Медицинская иммунология
2023, Т. 25, № 3,
стр. 435-440

Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2023, Vol. 25, № 3, pp. 435-440

СЛОЖНАЯ ИММУНОБИОЛОГИЯ ФАКТОРА НЕКРОЗА ОПУХОЛЕЙ И НОВАЯ АНТИ-TNF ТЕРАПИЯ

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Резюме. Фактор некроза опухолей (TNF) был открыт почти 50 лет назад как в «сывороточный фактор» у мышей после инфекций или инъекции бактериального липополисахарида (ЛПС) и обладающий ярким противоопухолевым эффектом. Молекулярное клонирование установило, что этой активностью обладает небольшой белок (17 кДа), принадлежащий к широкому множеству цитокинов. В силу особенности организации кодирующей последовательности TNF в геноме, все клетки, продуцирующие растворимый TNF, несут на своей поверхности и мембранно-связанный цитокин. Физиологические эффекты TNF опосредованы передачей сигналов через два типа высокоспецифичных рецепторов. Несмотря на гомеостатические и защитные функции TNF, в случае его избыточной системной или локальной продукции могут развиваться различные патологии – от септического шока до хронического воспаления. Поэтому в практической иммунотерапии нашли свое применение не агонисты TNF (от которых ожидали противоопухолевых эффектов), а антагонисты-блокаторы, которые оказались эффективными при лечении целого ряда аутоиммунных заболеваний с воспалительным компонентом. Наши исследования на мышах, основанные на технологиях обратной генетики и экспериментальных моделях заболеваний, выявили парадоксальное свойство TNF, состоящее в том, что одни клеточные источники этого цитокина (такие как миелоидные клетки) способствовали развитию заболеваний, а другие клетки (например, Т-лимфоциты) производили защитную форму того же цитокина. Имеется несколько возможных механистических объяснений этому явлению. Одно из них предполагает, что «патогенный» цитокин продуцируется в растворимом виде и может оказывать системные эффекты, действуя через TNFR1. При этом защитные эффекты связаны с мембранно-связанным TNF, который действует через TNFR2. Известно, что системная антицитокиновая терапия сопровождается нежелательными побочными эффектами, которые гипотетически могут быть объяснены нейтрализацией «полезных» функций конкретного цитокина. На основании этих соображений нами были разработаны прототипы блокаторов TNF, которые ограничивают биодоступность этого

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Образец цитирования:
С.А. Недоспасов «Сложная иммунобиология фактора некроза опухолей и новая анти-TNF терапия» // Медицинская иммунология, 2023. Т. 25, № 3. С. 435-440.
doi: 10.15789/1563-0625-COI-2860

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For citation:
S.A. Nedospasov "Complexity of immunobiology of tumor necrosis factor and novel anti-TNF therapy", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2023, Vol. 25, no. 3, pp. 435-440.
doi: 10.15789/1563-0625-COI-2860

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DOI: 10.15789/1563-0625-COI-2860

цитокина только из его главного «патогенного» источника – миелоидных клеток. Эти блокаторы, называемые MYSTI, представляют собой биспецифичные миниантитела, лишенные Fc-домена и связывающие как TNF, так и поверхностный маркер миелоидных клеток. MYSTI удерживает вновь синтезированный TNF на поверхности клетки-продуцента, а затем интернализует его. Этот новый тип иммунотерапевтических препаратов уже показал эффективность в ряде экспериментальных заболеваний.

Ключевые слова: цитокины, антицитокиновая терапия, биспецифичные антитела, мышиные модели, гуманизация

