TNF α kinoid vaccination-induced neutralizing antibodies to TNF α protect mice from autologous TNF α -driven chronic and acute inflammation

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The proinflammatory cytokine TNF α is a potent mediator of septic shock and a therapeutic target for chronic inflammatory pathologies including rheumatoid arthritis and Crohn's disease. As an alternative to anti-human TNF α (hTNF α) mAbs and other hTNF α blocker approved drugs, we developed an active anti-hTNF α immunotherapy, based on a vaccine comprised of a keyhole limpet hemocyanin-hTNF α heterocomplex immunogen (hTNF α kinoid) adjuvanted in incomplete Freund's adjuvant. In mice transgenic for hTNF α (TTg mice), hTNF α kinoid vaccination elicited high titers of Abs that neutralized hTNF α bioactivities but did not result in a cellular response to hTNF α . The vaccine was safe and effective in two experimental models. Kinoid-immunized but not control TTg mice resisted hTNF α -driven shock in one model and were prevented from spontaneous arthritis, inflammatory synovitis, and articular destruction in a second model. These data demonstrate an anti-cytokine induction of autoimmune protection against both acute and chronic hTNF α exposure. They show that active vaccination against a human cytokine can be achieved, and that the immune response can be effective and safe.

anticytokine vaccination | rheumatoid arthritis | TNF α therapeutic vaccination | TNF α -dependent shock

The pathogenesis of severe chronic diseases, including rheumatic diseases, autoimmunity, and cachexia, depend at least in part on cytokine dysregulation within altered tissues (1). In particular, the proinflammatory TNF α cytokine was shown to be critically involved in the pathogenesis of rheumatoid arthritis (RA) and further its inhibition by anti-TNF α Abs controls the disease in experimental models (2, 3). These data prompted anti-human TNF α (hTNF α) mAb therapy for RA in humans (4, 5). Efficacy in preventing RA of either mouse–human chimeric (infliximab; ref. 4) or fully human (adalimumab; ref. 5) mAbs was first demonstrated in the transgenic mouse model expressing hTNF α . These mice mimic the clinical and histopathological progression of RA in humans (6, 7). Thereafter, clinical trials using infliximab (8, 9) and adalimumab (10) were successfully completed. Today, these approved therapeutic mAbs, as well as soluble hTNF α receptor derivative (etanercept) specifically targeting hTNF α , are used in therapy of autoimmune diseases (11, 12). About 1 million patients to date have been treated with these TNF α blockers with clinical efficacy in RA (1), Crohn's disease (13), and psoriasis (14). Potential side effects, however, include the risk of infection (sepsis or tuberculosis; ref. 15) and the occurrence of anti-DNA autoAbs (16) and possibly lymphoma (17). Furthermore, these therapeutic approaches are expensive and cumbersome. These limitations prompted investigations of alternative strategies, including active anti-TNF α immunization, targeting TNF α pathogenic effects (18–20). In this report, we show that a biologically inactive but immunogenic hTNF α derivative, constituted of a keyhole limpet hemocyanin (KLH)-

hTNF α heterocomplex, called hTNF α kinoid (21), successfully triggers a B but not a T cell response to native hTNF α . Most importantly, this study also proved that induction of polyclonal autoAbs to hTNF α is safe and therapeutically effective in two experimental hTNF α -autologous models. Kinoid immunization inducing anti-TNF α Abs prevents acute TNF α -dependent lethal shock and chronic spontaneous destructive polyarthritis in hTNF α transgenic mice (TTg) mimicking RA.

Results

Biological Properties of hTNF α Kinoid. *Immunobiochemical characterization.* hTNF α kinoid is a KLH–hTNF α heterocomplex that migrated in SDS/PAGE as three bands exhibiting M_r of $\approx$ 250, 105, and 40 kDa (Fig. 1*a*). A rather large proportion of the kinoid did not migrate and is visualized at the level of the stacking gel. Both KLH and hTNF α were identified with specific antibodies (Fig. 1*b1* and *b2*). A similar diagram of migration was obtained with ¹²⁵I-labeled kinoid at either hTNF α or KLH, as shown in Fig. 1*c1* and *c2*. Four peaks were resolved on superose 6 gel filtration. One peak was in the exclusion volume, whereas the three others were of M_r 440, 158, and 13.7 kDa (Fig. 1*d*).

