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CUTTING EDGE

Cutting Edge: Anti-Tumor Necrosis Factor Therapy in Rheumatoid Arthritis Inhibits Memory B Lymphocytes via Effects on Lymphoid Germinal Centers and Follicular Dendritic Cell Networks[1]

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***Rheumatoid arthritis (RA) is mediated by a proinflammatory cytokine network with TNF at its apex. Accordingly, drugs that block TNF have demonstrated significant efficacy in the treatment of RA. A great deal of experimental evidence also strongly implicates B cells in the pathogenesis of RA. Yet, it remains unclear whether these two important players and the therapies that target them are mechanistically linked. In this study we demonstrate that RA patients on anti-TNF (etanercept) display a paucity of follicular dendritic cell networks and germinal center (GC) structures accompanied by a reduction in CD38$^+$ GC B cells and peripheral blood memory B cell lymphopenia compared with healthy controls and RA patients on methotrexate. This study provides initial evidence in humans to support the notion that anti-TNF treatment disrupts GC reactions at least in part via effects on follicular dendritic cells. The Journal of Immunology,* 2008, 180: 688–692.**

Rheumatoid arthritis (RA)[3] is a systemic autoinflammatory disorder manifested by aggressive synovitis that over time causes bone, tendon, and cartilage damage. Although different cell types may play pathogenic roles in RA, a prominent participation of the B cell has been recently highlighted. Thus, rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) autoantibodies are well-established indicators of disease and disease severity, and autoantibody-mediated models of RA have been described in the mouse (1–3). Moreover, B cells may provide a critical link between the development of tertiary lymphoid tissue within the inflamed synovium and the propagation of the autoimmune process. This contention is supported by the finding of germinal center (GC)-like structures within the inflamed RA synovium, the profound effect of B cell-borne lymphotoxin (LT) α on lymphoid architecture, and the observation that T cell activation in the RA synovium is dependent on the presence of B cells within these active GCs (4). Perhaps most strikingly, the key role of the B cell in disease pathogenesis has recently been demonstrated by the clinical efficacy of targeted B cell depletion with rituximab (5).

TNF-α has also been established as a central player in the pathogenesis of RA. The pathogenic significance of TNF has been demonstrated by the clinical efficacy of TNF blockade in the treatment of RA (6). Interestingly, TNF and LTα (also blocked by some anti-TNF drugs) may exert powerful direct and indirect influences over B cells (which express TNFR1 and TNFR2) and through B cells that may produce TNF and constitute the main source of LTα (7–9). Of note, in mice B cell-borne LTα is critical for the development and maintenance of spleen and lymph node microarchitecture (10). Signaling by TNF and LT is also required for the development of follicular dendritic cells (FDCs), the cells that are responsible for the initiation of GC structures and that specialize in displaying intact Ag for recognition by B cells (11, 12).

It is therefore reasonable to postulate that, at least in part, the efficacy of anti-TNF drugs could be mediated by anti-B cell effects and that, in turn, B cell depletion could mimic some anti-TNF effects by eliminating a major source of LTα and TNF. However, the precise in vivo effects of a blockade of TNF and LT signaling pathways on human B lymphocytes remain unclear. Herein, we provide the first evidence in humans that anti-TNF therapy may impair B cell function via effects on FDCs and disruption of GC formation and maintenance.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; FDC, follicular dendritic cell; GC, germinal center, LT, lymphotoxin; MTX, methotrexate; PB, peripheral blood.

Materials and Methods

Study population

Forty-five patients with RA, fulfilling the classification criteria of the American College of Rheumatology, were evaluated in three different study arms, methotrexate (MTX; $n = 17$), anti-TNF (etanercept; TNF receptor-Ig (p75) decoy; $n = 11$), and MTX/anti-TNF ($n = 17$) and compared with normal controls ($n = 22$). Patients were excluded if they were older than 70 years of age, on >10 mg of prednisone, or had a change in RA treatment within the prior 3 mo. Each study patient completed a health assessment questionnaire (score 0–3) and a disease activity score (DAS28; including a 28 joint count) was calculated (range 0–10; >5.1 indicates high disease activity, <3.2 indicates low disease activity) at the time of enrollment.

Sample procurement

Samples were obtained with informed consent using protocols approved by the University of Rochester Medical Center Institutional Review Board (Rochester, NY). Peripheral blood (PB) and tonsillectomy samples were obtained as before (13). Tonsil samples were acquired from consenting RA patients by triangular adenoid forceps biopsy and from normal controls via elective tonsillectomy.

