Characterization of Autoreactive T Cells to the Autoantigens Heterogeneous Nuclear Ribonucleoprotein A2 (RA33) and Filaggrin in Patients with Rheumatoid Arthritis

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The role of autoimmune reactions in the pathogenesis of rheumatoid arthritis (RA) is poorly understood. To address this issue we have investigated the spontaneous T cell response to two well-characterized humoral autoantigens in RA patients and controls: 1) the heterogeneous nuclear ribonucleoprotein A2, i.e., the RA33 Ag (A2/RA33), and 2) filaggrin in unmodified and citrullinated forms. In stimulation assays A2/RA33 induced proliferative responses in PBMC of almost 60% of the RA patients but in only 20% of the controls (patients with osteoarthritis or psoriatic arthritis and healthy individuals), with substantially stronger responses in RA patients (p < 0.00002). Furthermore, synovial T cells of seven RA patients investigated were also clearly responsive. In contrast, responses to filaggrin were rarely observed and did not differ between RA patients and controls. Analysis of A2/RA33-induced cytokine secretion revealed high IFN-γ and low IL-4 production in both RA and control PBMC, whereas IL-2 production was mainly observed in RA PBMC (p < 0.003). Moreover, A2/RA33-specific T cell clones from RA patients showed a strong Th1 phenotype and secreted higher amounts of IFN-γ than Th1 clones from controls (p < 0.04). Inhibition experiments performed with mAbs against MHC class II molecules showed A2/RA33-induced T cell responses to be largely HLA-DR restricted. Finally, immunohistochemical analyses revealed pronounced overexpression of A2/RA33 in synovial tissue of RA patients. Taken together, the presence of autoreactive Th1-like cells in RA patients in conjunction with synovial overexpression of A2/RA33 may indicate potential involvement of this autoantigen in the pathogenesis of RA. The Journal of Immunology, 2002, 169: 1068–1076.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease of unknown etiology characterized by hyperplasia of the synovial membrane, degradation of cartilage, and bone erosion. Overexpression of proinflammatory cytokines such as IL-1 and TNF-α is considered to drive the destructive processes, but the causes for this disregulated cytokine production are unknown. Although it is generally assumed that RA belongs to the group of systemic autoimmune diseases, the pathogenetic role of cellular and humoral autoimmune reactions is still incompletely understood (1, 2).

Already in early disease stages the inflamed rheumatoid synovial membrane is characterized by massive infiltration of T cells and other immune cells including B cells, macrophages, and mast cells (3–5). The majority of synovial T cells are CD4+ memory cells that typically express activation markers such as HLA-DR, CD69, or CD40L but, remarkably, may be deficient in CD28 expression (6). Cytokine analyses performed in situ revealed a predominance of IFN-γ over IL-4-producing T cells, although the numbers of cytokine-secreting T cells were found to be relatively low (7–9). Furthermore, the T cell repertoire of RA patients shows features of clonal expansion (6) and most T cell clones (TCC) obtained from synovial tissue or fluid could be functionally attributed to the Th1 subset (10–12), which is generally considered to constitute a driving force of pathologic autoimmune reactions (13–15). However, in vitro, synovial T cells generally show some features of anergy such as reduced responsiveness to mitogens or recall Ags, which has been attributed to the effects of chronic TNF-α exposure and oxidative stress in the joint (16, 17).

The abundance of T cells and the association of RA susceptibility with particular MHC class II alleles suggests an important if not pivotal role of T cells in the pathogenesis of RA, which has been widely discussed in recent years (2, 18–22). Moreover, the presence of high-titer rheumatoid factor (RF) and other autoantibodies (Aab) in the sera of RA patients is considered a further indication for involvement of autoimmune processes, although direct evidence for a role of Aab in the pathophysiology of RA is scarce (23). Based on these observations, an autoimmune model of RA pathogenesis has been suggested in which autoreactive T cells are initially activated by Abs presented via disease-associated HLA molecules (2). These T cells subsequently activate autoreactive B cells, macrophages, and synoviocytes, which may further

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stimulate T cell responses. Thus, a vicious circle is induced and maintained that leads to chronic inflammation and finally results in irreversible destruction of the joint.

