Modulation of Anti-Tumor Necrosis Factor Alpha (TNF-α) Antibody Secretion in Mice Immunized with TNF-α Kinoid

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Tumor necrosis factor (TNF) is a well-established therapeutic target in several chronic inflammatory diseases, including rheumatoid arthritis (RA), psoriasis, and Crohn’s disease (1, 14). For treatment of RA, two classes of TNF-α-blocking agents have been developed so far: a soluble TNF-α receptor (etanercept) and TNF-binding monoclonal antibodies (MAbs) or MAb fragments, such as infliximab, adalimumab, golimumab, and certolizumab. These biologic drugs show rapid and substantial therapeutic efficacy in most patients and in experimental models (12). TNF-α is not the only compound involved in the pathophysiology of RA, and better disease control is often achieved when TNF-α antagonism is associated with an immunosuppressant agent such as methotrexate. Indeed, infliximab treatment induces the re-emergence of a discrete regulatory T cell subtype in RA patients and inhibits Th1 and Th17 accumulation in the joints (3, 4, 23, 24).

Current TNF-α-targeting strategies have several drawbacks. First, anti-TNF-α agents raise some concern because of the role of TNF-α in controlling infections and tumors. Second, primary and secondary failures are not infrequent: in clinical trials, less than 50% of responder patients attained disease remission (27). The risk of antidrug antibody (ADA) production, with possible loss of efficacy and side effects, is inherent in the use of current anti-TNF-α agents, especially monoclonal antibodies (2). Third, treatments with biologics have high costs for the community, which precludes their usage in some countries (12). Thus, there is a need to develop new drugs to neutralize TNF-α.

A promising alternative strategy consists of active immunotherapy against TNF-α, i.e., anti-TNF-α vaccination. This technique leads to the production of neutralizing polyclonal antibodies by the patient and avoids the possible loss of efficacy by production of antidrug antibodies. Over the last decade, several active anti-TNF-α immunotherapies using TNF-α derivatives as immunogens have been developed and tested in experimental models of RA (5, 7, 8, 26). Immunogens must be capable of disrupting B cell but not T cell tolerance of self cytokines, thereby eliciting the production of neutralizing antibodies at high titers. Recently, we developed a heterocomplex vaccine, called human TNF-α kinoid (TNF-K), consisting of biologically inactive but immunogenic human TNF-α (hTNF-α) conjugated to a carrier protein, keyhole limpet hemocyanin (KLH) (20). Since antibodies generated by TNF-K immunization target only hTNF-α, we tested TNF-K in hTNF-α-transgenic (TTg) mice, which overexpress hTNF-α and develop a spontaneous arthritis at 6 to 8 weeks of age (19). In the TTg mouse model, we showed first that an early anti-hTNF-α immunization protected TTg mice from developing arthritis (9). We were subsequently able to show that TNF-α is efficacious against established arthritis, inducing a transient hTNF-α blockade with reversible effects on arthritis (10). These results contributed to the initiation of two clinical trials in Crohn’s disease (EudraCT number 2010-019996-32) and RA (EudraCT number 2009-012041-35).

The objective of the present study was to further investigate the immune effect of TNF-K in the context of coadministration of immunosuppressant drugs. We first aimed at studying the effect on the response to the kinoid of coadministration with various immunosuppressant agents, such as MTX and corticosteroids,
which are currently used in clinical practice during the treatment of RA and other TNF-dependent diseases. Then, we investigated the impact of a high dose of an immunosuppressant agent on the response to TNF-K immunization.

**MATERIALS AND METHODS**

**Mice.** Seventy-three female BALB/c mice (6 weeks old) were purchased from Janvier Laboratory (Le Genest-St-Isle, France). *In vivo* experiments complied with the recommendations for animal experimentation issued by the Institutes of Laboratory Animal Resources committee and by the local Ethics Committee on Animal Care and Experimentation. Mice were randomly distributed into nine groups of 8 mice each (except for group D1, which had 9 mice) and identified according to the study design described in Table 1. The first five groups of mice (A1, A2, B1, B2, and C) were included in part I of the study, while the last four groups (D1 to D4) were included in part II.

**Immunogens and administration.** Human TNF-α koidin (TNF-K; 40 mg, scale of vaccine production; batch G) was provided by NeoVacs (Paris, France). TNF-K was emulsified with Montanide ISA 51 VG (Microvette, Sarstedt, France). The tubes were kept at room temperature for 10 min (at 4°C). TNF-K emulsions were aseptically prepared under a laminar flow hood and kept at 2 to 8°C for at least 1 h and no more than 4 h before injection. Animals were injected intramuscularly with 40 mg/kg of TNF-K at days 0, 7, 28, and 49 for part I of the study and days 0, 7, and 28 for part II.