Flow cytometric analysis

Single cell suspensions of Ficoll-isolated mononuclear cells (10^6/sample) were labeled at 4°C with predetermined optimal concentrations of fluorophore-conjugated mAbs and pair-matched isotype controls. Analyses were performed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software with CD19 gating for identification of B cells (14). B cells were classified along a developmental pathway based on the expression of defined surface markers as follows: immature ($CD38^{3+}CD24^{3+}IgD^-$), transitional-1 ($CD38^{3+}CD24^{3+}IgD^+$), transitional-2 ($CD38^{2+}CD24^{2+}IgD^+$), naive ($CD38^+CD24^{low}IgD^+CD27^-$), pre-germinal center ($CD38^{2+}CD24^{low}IgD^+$), nonswitched memory ($CD27^+IgD^+$), and switched memory ($CD27^+IgD^-$) (15–20). B cells were also defined according to their Bm5 phenotype within the Bm1–Bm5 classification (16).

Immunohistochemistry of tonsillar tissue

Serial tonsil sections (obtained from at least five different levels of the tissue blocks) were stained using a Dako LSAB2 System according to the manufacturer's instructions (DakoCytomation) (20). FDC networks ($CNA.42^+$) and primary (homogeneous IgD staining and paucity of T cell markers) and secondary (GC) (asymmetric IgD staining and light/dark zone (CD23/Ki67) polarization) follicles were carefully enumerated by morphometric analysis (area occupied per mm^2 tissue) using ImageJ software to discriminate positive staining after imaging on a Leica microscope (20, 21). Quantitation and definitions were as follows: FDC percentage = FDC area/total lymphoid tissue area; FDC/IgD ratio = FDC area/IgD area; GC percentage = GC FDC area/total lymphoid tissue area.

Statistical analysis

Analysis for statistical significance was conducted using the two-group comparison by *t* test as well as nonparametric ANOVA (Kruskall-Wallis) for comparison of groups. Multivariate regression analysis on log-transformed data for analysis of potential confounding variables was also conducted.

Results and Discussion

Anti-TNF therapy is associated with reduced $CD27^+$ memory B cells in PB

We compared the percentage of B cells (Fig. 1*A*) in the PB of healthy controls ($n = 22$) with a cohort of RA patients treated either with anti-TNF ($n = 28$) or MTX ($n = 17$). Anti-TNF treated patients were characterized by a highly significant decrease (ANOVA) in the percentage of total $CD27^+$ memory B cells (22.5 ± 9.7 for anti-TNF alone and 22.8 ± 10.2 for anti-TNF/MTX) as compared with normal controls (31.7 ± 6.8, $p = 0.004$) and the MTX only group (37.3 ± 18.5, $p = 0.006$). A significant difference (Student's *t* test) was also seen when comparing the anti-TNF arm alone to the MTX group ($p = 0.01$) and the anti-TNF/MTX arm alone to MTX ($p = 0.009$) (Fig. 1*B*). Of note, significant reductions in both IgM ($p = 0.019$) and switched ($p = 0.019$) $CD27^+$ memory B cells were observed in the group of patients on anti-TNF compared with

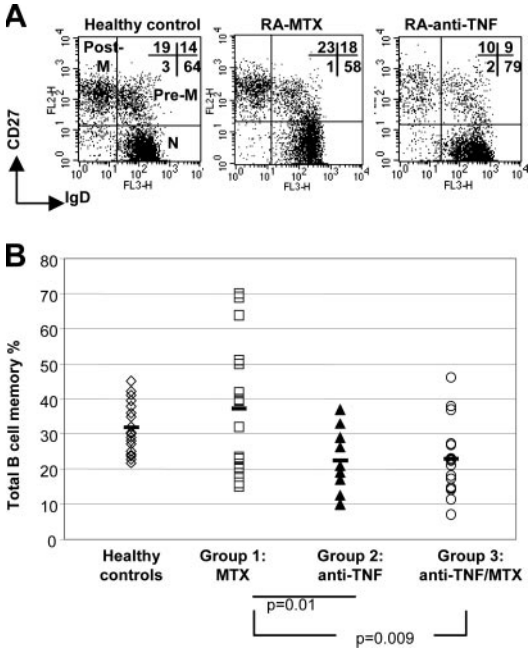

FIGURE 1. Anti-TNF treated RA patients have reduced fractions of PB memory B cells. *A*, Representative phenotypic analysis of B cells in human PB of healthy control (*left*), RA patient on MTX (*middle*), or RA patient on anti-TNF (*right*). PBMCs were stained with Abs to CD19, CD27, and IgD and analyzed by multicolor flow cytometry. Data are presented as dot plots on CD19-gated B cells. B cell subsets are defined as indicated (*N*, naïve; pre-M, unswitched memory; post-M, switched memory), with the numbers in each dot plot representing the percentage of B cells in each subset. *B*, Percentage of memory B cells. Mean values ± SD are indicated for each group.