The search for Ags triggering pathogenic T cell responses has led to the identification of a number of joint-specific candidate proteins including collagen, cartilage link protein, and gp39 (23, 24). However, even though arthritis may be induced in susceptible mouse and rat strains by immunization with these Ags, their pathogenic role has remained elusive. In addition to joint-specific Ags, several other proteins have been identified as targets of Aab of patients with RA. Interestingly, even though these Ags are more or less ubiquitously expressed, the Aab directed to them seem to be more specific for RA than anti-collagen Aab or RF (23). These include the following: Aab to the heterogeneous nuclear ribonucleoprotein (hnRNP) A2, also known as the RA33 Ag (A2/RA33) (25); anti-keratin Aab, which recognizes the cytkeratin-aggregating protein filagrin (Fil) (26); anti-Sa Aab, whose target moiety is still unknown (27); and Aab to the H chain binding stress protein (28).

Aab to A2/RA33 can be detected in about one-third of RA patients and may also occur in patients with systemic lupus erythematosus or mixed connective tissue disease, usually in association with anti-scleroosomal Aab (such as anti-Sm or anti-U1RNP) which are not found in RA (29, 30). A2/RA33 is an abundant mRNA binding protein that, like other hnRNP proteins, shows a modular structure consisting of two conserved RNA binding domains and a glycine-rich auxiliary domain assumed to be involved in interactions with other proteins (31, 32). It has a predominant nuclear localization and exerts multiple functions, including regulation of alternative splicing and transport of mRNA (33, 34). A2/RA33 appears to be ubiquitously expressed, although the level of expression may greatly vary between different tissues (35).

Anti-keratin or anti-Fil Aab (AFA), respectively, can be detected by indirect immunofluorescence or immunoblotting in 40–50% of RA patients (36). They are rather specific for RA and recognize the citrullinated form of Fil (cFil), which is generated by posttranslational deimination of certain arginine residues (37). Fil is exclusively expressed in terminally differentiated epithelial cells, where it is involved in aggregation of cytkeratin filaments and presumably plays a role during apoptosis (38). Remarkably, when citrullinated peptides were used instead of the whole protein, >60% of the patients were found to be reactive, even with peptides not derived from Fil (39, 40). This has led to speculations on the existence of other citrullinated targets, and recently strong evidence has been provided that such proteins are indeed present in synovial tissue of RA patients (41).

Anti-A2/RA33 and AFA are already present in early stages of the disease (40, 42–44) and are predominantly of the IgG isotype, which is indicative of T cell-driven processes. To gain more insight into such processes and their potential pathogenetic role, we investigated the spontaneous T cell responses to A2/RA33 and Fil in patients with RA and in control subjects including patients with osteoarthritis (OA) and psoriatic arthritis (PSA) and healthy persons. The data obtained suggest that A2/RA33 may constitute an important T cell autoantigen in patients with RA, whereas Fil does not seem to be a major T cell autoantigen and therefore presumably does not drive the humoral autoimmune response to citrullinated Ags.

Materials and Methods

Patients and controls

Peripheral blood from 50 patients with RA (37 female, 13 male, mean age 55 ± 17.7 years) classified according to the established criteria of the American College of Rheumatology was drawn into heparinized test tubes. Informed consent was obtained from all patients. At the time of the investigations, patients were treated with nonsteroidal anti-inflammatory drugs (n = 32), disease-modifying antirheumatic drugs (n = 34), and/or low-dose glucocorticoids (n = 18). While 37 patients had moderately active disease with fewer than five swollen joints and C-reactive protein (CRP) ≤2 mg/dl, 13 patients had active RA (i.e., more than six swollen joints and CRP >2 mg/dl). Synovial fluids were obtained during routine joint tapping from a number of patients with RA after heparinized tubes. The control population consisted of 18 patients with OA (all female, mean age 59.2 ± 12 years), 11 patients with PSA (four male, seven female, mean age 45 ± 17 years), and 21 healthy individuals (10 female, 11 male, mean age 39.3 ± 13.4 years), including two otherwise healthy persons with osteoarthritis. Four of the PSA patients had active disease with more than two swollen joints and/or elevated serum CRP levels. HLA-DR genotyping as assessed by PCR revealed that 66% of the RA patients and 30% of the tested controls carried HLA-DR4 and/or DR1.