**Bleeding.** Blood was collected by retro-orbital sinus puncture at days −6, 32, 40, and 60 and by heart puncture at day 70 for anti-hTNF-α and anti-KLH antibody (Ab) titration, as well as anti-hTNF-α Ab neutralization assessment. The blood was directly transferred to gel separator tubes (Microvette, Sarstedt, France). The tubes were kept at room temperature for at least 20 min and then centrifuged at 1,800 × g for 10 min (at 4°C). Sera were stored at −80°C until use.

**Immunosuppressant administration.** Animals were injected intraperitoneally (IP) with the immunomodulators cyclophosphamide (Cyc), methylprednisolone (MP), and methotrexate (MTX).

**Study part I.** To investigate the potential impediment to the induction of antibodies after immunization by immunomodulator coadministration, immunomodulators were administered at a chronic dose (MP, 0.2 mg/kg; MTX, 1 mg/kg) three times per week for 9 weeks (except one time missed for all groups) before and during the TNF-K immunization from day −6 to day 4 to day 67.

**Study part II.** To investigate the possibility of influencing the production of antibodies after immunization, several immunomodulators were administered in short courses at high doses 7 days after the last booster dose of TNF-K. Two (Cyc) or four (MP and MTX) injections of immunomodulators or phosphate-buffered saline (PBS) were given. Cyc (200 mg/kg) was injected at days 35 and 44. MP (5 mg/kg), MTX (2.5 mg/kg), and PBS were injected at days 35, 38, 41, and 44.

**Serum analyses.** Serum samples were analyzed for anti-hTNF-α- and anti-KLH-antibody titers by enzyme-linked immunosorbent assay (ELISA) and for neutralizing capacity by an L929 bioassay.

**RESULTS**

**Absence of influence of concomitant low doses of immunosuppressants on anti-hTNF-α and anti-KLH Ab levels.** The first part of the study aimed at determining whether the effects of TNF-K immunizations were modified by concomitant and repeated immunosuppressive treatments in BALB/c mice. As shown in Fig. 1A, high levels of anti-hTNF-α Abs were

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**TABLE 1 Experimental design**

| Study part | Group | Immunosuppressant | Dose of TNF-K (μg) or immunosuppressant (mg/kg)* | Day 0 | Day 2 | Day 4 | Day 7 | 3×/week for 9 weeks | Day 28 | Day 35 | Day 38 | Day 41 | Day 44 | Day 49 |
|------------|-------|-------------------|-------------------------------------------------|-------|-------|-------|-------|---------------------|-------|-------|-------|-------|-------|-------|
| I          | A1    | MTX               | 1                                               | 4     | −     | 1     | 4     | 4                   | −     | −     | −     | −     | −     | −     |
|            | A2    | MTX               | 1                                               | 1     | 1     | 4     | 1     | 4                   | 4     | 4     | 4     | 4     | 4     | 4     |
|            | B1    | MP                | −                                               | 4     | −     | 0.2   | 4     | 0.2                 | 4     | 4     | 4     | 4     | 4     | 4     |
|            | B2    | MP                | 0.2                                             | 0.2   | 0.2   | 4     | 0.2   | 4                   | 4     | 4     | 4     | 4     | 4     | 4     |
|            | C     | PBS               | /                                               | /     | /     | 4     | /     | /                   | 4     | 4     | 4     | 4     | 4     | 4     |
| II         | D1    | PBS               | 4                                               | 4     | 4     | 4     | /     | /                   | /     | /     | /     | /     | /     | /     |
|            | D2    | CYC               | 4                                               | 4     | 4     | 200   | −     | −                   | −     | −     | −     | −     | −     | −     |
|            | D3    | MP                | 4                                               | 5     | 5     | 5     | 5     | 5                   | 5     | 5     | 5     | 5     | 5     | 5     |
|            | D4    | MTX               | 4                                               | 2.5   | 2.5   | 2.5   | 2.5   | −                   | −     | −     | −     | −     | −     | −     |

* Immunization with TNF-K was by the intramuscular route, and administration of immunosuppressant was by the intraperitoneal route. BALB/c mice were sacrificed at day 70.

Shading indicates TNF-K immunization; other columns show doses of immunosuppressant. −, no administration; /, PBS injection.
detected in all groups, with a significant production peak at day 60 ($P < 0.001$ for within-group variation) and a significant decrease at day 70 for all groups ($P < 0.001$ for within-group variation). Levels of anti-hTNF-$\alpha$ Abs were not significantly affected by the different treatments versus the control PBS group (between-group variation and the two factors’ interaction were not significant). A decrease in anti-hTNF-$\alpha$ IgG titers was noted between MP-treated groups at day 60, when MP treatment started prior to TNF-K immunization (day $-6$ versus day 2), that did not reach statistical significance.