Absence of TNF α bioactivity. The kinoid was devoid of any TNF α -induced cytotoxicity, as assessed by standard L929 test even at the highest concentrations (Fig. 1*e*). hTNF α kinoid bound to both the hTNF α receptor I (p55) and receptor II (p75) despite its desirable lack of bioactivity (Fig. 1*f*).

Kinoid Induction of Anti-TNF α Abs in TTg Mice. In TTg mice, immunization with TNF α kinoid induced high titers of seric anti-TNF α Abs, whereas immunization with inactivated hTNF α or with control KLH failed to elicit any detectable anti-hTNF α Abs. By contrast, in BALB/c mice, both immunizations with kinoid or with inactivated hTNF α elicited anti-hTNF α Abs (Fig. 2*a*). In TTg and BALB/c mice, KLH but also kinoid immunizations resulted in the generation of anti-KLH Abs (not shown).

As assessed by standard L929 cytotoxicity tests, hyperimmune sera at high dilution from kinoid-immunized TTg mice neutralized TNF α bioactivity even at high dilution, whereas there were no effects of sera from KLH-immunized mice (Fig. 2*b*). The

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Conflict of interest statement: H.L.B. is a scientist, R.G. a consultant, and D.Z. a shareholder of Neovacs, Incorporated.

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Abbreviations: KLH, keyhole limpet hemocyanin; RA, rheumatoid arthritis; hTNF α , human TNF α ; TTg, hTNF α transgenic.

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ing, as shown by the reversal of arthritis in hTNF α -vaccinated TTg mice. This long-term observation demonstrates a long-lasting efficiency of an active anti-hTNF α immunotherapy.

In conclusion, these experiments suggest that active immunization against an endogenous cytokine may be a feasible approach in treating pathologies associated with high-TNF α production. They may represent an alternative strategy to passive anti-hTNF α mAb therapy or drugs that block TNF α activity currently used in patients with RA and Crohn's disease. TNF α kinoid vaccination would have the following advantages. First, the generation of polyclonal antibodies by active immunization is unlikely to generate antiidiotypic antibodies, thereby limiting the rate of therapeutic failure (29). Second, the potential low frequency of boost immunization would foster patient compliance. Finally, the proposed therapeutic approach would represent a nonnegligible cost advantage.

Materials and Methods

Reagents. KLH was purchased from Intracel, except when mentioned; hTNF α was from Boehringer Ingelheim (Mannheim, Germany); murine TNF α was from Cytolab (Rehovot, Israel); hTNF α receptors RI and RII, hTNF α , murine IL-2, and the murine IFN γ ELISA kit were from R&D Systems (Lille, France); and ISA51 adjuvant was from Seppic (Paris, France).

Animals. Four- to five-week-old female and male heterozygous TTg (1006-T) were purchased from Taconic Farms (Germantown, NY); 6- to 8-week-old female C57BL/6 and BALB/c mice were from Charles River Breeding Laboratory (L'Arbresles, France). Mice were maintained under pathogen-free conditions.

Preparation of Immunogen. The KLH-hTNF α heterocomplex was prepared as follows. hTNF α (1 mg) in 1 ml of 0.5 M EDTA/0.1 M phosphate buffer, pH 7.8 (working buffer) was treated with 1% DMSO for 30 min. First KLH (0.430 mg) and then glutaraldehyde (0.026 M) were added to this solution. After 45-min incubation at 4°C, the preparation was dialyzed against the working buffer and then treated with formaldehyde (66 mM) for 6 days at 37°C. After quenching with glycine (0.1 M) and subsequent dialysis against Dulbecco's PBS, the preparation was stored at 4°C. The heterocomplex was also prepared with either

radiolabeled TNF or KLH proteins using Iodogen (Pierce, Rockford, IL). Native KLH and hTNF α treated as described above were used as control and referenced in the text as KLH and inactivated hTNF α .