MTX. Patients in the anti-TNF, MTX, and combination therapy groups were fully comparable in terms of age, disease activity measures (erythrocyte sedimentation rates, health assessment questionnaire scores, and disease activity scores), and other disease characteristics (Table I and data not shown). Moreover, the effect of anti-TNF treatment was maintained when potential confounding variables such as age, disease duration, and severity were introduced on multivariate regression analysis (data not shown).

Table I. *Select patient characteristics and histologic quantitation*[a]

	Healthy	RA: MTX	RA: Anti-TNF
HAQ (0–3)		0.24 (0.22)	0.3 (0.47)
DAS (0–9.3)		3.69 (1.0)	3.3 (1.3)
RF^+ (%)		77%	79%
GC (Bm3/4)	33 ± 12	27 ± 18	13 ± 9*
FDC (%)	12.2 ± 4.8	11.9 ± 5.1	7.5 ± 1.6**
FDC/IgD	0.63 ± 0.29	0.71 ± 0.10	0.42 ± 0.14***
GC (%)	8.4 ± 3.6	9 ± 5.2	2.3 ± 1.6****
1°:2°	2.8:13.2	1:14	4.7:7.3

[a] The first three rows from the top summarize select patient characteristics (HAQ, health assessment questionnaire; DAS, disease activity score) with mean ± SD or positive percentage (RF, rheumatoid factor) in RA on MTX ($n = 17$) and RA on anti-TNF ($n = 28$) recruited for PB analysis. The next four rows characterize tonsil tissue from healthy subjects ($n = 25$ for flow cytometry analysis of GC cells), RA on MTX ($n = 2$), and RA on anti-TNF ($n = 4$). FDC network area is quantitated as a percentage of the total tissue area (FDC (%)) or relative to the IgD^+ areas (FDC/IgD) and is based on morphometric analysis of immunohistochemistry stains. The fraction of the total tissue area occupied by GC FDC networks (GC (%)) and the mean primary number (1°) to secondary follicle number (2°) are shown. The statistical significance of the differences is indicated as follows: *, $p = 0.003$ compared to healthy controls; **, $p = 0.036$ compared to healthy controls; ***, $p < 0.0001$ compared to RA MTX; and ****, $p < 0.0001$ compared to RA MTX and healthy controls.

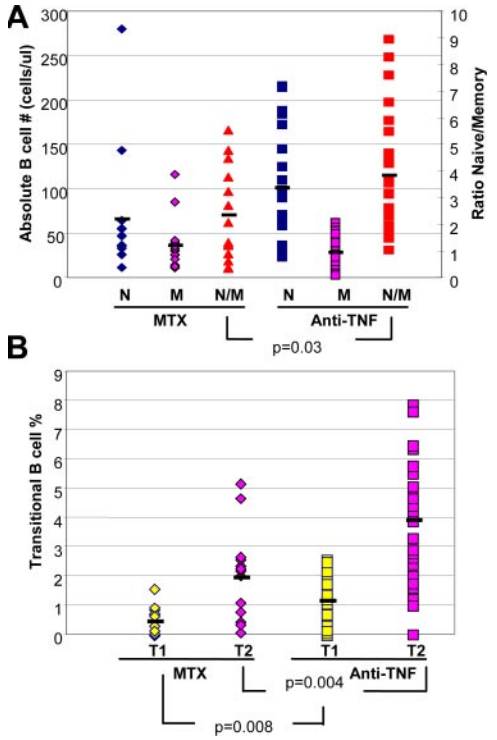

FIGURE 2. TNF blockade increases PB transitional and naive B cells while decreasing memory B cell numbers. *A*, Absolute numbers of naive (N) and memory (M) B cells were calculated from the fractions defined in Fig. 1, the percentage of lymphocytes, and the percentage of CD19 cells identified on flow cytometry. The increase in naive and the decrease in memory B cell numbers was reflected in the highly significant increase in the ratio of absolute naive to memory B cells with anti-TNF. *B*, Percentage of transitional B cells was determined by staining PBMCs with Abs to CD19, CD24, and CD38 as described in *Materials and Methods*. Mean values are indicated by the horizontal bars.