Antigens

Recombinant full-length hnRNP-A2/RA33 was used throughout this study. The cDNA encoding A2/RA33 was cloned into a ligation-independent cloning into the pET-30 LIC vector (Novagen, Madison, WI) and expressed as His-tagged fusion protein. Purification from bacterial lysates was achieved by nickel-nitriiotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) followed by polymyxin B Sepharose adsorption (Bio-Rad, Hercules, CA) and anion exchange chromatography on DEAE Sepharose (Pharmacia Biotech, Uppsala, Sweden). Endotoxin content was determined by the limypholytic amebocyte lysate assay (BioWhittaker, Verviers, Belgium). By this procedure a >99% pure, endotoxin-free preparation was obtained. The optimum concentration for proliferation assays was found to be 0.35 μg/ml.

Recombinant His-tagged Fil and cFil were a kind gift from Dr. A. Union (Innogenetics, Ghent, Belgium) and were used at a concentration of 2.5 μg/ml. In addition, two synthetic cFil-derived peptides as well their unmodified boforms were provided and used at 2.5 μg/ml. These peptides were selected on the basis of recognition by AFA (46) and had the following sequences (X = citrulline, i.e., deaminated arginine): HSAS QDGQDTILXGHPGSS (filipep 1) and DSGHXGYSGQSAQDNEGH (filipep 2).

Tetanus toxoid (TT) as control Ag was obtained from Pasteur Merieux Connaught (Willowdale, Ontario, Canada) and was used at a concentration of 0.5 μM as previously described (47).

Ab detection

RF was determined by nephelometry. Anti-A2/RA33 Aab were detected by ELISA (IMTEC, Berlin, Germany) and by immunoblotting as described (29). AFA were detected by an ELISA (Eurodiagnostics, Amherst, The Netherlands) using cFil-derived peptides (39). RF, anti-A2/RA33 Aab, and AFA were present in 57, 22, and 50% of the RA patients, respectively.

T cell stimulation assays

PBMC or synovial fluid mononuclear cells (SFMC) were isolated from fresh heparinized blood or synovial fluid samples of RA patients and controls by centrifugation on Ficoll-Hypaque (Pharmacia Biotech). After washing and counting, cells were either immediately used or frozen in RPMI medium containing 10% DMSO and 20% FCS. Cells were cultured for 5 days at 37°C in triplicate in 96-well plates (Costar, Cambridge, MA) in a total of 200 μl (10 5 cells/well) in the presence of the Ags. Culture medium was a mixed of Ultra Culture serum-free medium (BioWhittaker, Walkersville, MD) containing 2 mM glutamine and 0.02 mM 2-ME supplemented with 100 U/ml penicillin/streptomycin (Life Technologies, Paisley, U.K.). PHA (Life Technologies) and IL-2 (Roche Molecular Biochemicals, Mannheim, Germany) were used as polyclonal stimuli. During the last 16 h of culture, 0.5 μCi/well [3 H]Tdr (Amersham Biosciences Biochemicals, Freiburg, Germany) was added and the incorporated radioactivity was measured by scintillation counting. Results were expressed as stimulation index (SI) defined as the ratio of mean cpm obtained in cultures with Ag to mean cpm obtained in cultures incubated in the absence of Ag. SI ≥2 and Δcpm >1000 (mean cpm obtained in cultures with Ag minus mean cpm obtained in cultures incubated in the absence of Ag) was regarded as a positive response.