SDS/PAGE, Silver Staining, and Immunoblotting. SDS/PAGE analysis under nonreducing conditions was carried out according to Laemmli (30). On a 12% SDS/PAGE, the proteins were revealed by silver staining, autoradiography (31), or immunoblotting analysis by using the appropriate antibodies.

Gel-Filtration Chromatography. The heterocomplex was loaded on a Superose 6 10/300 GL gel-filtration column (Amersham Bioscience, Orsay, France) preequilibrated with DPBS and eluted with the same buffer as used for the equilibration, at 0.2 ml/min.

Cell Cultures. Mouse L929 cell line (American Type Culture Collection, Manassas, VA; CCL 1) was cultured in RPMI medium 1640 containing 10% FCS. Purified murine splenocytes were resuspended in RPMI medium containing 5% FCS and 50 μ M 2-mercaptoethanol and incubated in culture medium with or without KLH (30 μ g/ml), hTNF α (3 μ g/ml), and murine TNF α (1 μ g/ml) antigens.

T Cell Proliferation Assay. Antigen-activated splenocytes were cultured for 4 days and then pulsed for 18 h with 3H-Thymidine. Cells were harvested 18 h later on filter mats, and thymidine incorporation into DNA was quantified by using a β counter. The stimulation index (SI) was expressed as [(mean of cpm from stimulated cells)–(mean of cpm from unstimulated cells)]/(mean of cpm from unstimulated cells). SI values >2 were considered positive.

hTNF α and Receptors Assays. Direct receptor-binding assay. The heterocomplex and hTNF α binding to their target receptors were measured by using precoated plates with 50 ng/well of hTNF α RI or RII. Serial dilutions of the samples were incubated with their receptor and bound hTNF α were detected by using biotinylated goat polyclonal anti-hTNF α Ab (R&D Systems). To evaluate the capacity of serum or IgG to inhibit the binding of hTNF α to its receptors, hTNF α was pretreated with serial dilution of tested sera or IgG before being transferred to the plate onto which hTNF α RI and/or RII were immobilized. The blocking titer was expressed as the reciprocal of the serum dilution neutralizing 50% of hTNF α binding.

Anti-hTNF α Ab titer assay. Specific anti-hTNF α Ab titer in sera of immunized and control mice was determined by a direct ELISA. Precoated ELISA plates with 50 ng/well hTNF α were incubated with serial dilutions of sera from immunized and control mice. Specific IgG were detected by using peroxidase rabbit anti-mouse IgG (Zymed, Carlsbad, CA). Endpoint titers were expressed as the reciprocal of the highest sample dilution giving an OD of 0.3.

Cytokine quantification. hTNF α , murine IL-2, and murine IFN- γ were determined in the serum and culture supernatant by ELISA.

hTNF α Bioassay. hTNF α activity was assessed by using the L929 cytotoxicity assay (32). Serial dilutions of hTNF α and heterocomplex were incubated for 18 h with L929 cells in the presence of actinomycin D (1 μ g/ml) and the number of surviving cells determined by the MTT dying assay. The ability of hyperimmune serum or IgG to neutralize hTNF α activity was similarly determined after incubating sera and IgG with hTNF α . The neutralizing titer was expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNF α activity.

Fig. 5. Vaccination with TNF α kinoid improves synovitis and articular destruction in TTg. Examples of histological sections stained with H&E are shown. (a and b) Ankles in vaccinated mice (a; normal aspect) and controls [b; with synovitis (score = 2) and destruction (score = 1.5)]. (c and d) Knees in vaccinated mice (c; normal) and controls [d; with synovitis (score = 1.5) and articular destruction (score = 1.5)]. (Magnification: a and b, $\times 100$; c and d, $\times 40$.)