The percentage of memory B cells was proportional to the absolute number of memory B cells (e.g., $R^2 = 0.75$ for anti-TNF treated subjects), with reductions seen in both in the anti-TNF-treated groups compared with MTX-treated RA controls. However, there was considerable variability in total absolute peripheral B cell numbers such that the differences between groups for memory B cell numbers were not significant. Anti-TNF patients also had an increase in both the fractions and the absolute numbers of PB naive B cells compared with MTX patients ($p = 0.004$ and 0.07, respectively). The increased numbers of naive B cells and the decreased numbers of memory B cells were reflected in the statistically significant higher ratio of absolute naive/memory B cells in anti-TNF patients (3.8 ± 2.2) compared with the MTX group (2.3 ± 1.8, $p = 0.03$) (Fig. 2*A*). Additionally, the percentage of transitional B cells was increased in the PB with TNF blockade (Fig. 2*B*). As TNF has been described to mobilize murine bone marrow B cells, this result was unexpected (22) but may be explained by the decreased generation of memory B cells from earlier precursors or other effects on B cell generation in the bone marrow, survival, and differentiation (23).

Lymphoid architecture is altered in patients on anti-TNF therapy

To address whether the decrease observed in PB memory B cell fractions was due to decreased generation in secondary lymphoid tissue and alteration of the lymphoid architecture by TNF blockade, tonsil biopsies from patients with RA on MTX or anti-TNF therapy were obtained and compared with normal healthy controls. Whereas age-matched normal controls ($n = 5$) showed tight networks of FDC staining (CNA.42-positive areas), patients treated with etanercept ($n = 4$) had a remarkable decrease in FDC staining (Fig. 3*A*). To control for patient to patient variability in sample size and lymphocyte content, the FDC area was carefully quantitated and normalized relative to the size (FDC percentage) and immunologic activity (FDC/IgD ratio) of the lymphoid compartment (Table I), with significant reductions observed in all measures in patients on anti-TNF.

Attenuation of GC reactions by anti-TNF therapy

The GC of secondary follicles were identified as polarized structures, with an IgD negative center, various ratios of light ($CD23^{2+}$) to dark ($Ki67^{+}$) zone area, and a mantle of IgD-positive naive B cells (Fig. 3*B*). Strikingly, GCs were significantly reduced in patients on anti-TNF, both in total number (when expressed as a ratio of secondary follicles to primary follicles; $p = 0.05$ compared with normal controls or the total number of secondary follicles, $p = 0.05$ compared with normal controls, and $p = 0.02$ compared with MTX) and size. Thus, the overall area of lymphoid tissue occupied by GCs was significantly reduced with anti-TNF (GC percentage in Table I). Primary follicles were not reduced (Fig. 3*B*). In accord with the immunohistochemistry data, RA patients on anti-TNF therapy had a significant decrease in tonsil B cells of the GC phenotype by flow cytometry (Fig. 3*C* and Table I). Interestingly, however, the Bm5 memory compartment in the tonsil does not appear to be decreased with anti-TNF (Fig. 3*C*). Yet, given the significantly diminished contribution of GC cells to the composition of the tonsil, the total numbers of memory B cells must have decreased to explain the lack of increase in the frequency of memory cells (in contrast to naive B cells). Furthermore, one must bear in mind that the output of the GC is heterogeneous and may include a nonrecirculating long-lived population that would not be affected by anti-TNF and a recirculating population that may be more representative of ongoing GC activity. Further characterization of differences in tissue memory induced by anti-TNF is underway.

In conclusion, the data presented here indicate that anti-TNF therapy alters B cell populations and also likely impacts the ability of B cells to enter or survive a GC reaction. In combination, this provides new insight into the biology of TNF blockade in humans within the context of RA, implicating effects on B lymphocytes. Our results are consistent with studies from knockout mice and mice expressing a TNF receptor-Ig (p55) decoy, demonstrating that the maintenance of FDCs and GCs requires ongoing and coordinated signaling via TNF and LTα (11, 12, 24). In our study, the decrease in FDC networks correlated with the loss of GCs but appeared to be uncoupled from the maintenance of primary follicles. This is in contrast to results in primates undergoing LT blockade, where the loss of FDC networks was uncoupled from the maintenance of GC integrity (25). Nevertheless, in these latter studies GC function was impaired due to the lack of immune complex trapping by FDCs despite the maintenance of GC structures. The profound inhibition of GCs observed in our present study may be explained by the combination of TNF-α and LTα blockade mediated by the TNF receptor-Ig (p75) decoy etanercept (26), although the relative role of TNF-α vs LTα blockade in the