Cytokines were measured by ELISA (BioSource, Fleurus, Belgium) in supernatants (SN) after a 24-h incubation with A2/RA33 or 0.5 U/ml TT as control Ag. Detection limits were 5 pg/ml for IL-2, 4 pg/ml for IL-4, and 9 pg/ml for IFN-γ.

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Blocking experiments
Inhibition of Ag-induced T cell activation was investigated by incubating PBMC with A2/RA33 in the presence or absence of 5 µg of mAb specific for HLA-DR (clone G4-6-6) or HLA-DR/DP/DQ (clone TU 39), respectively (BD PharMingen, San Diego, CA) known to block MLR. An isotype-matched mAb to β-galactosidase was used as control Ab (Zymed Laboratories, San Francisco, CA). Proliferation was measured by [3H]Tdr uptake and IFN-γ production was measured by ELISA.

T cell lines and clones
Ag-specific T cell lines were obtained using a previously established protocol (48, 49). In brief, 2 × 10^6 PBMC were stimulated with A2/RA33 for 5 days in 24-well flat-bottom culture plates. On day 5 of culture 20 U/ml IL-2 was added and the culture was continued for an additional 7 days. To generate TCC, T cell lines were restimulated with A2/RA33 and after 2 more days viable T cell blasts were separated by Ficoll-Hypaque and seeded in limiting dilution (0.5 cells/well) in 96-well plates. T cell blasts were cultured in the presence of 1 × 10^5 irradiated (5000 rad) allogeneic PBMC as feeder cells, 0.5 µg/ml PHA, and 20 U/ml IL-2 in medium containing 1% heat-inactivated human AB sera. Growing microcultures were then expanded at weekly intervals with fresh feeder cells in the presence of IL-2. The specificity of TCC was assessed by proliferation assays incubating 2 × 10^5 T cell blasts with A2/RA33 (0.35 µg/ml) in the presence of 10^5 autologous irradiated PBMC. After a 48-h incubation and pulsing with [3H]Tdr for additional 16 h, cells were harvested and the incorporated radioactivity was measured by scintillation counting. Production of IL-4 and IFN-γ was measured by ELISA in SN collected after 24 h of incubation.

For phenotyping, cloned T cells (5 × 10^-4–10^1) were washed twice with ice-cold FACS buffer (PBS, 5% FCS, 0.01% NaN_3) by centrifugation for 5 min at 1000 × g. The washed cells were incubated for 30 min at 4°C with a FITC- or PE-conjugated mAb (BD PharMingen). Anti-TCRαβ and anti-CD4 mAb were FITC conjugated, and anti-TCRγδ and anti-CD8 mAb were PE conjugated. Afterward, cells were washed again in FACS buffer and analyzed with a FACSscan flow cytometer (BD Biosciences, Franklin Lakes, NJ) as substrate leading to brown staining of A2/RA33-expressing cells. Finally, slides were counterstained with hematoxylin (Merck, Darmstadt, Germany). To investigate expression of A2/RA33 in macrophages, tissues were double stained with 10D1 and an anti-CD68 mAb (DAKO, Glostrup, Denmark) using an alkaline phosphatase-based detection system (DAKO) leading to blue staining of the CD68-positive cells. Additional double stainings were performed with an anti-CD3 mAb (BD PharMingen) and the fibroblast-specific mAb ASO_2 (Dianova, Hamburg, Germany). Iso-type-matched mAb (DAKO) served as negative controls.

Statistical analysis
Unless stated otherwise, SI or cytokine concentrations, respectively, are indicated as mean ± SD. Student’s t test was used to determine differences between groups. Where appropriate, Bonferroni corrections were done. A p value <0.05 was regarded as significant.