As a control, levels of anti-KLH Abs were studied (Fig. 1B). High levels of anti-KLH Abs were detected in sera of immunized mice with a production peak at day 60 for all groups ($P < 0.001$ for within-group variation), except for group A2 (MTX). A significant decrease in anti-KLH Abs was observed at day 70 for all groups ($P < 0.001$ for within-group variation), except for group A2, without any difference in anti-KLH titers for MTX and MP versus PBS, whatever the dose or the time schedule applied (inter-group variation was not significant).

Analysis of the neutralizing capacity of anti-hTNF-$\alpha$ Abs in the sera of BALB/c mice undergoing repeated administrations of immunosuppressive treatments showed high titers of hTNF-$\alpha$-neutralizing antibodies for all groups, with a production peak at day 60 ($P < 0.001$ for within-group variation) and a decrease at day 70 ($P < 0.001$ for within-group variation) for all groups (Fig. 2). No significant dose or time schedule effects of concomitant administration of either MP or MTX on neutralization capacity were observed. MTX-treated groups had lower NC$_{50}$ values (i.e., the reciprocal of the serum dilution that neutralizes 50% of hTNF-$\alpha$ activity) than the PBS group, but these differences were not statistically significant. To estimate the effect size of the difference, we calculated the 95% confidence interval (CI) for the difference in the means of the two groups displaying the highest difference in NC$_{50}$ values (groups A1 and C; both displayed a normal distribution of values). The 95% CI included 0 ($-4,966$ to $43,724$).

As a control, levels of anti-KLH Abs were studied (Fig. 3B). High levels of anti-KLH Abs were detected in sera of immunized BALB/c mice. As shown in Fig. 3A, high levels of anti-hTNF-$\alpha$ Abs were detected in sera of immunized mice with a peak at day 60 for the MP, MTX, and PBS groups ($P < 0.001$ for within-group variation), and then a decrease in anti-hTNF-$\alpha$ Ab was observed at day 70 ($P < 0.001$ for within-group variation). Conversely, mice receiving a high dose of CYC had a production peak at day 40 for anti-hTNF-$\alpha$ Abs ($P < 0.01$). They had overall lower Ab production throughout the experiment ($P < 0.01$ for AUC comparison) and an early decrease at days 60 and 70 compared to all other groups ($P < 0.001$ for within- and between-group variation). For MP and MTX treatments, a slight increase in Ab levels versus those in the PBS group that did not reach statistical significance was observed at days 60 and 70. We calculated the 95% CI for the difference in the means of the two groups displaying the highest difference in Ab levels (MP and PBS groups; both displayed a normal distribution of values). The 95% CI included 0 ($-4,966$ to $43,724$).

Influence of high doses of immunosuppressant on anti-hTNF-$\alpha$ and anti-KLH Ab levels. In the second part of this study, we aimed at determining whether a high dose of immunosuppressants significantly impacted the production of antibodies after TNF-K immunizations in BALB/c mice.
mice, with a peak at day 40 for all groups ($P < 0.001$ for within-group variation). Then, a significant decrease in anti-KLH Abs was observed from day 40 to day 60 and from day 60 to day 70 for all groups ($P < 0.001$ for within-group variation). Administration of a high dose of immunosuppressant between days 35 and 44 had no effect on anti-KLH Ab levels compared to the PBS control group.

The neutralization capacity of anti-hTNF-α Abs was investigated with an L929 bioassay. As shown in Fig. 4, hTNF-α-neutralizing Ab was first detected at day 40, with a production peak at day 60 (except for the CYC group). Then, a decrease was observed at day 70 for all groups ($P < 0.001$ for within-group variation). A high heterogeneity in responses was observed for all groups. Mice receiving a high dose of CYC exhibited a significant strong decrease at day 60 and 70 of neutralizing capacity compared to all other groups ($P < 0.05$ and $P < 0.001$ at day 60 and at day 70 for within- and between-group variation, respectively). Administration of MP (5 mg/kg) or MTX (2.5 mg/kg) after TNF-K immunization did not affect the neutralizing capacity of anti-hTNF-α Abs.

**FIG 3** Effect of short-term immunosuppressant treatment on anti-hTNF-α (A) and anti-KLH (B) Ab titers in sera of TNF-K-immunized mice, showing an overall lower anti-hTNF-α Ab production in the CYC group ($P < 0.01$) and a significant reduction in Ab levels at days 60 and 70 compared to all other groups ($P < 0.001$). Mice received three TNF-K immunizations (days 0, 7, and 28) before high-dose immunosuppressant administration. Mice received 2 (CYC) or 4 (MP, MTX, and PBS) doses between day 35 and day 44. Anti-KLH and anti-hTNF-α Abs in sera were quantified by ELISA. Results are expressed as the dilution factor giving half-maximal absorbance (medians and interquartile ranges are reported).