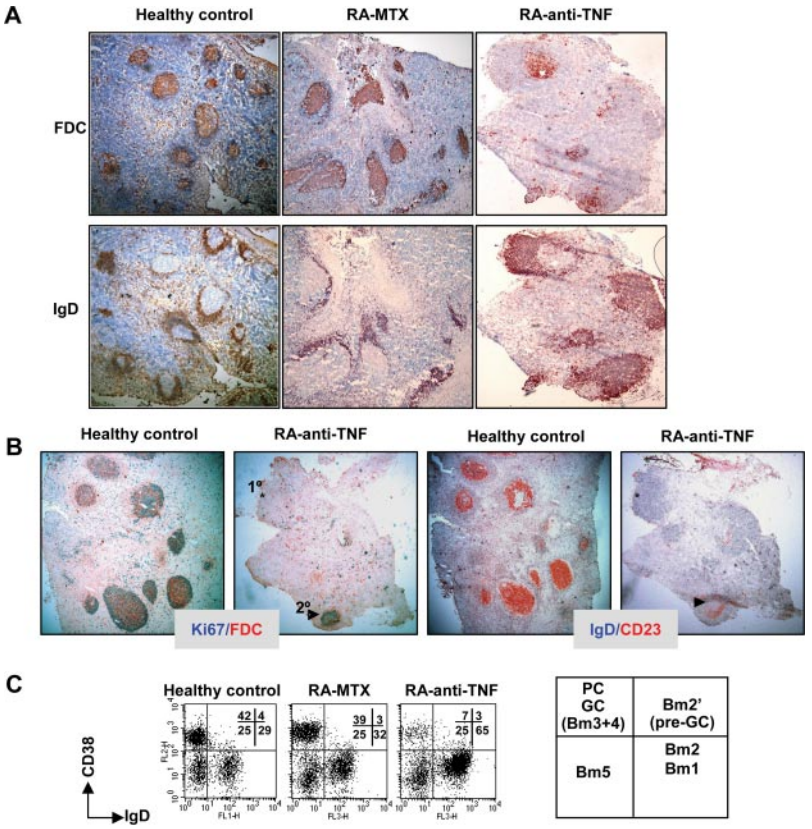

FIGURE 3. Anti-TNF treatment inhibits GC reactions and FDC networks in peripheral lymphoid tissue. *A*, Immunohistochemistry of tonsillar tissue in a normal control, a RA patient on MTX, and a RA patient on anti-TNF. FDCs are labeled with the CNA.42 FDC-specific Ab in the *top panels*. Images represent serial sections. *B*, Primary follicles are indicated by diffuse IgD staining (*) (also distinguished by the absence of polarized dark/light zones and the paucity of T cells; data not shown for the latter) and secondary follicles (arrowhead points to GC) by polarization with peripheral IgD staining (follicular naive B cells), dark zone staining for Ki67, and light zone staining with anti-CD23 (arrowhead) (CD23 is also expressed at a low level on follicular B cells in the mantle). *C*, Phenotypic analysis of B cells in human tonsil.

effects observed remains to be defined. It is also important to recognize that there may be critical differences between the TNF/LT axis in humans compared with other species given strong evidence for the existence of LTα homotrimers with biologic activity in humans (27). This provides a compelling reason to further study the effects of TNF blockade on human B cell follicle formation and function. Although we suggest that TNF/LT blockade directly inhibits FDCs, the relative contribution of other mechanisms of inhibition of GC responses, either mediated directly via B cells or indirectly through other cell types, also remains to be elucidated.

Regardless, the results have several important implications. FDC-like cells and GC structures appear in the synovial microenvironment of some RA patients and, hence, elimination of these structures could potentially interrupt tissue-specific, pathogenic B cell activation. Our findings also suggest a convergence of mechanisms between TNF blockade and B cell depletion therapy, as both seemingly divergent approaches can reduce memory B cells (15). Moreover, B cell depletion may mimic TNF blockade by eliminating LTα-bearing and TNF-secreting B cells (7–9). Thus, we have recently demonstrated a prolonged delay in memory B cell reconstitution and the disorganization of lymphoid architecture after rituximab-mediated B cell depletion in systemic lupus erythematosus patients experiencing sustained clinical remission (20). Further longitudinal studies are necessary to define the kinetics of the inhibition of memory B cells and FDC networks in RA patients treated with anti-TNF agents, the potential synergy between B cell depletion and TNF blockade, and the impact of these therapies on protective humoral immunity and pathogenic immunity in the synovium.

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Disclosures

The authors have no financial conflict of interest.

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