COMPLEXITY OF IMMUNOBIOLOGY OF TUMOR NECROSIS FACTOR AND NOVEL ANTI-TNF THERAPY

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Abstract. Tumor Necrosis Factor (TNF) was discovered almost 50 years ago as “serum factor” detected in mice following infections or administration of bacterial lipopolysaccharide (LPS), with a remarkable anti-tumor effect. Molecular cloning showed that this activity is mediated by a small protein (17 kDa), which belongs to a wide plethora of cytokines. Due to the particular organization of the TNF gene coding sequence, all cells producing soluble TNF also carry a membrane-bound cytokine on their surface. The physiological effects of TNF are mediated by signaling through two types of highly specific receptors. Despite established protective and homeostatic functions of TNF, when overproduced systemically or locally, it can trigger pathologies ranging from septic shock to autoimmune diseases. Therefore, in clinical immunotherapy there were not the TNF agonists, which were expected to induce anti-tumor effects, but rather the antagonistic blockers, that proved effective in a wide range of autoimmune diseases with an inflammatory component. Our studies in mice based on the technologies of reverse genetics and experimental disease models, revealed a paradoxical feature of TNF: some cellular sources of this cytokine (such as myeloid cells) promoted diseases, while other cell types (such as T lymphocytes) produced a protective form of the same cytokine. There are several possible mechanistic explanations for this phenomenon. On the one hand, the "pathogenic" cytokine is produced in a soluble form and can exert systemic effects via broadly expressed TNFR1. On the other hand, protective functions are mediated by the membrane-bound TNF via TNFR2. Systemic anti-cytokine therapy is known to be accompanied by undesirable side effects, which can hypothetically be explained by the neutralization of these protective functions. Thus, we developed prototypes of TNF blockers which limit the bioavailability of this cytokine only from its main “pathogenic” source – myeloid cells. This type of inhibitors, called MYSTI, represent bispecific mini-antibodies binding both TNF and a surface marker on myeloid cells and lacking the Fc domain. MYSTI retain newly synthesized TNF on the surface of the producing cell and then internalize it. This novel type of immunotherapy drug has already shown efficacy in a number of experimental disease models.

Keywords: cytokines, anti-cytokine therapy, bispecific antibodies, mouse models, humanization

This study is supported by Russian Science Foundation, grant #19-75-30032.

Introduction

Tumor Necrosis Factor (TNF) was discovered due to its anti-tumor effect in mice [3]. Molecular cloning identified a small protein (17 kDa) that can recapitulate TNF activity *in vivo* [14]. It was later found that TNF is the founding member of a family of cytokines that play an important role in the immune regulation [9].

TNF gene is linked to class III region of the MHC [11, 15] and is flanked by two related lymphotoxin genes, the other members of TNF family [12]. TNF is transcriptionally regulated in response to stimulation of various receptors of the innate immune system, such as TLR4. The primary product of TNF gene is a 26 kDa membrane-bound form of this cytokine. Analysis of TNF-deficient mice ruled out TNF as an enigmatic player in anti-tumor defense, but at the same time uncovered several unexpected functions, including proinflammatory activity [10, 13]. These functions are mediated by two distinct receptors, TNFR1 (the main receptor for the soluble TNF that is promiscuously expressed) and TNFR2 (responding to membrane-bound TNF and expressed on hematopoietic cells) [9]. Therefore, TNF signaling turned out to be more complex than originally proposed and includes three distinct signals: by soluble TNF through TNFR1, by membrane-bound TNF through TNFR2 and the “reverse signaling” by membrane-bound TNF into TNF-expressing cell (Figure 1). Unexpectedly, TNF was identified as an obligatory mediator of a number of disease states, including septic shock [2] and several autoimmune diseases with an inflammatory component. As a result, anti-TNF therapy was established as an effective treatment for rheumatoid arthritis [6] and several other diseases. However, such therapy cannot be free from unwanted side effects due to TNF role in protection from infections and its several homeostatic functions.

Materials and methods

Mice. TNF-humanized hTNFKI mice (6-8 weeks) were housed in SPF conditions at the Animal Facility of the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS. All manipulations with animals were carried out in accordance with the protocols approved by the Bioethics Committee of the EIMB RAS.

Primary cultures of bone marrow-derived macrophages (BMDM) and TNF-retention *in vitro*. Bone marrow was isolated from tibias and femurs of hTNFKI mice. For BMDMs differentiation bone marrow cells were cultured on non-treated cell culture plates in DMEM containing 30% L929 conditioned medium as the source of M-CSF, 20% FBS (Capricorn Scientific), L-glutamine and antibiotics (Pen/Strep, ThermoFisher). To analyze TNF production, cells were activated with LPS 10 ng/mL (O111:B4) for 4 hrs. Cytokine release in culture supernatants of stimulated BMDM, as well as BMDM preincubated with MYSTI for TNF-retention experiments, was measured by enzyme-linked immunosorbent assay (ELISA) using ready-made commercial kits "Human TNFalpha ELISA Ready-SET-Go" (ThermoFisher) according to the manufacturer's protocol.

