (5, 6, 7). It is worth noting that by creating antibodies against TNF and LT, it was discovered that these proteins are immunologically distinct, with TNF-α produced by macrophages while TNF-β by lymphocytes (37, 38). The full-length cDNAs for TNF-α and TNF- β were prepared and isolated by using an already-defined amino acid sequence (10, 11). After the discovery of TNF-α the next big challenge for the scientist was the identification of cell surface receptors. In 1985, it was reported that radiolabeled recombinant TNF-α and LT were bound to a single class of receptors on carcinoma cells (8). Chromatographic purification of a binding protein, now known to be TNFR1 (also known as TNFRSF1A), was achieved in 1989 (39), and a soluble form of the TNFR2(also known as TNFRSF1B) was purified in 1990 (40) (Table 1). These receptors are now identified by CD numbers as well: TNFR1 is CD120a and TNFR2is CD120b implying that they are both located on hematopoietic cells. TNFR1 has a far broader distribution than TNFR2, being practically expressed by every cell in the body (41). Genes for both TNF receptors were cloned in 1990 (reviewed in 41). The cloning of genes encoding TNF and TNF receptors enabled the development of several research tools, including gene-deleted mice that will further open the door for enormous amounts of research and discoveries regarding the potential roles of TNF-α.

TNF-α and tumor necrosis

In the years following its discovery, TNF-α cytotoxic effects were demonstrated in various animal cancer models as well as in human and murine-transformed cells in vitro (42-47).

Simultaneously, a similar T-cell immune response to the curative effects of endotoxin therapy was demonstrated (48, 49) were findings of the influence of TNF/IL-2/α-interferon combination therapy on cancer progression and metastasis (50, 51).

Furthermore, high dosages of human recombinant TNF-α caused necrosis of both syngeneic and xenografted tumors, but only with numerous local injections; otherwise, the chance of regrowth at the lesion's perimeter was greatly enhanced. (10, 46, 52, 53). An exception of this observation was the transplantable murine tumor Meth A sarcoma. In this type of tumor the systemic administration of TNF-α consistently caused hemorrhagic necrosis (10, 54, 55). The finding that tumor necrosis caused by TNF-α was hemorrhagic in nature, sparked intense attention and provoked further research. Mantovani & Dejana, in 1989 reported that in combination with cytokine interleukin-1 (IL-1), TNF-α was able to activate endothelial cells in a gene expression-dependent way, leading to a significant change in tumor vasculature (56) (Table 1).

Immunocytokines have recently been generated by the approach of fusion proteins between cytokines and antibody fragments, and those using TNF have been studied in vitro, in mouse models (57, 58, 59, 60), and clinical trials in humans with encouraging results. Moreover, TNF holds promising synergistic potential when combined with cancer immunotherapy, chemotherapy, anti-angiogenic therapy, or even with other immunocytokines (61-65).

TNF-α and tumor progression

Not all findings supported the hypothesis of TNF-α as a newly discovered miraculous molecule against cancer. In 1987, Spriggs et al. reported that TNF-α could induce a breast cancer cell line to produce more TNF-α (66). Other studies showed that TNF-α might induce the growth and enhance the

progression of tumor metastasis (67, 68). This was followed by reports that TNF-α mRNA and protein could be detected in malignant and stromal cells in human cancer biopsies (69, 70, 71) and that levels of plasma TNF-α were increased in some cancer patients, especially those with poor prognosis (reviewed in 72, 73). In 1987, Leibovich et al. were the first to prove that TNF-α might stimulate tumor growth acting as a potent inducer of new blood vessel growth (angiogenesis) (Table 1). Namely, in vivo at very low doses, TNF-α induces capillary blood vessel formation in the rat cornea and the developing chick chorioallantoic membrane. Moreover, in vitro, TNF-α stimulates chemotaxis of bovine adrenal capillary endothelial cells and induces cultures of these cells, grown on type-1 collagen gels, to form capillary-tube-like structures. In addition, the authors show that the angiogenic activity of TNF-α, produced by activated murine peritoneal macrophages, is completely neutralized by a polyclonal antibody to TNF-α. The authors conclude that TNF-α can have multiple roles. For example, in inflammation and wound repair, due to the angiogenic activity TNF-α could augment repair. On the other hand, in cancer TNF-α can have dual role, might both stimulate tumor development through the angiogenic activity and participate in tumor destruction by direct cytotoxicity (74).