**FIG 4** Effect of short-term immunosuppressant treatment on hTNF-α-neutralizing capacities in sera of TNF-K-immunized mice, evaluated by an L929 bioassay. An overall lower rate of production of anti-hTNF-α-neutralizing Ab was found in the CYC group ($P < 0.05$), with a significant decrease at day 60 and day 70, resulting in lower neutralizing capacity than in all other groups ($P < 0.001$). Neutralizing titers are expressed as the reciprocal of the serum dilution that neutralizes 50% of hTNF-α activity (medians and interquartile ranges are reported).

**DISCUSSION**

In the present study, we showed that anti-TNF antibodies induced by TNF-K immunization were not significantly influenced by a concomitant low-dose immunosuppressive treatment, such as methylprednisolone or methotrexate. Our study, performed in mice, is relevant to the current scenario of anti-TNF administration in humans; in most cases, corticosteroids and immunomodulatory agents are associated to enhance the clinical efficacy of the treatment. A potential side effect of immunosuppressive drugs is a reduction in the response to vaccinations (17, 18, 29). It was of high interest to explore the influence of this class of treatments on the anti-TNF antibody response to TNF-K. We observed a slight modification of anti-TNF Ab levels in BALB/c mice treated with concomitant MTX or MP and TNF-K that was not statistically significant, despite the fact that the doses of MTX we used in mice were 10-fold higher than those used in RA patients (0.3 mg/kg/week). Similarly, despite the high doses of MTX we used, the difference in TNF-neutralizing capacity between MTX- and PBS-treated groups had a modest effect size.

The timing of the start of immunosuppressive treatment did not influence the production of neutralizing anti-hTNF-α Abs. Taken together, these results showed that the use of low doses of MP or MTX during TNF-K immunization does not alter titers of anti-hTNF-α Abs or their neutralizing capacity. This is consistent with a previous study performed with hTNF-α transgenic mice developing arthritis, in which no alteration in the production and neutralizing capacity was observed after TNF-K immunization in the presence of MTX (9). In this context, we could speculate that TNF-K should preserve its efficacy even in patients on concomitant disease-modifying antirheumatic drugs (DMARDs), such as MTX at the doses used in RA, or patients on steroids.

Another point addressed in our study was the reversibility of the anti-TNF-α Ab response to TNF-K. Previous studies consistently demonstrated a bell curve response of Abs to anticytokine vaccines with an ~12- to 16-week cycle of response (9, 25, 28, 30), slightly longer than the duration of action of infliximab in humans.

On the other hand, we show here that partial acute immunosuppression with high doses of CYC efficiently blocked at least in part the immunological response induced by TNF-K immunization in mice. Indeed, CYC significantly reduced both the levels of...
anti-hTNF-α Abs and their hTNF-α-neutralizing capacity. This CYC inhibitory effect on Ab titers was not observed with the two other immunosuppressive agents (MP and MTX). Indeed, an increase, though not statistically significant, in production of anti-hTNF-α Abs was observed with both MP and MTX treatments, once again with a modest effect size. Furthermore, the neutralizing capacity was not affected by MP or MTX treatment. Moreover, this discrepancy in regulating anti-hTNF-α Ab production in the presence of CYC and MP or MTX treatments may be due to the implications of different modulatory molecular pathways of the immune response. MP is a glucocorticoid that exerts its anti-inflammatory effect by acting on selective genes, whereas MTX exerts its effect by two different pathways depending on the dose. High doses of MTX exert a cytostatic effect by blocking purine and thymidine synthesis, whereas low doses of MTX in humans (5 to 30 mg/week) inhibit inflammation via inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, leading to the accumulation of extracellular adenosine, which inhibits neutrophil activity. CYC is an alkylating agent that cross-links DNA and causes cytotoxicity in various cell types. In contrast to its well-known immunosuppressive effect at high doses (6), low doses of CYC are associated with immune response enhancement via specific targeting of regulatory T cells (21).

Our experimental results with CYC showed a pharmacological effect on Ab response. Even if high-dose CYC treatment, in order to reverse the effect of TNF-K immunization, is not applicable in a human clinical setting due to safety concerns, CYC served as a model of a drug with potent immunosuppressant activity. Further studies more specifically targeting memory B cells are needed to define a useful immunomodulator in RA patients treated with an active immunization strategy. Of note, recent data from antihumoral therapies in organ transplantation recipients have shown that proteasome inhibition by bortezomib depletes plasma cells, the source of Ab production (11). Additional studies to determine whether this drug might modulate Ab production after anti-TNF-α immunization could be warranted.

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