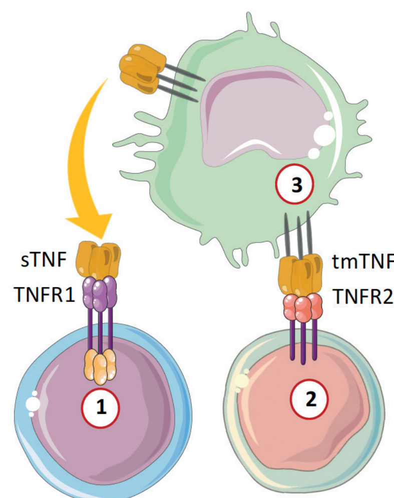

Figure 1. Complexity of signaling patterns induced by TNF

Note. Signal 1 is triggered by soluble TNF through TNFR1, signal 2 is triggered by membrane-bound TNF through TNFR2. Signal 3 is triggered through intracellular part of transmembrane TNF inside the producing cell

Administration of MYSTI and Infliximab. Anti-TNF reagents were administered i.p. at doses 1.5-3 mcg/g of body weight every day for MYSTI and every three days for Infliximab.

Germinal center (GC) formation. Induction of full-scope immune response was carried out by immunizing mice with 2×10^6 SRBC via i.p. injection in 200 mcl of PBS. Assessment of germinal center B cells and GC formation was carried out on day 8 following immunization by flow cytometry and IHC.

LPS/D-gal hepatotoxicity model. Mice were injected with MYSTI or Infliximab 30 min or 12-48 hours prior to of acute hepatotoxicity, which was induced by i.p. administration of 400 ng/g LPS (Sigma-Aldrich, L2630) and 800 μg/g D-Galactosamine (Sigma-Aldrich, G1639). Control group received vehicle buffer only (PBS). Mice were observed for 800 min after LPS/D-Gal injection. Moribund animals were euthanized, and time of death was noted.

Collagen antibody-induced arthritis. For induction of arthritis with arthritogenic antibodies, a cocktail containing five monoclonal antibodies against collagen type II and LPS from *E. coli* O111:B4 as adjuvant were injected in accordance with the manufacturer's protocol (Chondrex, USA, cat #53040). Clinical assessment for each paw was determined based on the scale as previously reported [8].

DSS-induced colitis. Mice were subjected to 5% DSS in drinking water for five consecutive days. Body weight changes and survival was measured during the three weeks following colitis induction. Colon length, weight and spleen index were measured upon termination of the experiment.

Experimental autoimmune encephalomyelitis. Mice were s.c. immunized with 50 μg of MOG_{35-55} peptide (Gene Script) emulsified in complete Freund's adjuvant (CFA) supplemented with 5 mg/mL *Mycobacterium tuberculosis* (Difco), followed by 150 ng of Pertussis toxin (List Biological Laboratories) administration on day 0 and 2. Mice were scored daily, and clinical signs were assessed according to standard protocol.

Results and discussion

Systemic anti-cytokine therapy may have undesirable side effects due to neutralization of the "beneficial" functions of a particular cytokine, which underlie its evolutionary selection, in particular, the role in protection against infections.