In 1989, while studying intraperitoneal xenografts of ovarian cancer cells. Malik et al., showed that TNF-α treatment could transform ascitic free-floating tumor cells into solid peritoneal deposits with extensive stroma and blood vessels (75), which was further confirmed in various studies (75, 67).

The first TNF-knockout mouse was created in 1996 (76), which enabled more profound studies and gave much needed answers regarding the role of TNF-α in cancer. Shortly after the creation of Tnf–/– knockout mice, a paper was published in which was shown that, when treated with a skin carcinogen, Tnf–/– mice developed fewer, not more, tumors (77). This finding was followed by studies in models of lung and liver carcinoma showing a decreased metastasis burden, rather than augmentation, in Tnfr1–/– mice compared with normal counterparts (78, 79).

It is now acquired that many malignant cells constitutively produce small amounts of TNF-α, enhancing the growth and spread of syngeneic, xenogeneic and carcinogen-induced tumors of the skin, ovary, pancreas, pleural cavity and bowel (41). Indeed, a huge number of inflammatory cells and products/mediators of inflammation are detected in tumor microenvironment (80). There is substantial evidence that inflammation itself increases the risk for cancer development favoring proliferation and survival of malignant cells, angiogenesis and dissemination (80, 81). TNF-α is one of the major mediators of cancer-related inflammation (72). The research of the past 30 years has just begun to reveal its mechanisms of actions.

Other roles of TNF-α

Since its discovery numerous roles of TNF-α have been unveiled. It has been shown that TNF-α exerts some non-cytotoxic effects on normal cells such as mitogenic effect on both mouse and human untransformed fibroblasts (82) and, in the case of a precursor to a cytotoxic T cell can replace the specific action of IL-1 (83, 84). At least in human systems TNF, in synergism with interferon (IFN)-γ, activates neutrophils in vitro (85). TNF is also identical to cachectin (86, 87, 88). TNF directly or indirectly influences gene expression in untransformed target cells. For example, it enhances the synthesis of class-I HLA-antigens in vascular endothelial cells and dermal fibroblasts (89) and induces other surface antigen in endothelial cells (90).

However the multiple roles TNF-α have the negative side as well. The inappropriate or excessive activation of TNF-α signaling is associated with chronic inflammation and can eventually lead to the development of pathological complications such as autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, psoriasis, autoimmune uveitis, multiple sclerosis, systemic lupus (18). The role of TNF-α in these diseases has not been entirely understood; however, it is generally known to contribute to the progression of disease when excessively produced by activating and accumulating specific cell types causing tissue structure deformation (reviewed in 91). Understanding of the TNF-α signaling mechanism has been expanded and applied for the

treatment of autoimmune diseases, which has resulted in the development of effective therapeutic tools, including TNF-α inhibitors. Thus, one of the standard treatments for most of these disorders is systemic TNF neutralization nevertheless is connected with an increased risk of non-melanoma skin cancer (18).

In recent years, it has become clear that TNF-α drives inflammatory responses through direct mechanism of induction of expression of specific inflammatory genes, but also indirectly by inducing cell death (92), hence participating in numerous pathological conditions in the human body. For instance, in the brain, TNF-α induce pro-inflammatory signals, implicated in depression, bipolar disorder, epilepsy, Alzheimer's disease, and Parkinson's disease. TNF-α, along with other inflammatory molecules, has a pivotal role in the initiation and progression of several cardiovascular and pulmonary diseases, including asthma, chronic bronchitis, chronic obstructive pulmonary disease, acute lung injury, and acute respiratory distress syndrome (15).

Conclusion

TNF-α was initially considered the most promising agent for cancer treatment, but accumulating evidence changed this perception. Nowadays we know that inflammation bears both positive and negative effects on cancer and other diseases depending on specific conditions, target cells and microenvironment. The challenge is to harness the helpful aspects of inflammation and propagate them towards positive outcomes in cancer or in other diseases. The question if the TNF-α could be the key for resolving this challenge, remains open even after almost one century of intensive research.

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