In this study we used engineered mice with humanization of the TNF system to compare the efficacy of two types of TNF inhibitors in several experimental disease models. We also addressed possible homeostatic effects of TNF inhibitors on microarchitecture of peripheral lymphoid tissues, known to be TNF-dependent. Earlier we used a panel of engineered mice with TNF deletions in specific cell types [7, 8] to define cellular sources of "deleterious" TNF in several experimental diseases. Surprisingly, in experimental arthritis "pathogenic" TNF is produced by myeloid cells, such as macrophages, while TNF produced by T lymphocytes provides protection [8]. Based on this unexpected observation we proposed a new type of anti-TNF therapy that relies on pharmacological neutralization of TNF from myeloid cells – the main source of pathogenic TNF. This therapeutic strategy is based on the administration of bispecific mini-antibodies that retain newly synthesized soluble TNF on the surface of macrophages and prevent it from diffusing out and triggering systemic TNFR1-mediated events [4, 5]. Such reagents, called MYSTI (from myeloid cell-specific TNF inhibitors), lead to retention and subsequent TNF internalization *in vitro* in the model of bone marrow-derived macrophages activated with LPS [4]. Side-by-side comparison with the efficacy of chimeric full-size therapeutic antibody, Infliximab, and MYSTI *in vivo* was performed throughout this study (Figure 2).

Specifically, both types of inhibitors protected mice from lethal hepatotoxicity and ameliorated experimental arthritis. However, only MYSTI was protective in experimental colitis and, unlike Infliximab, did not interfere with germinal center formation in SRBC-immunized mice. The lack of

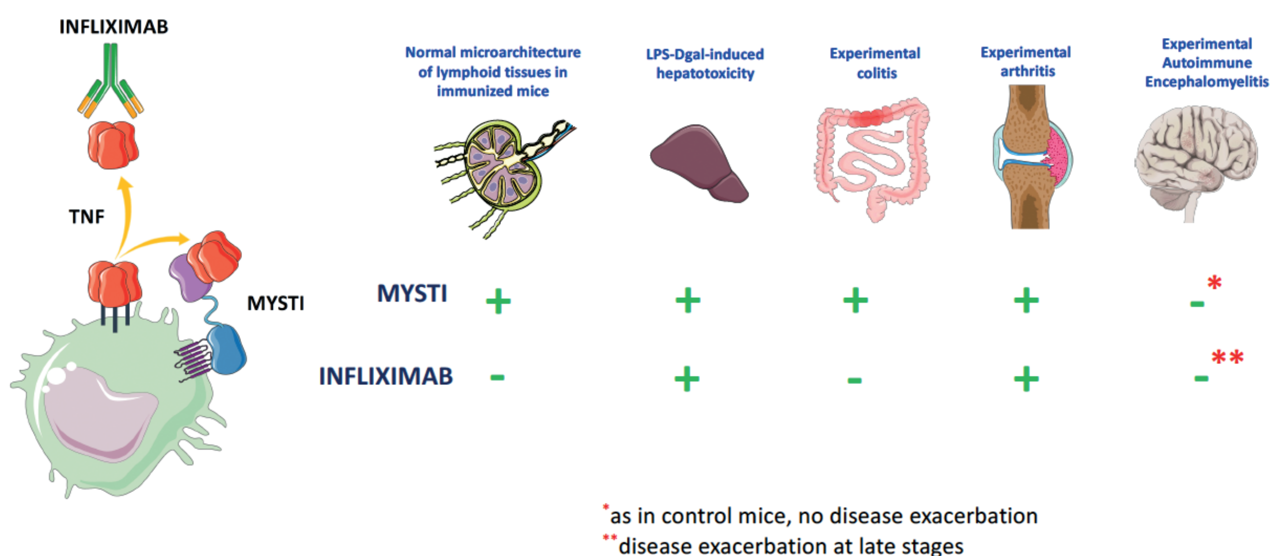

Figure 2. Comparison of the efficacy of systemic and myeloid cell-restricted TNF neutralization after immunization and in several disease models in humanized mice

staining for germinal center markers were previously noted in patients undergoing systemic anti-TNF therapy [1]. In EAE, a mouse model for multiple sclerosis (MS), both reagents failed to protect mice from disease development, but only Infliximab caused exacerbation at the late stage of the disease, reminiscent of the effects in patients that led to termination of a clinical trial with another systemic blocker in MS.

Conclusion

MYSTI represents a promising platform for further preclinical and clinical drug development.

Acknowledgments

I am greatly indebted to my colleagues Andrei Kruglov, Marina Drutskaya and Grigory Efimov, as well as to other members of my laboratory.

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Поступила 24.04.2023
Принята к печати 30.04.2023

Received 24.04.2023
Accepted 30.04.2023