Results
Proliferative responses of PBMC to A2/RA33
Cellular reactivity against A2/RA33 was investigated by measuring proliferation of PBMC obtained from 50 RA patients and 50 controls, including 18 patients with OA, 11 patients with PSA, and 21 healthy subjects. As shown in Fig. 1, pronounced responses were observed in 58% of the RA patients and in 20% of the controls (six healthy subjects, three OA patients, and one PSA patient). In RA patients mean SI was 4 ± 3.5 (median SI = 2.6), whereas in controls mean SI amounted to 1.5 ± 1.1 (median SI = 1.1). This difference was highly significant (p < 0.00002), while SI did not differ among the three control groups (see Fig. 1). Moreover, SI ≥4 were seen in 18 RA patients (maximum SI = 15) but in only two controls (maximum SI = 5.8).

In RA patients the T cell reaction did not correlate with the presence of anti-A2/RA33 Aab, which were detected in 22% of the RA patients but in neither healthy controls nor patients with OA or PSA, confirming previous observations (29, 44). Thus, T cell reactivity to A2/RA33 was seen in 8 of the 11 (73%) anti-A2/RA33 Aab-positive patients and also in 18 of the 39 (46%) anti-A2/RA33-negative patients. Although 73% of the reactive RA patients vs 57% of the nonreactive ones carried HLA-DR4 and/or DR1, this association did not reach the level of statistical significance.

Proliferative responses of synovial T cells to A2/RA33
To address whether cellular reactivity against A2/RA33 was also present in the synovial compartment, proliferation was measured in SFMC obtained from seven RA patients; proliferation of the corresponding PBMC was determined in parallel. In six patients, both SFMC and PBMC proliferated significantly, while in one patient only SFMC were responsive (Fig. 2). Of note, with one exception (Fig. 2), proliferation of SFMC was not significantly higher than that of PBMC from the same patients. In patients 1, 5, and 7 proliferation responses in SFMC were significantly lower than in their corresponding PBMC controls. The proliferation response in patient 6 was not different between SFMC and PBMC controls. This was consistent with the observation that proliferation responses in SFMC were lower than in PBMC from the same patients. In patient 4 proliferation responses in SFMC were significantly higher than in their corresponding PBMC controls. This was consistent with the observation that proliferation responses in SFMC were higher than in PBMC from the same patients.

Statistical analysis
Unless stated otherwise, SI or cytokine concentrations, respectively, are indicated as mean ± SD. Student’s t test was used to determine differences between groups. Where appropriate, Bonferroni corrections were done. A p value <0.05 was regarded as significant.
exception, the SI of SFMC was always higher than the SI of the corresponding PBMC, although the difference was not significant.

Cytokine secretion by PBMC in response to A2/RA33
To further characterize A2/RA33-induced T cell responses, the cytokines IL-2, IL-4, and IFN-γ were measured by ELISA in culture SN of PBMC from 15 responsive RA patients and eight control subjects stimulated with A2/RA33 or the control Ag TT, respectively (Fig. 3). These investigations revealed significantly higher IL-2 production by PBMC of RA patients (10.3 ± 9.1 vs 4.1 ± 3.3 pg/ml, p < 0.03), with IL-2 detectable in 10 SN of RA patients and in three SN of controls (Fig. 3A). A similar result was obtained with IL-4, but here the difference between the two groups did not reach the level of statistical significance (Fig. 3B). In contrast to IL-2 and IL-4, high IFN-γ levels were measured in virtually all SN of both RA patients and controls (Fig. 3C). Levels were similar in both groups (p = NS) and were an order of magnitude higher than IL-4 levels. However, when A2/RA33-induced IFN-γ production was compared with TT-induced production, a remarkable difference became apparent: thus, in SN of RA patients IFN-γ levels were markedly higher upon A2/RA33 stimulation than upon stimulation with TT (484 ± 379 vs 117 ± 97 pg/ml, p < 0.003), whereas PBMC from controls responded to both Ags in a comparable manner (875 ± 672 vs 1376 ± 1918 pg/ml, p = NS). Therefore, TT-induced IFN-γ production was significantly higher in controls than in RA patients (p < 0.02). Concerning IL-4, A2/RA33 induced a stronger response than TT only in RA patients, although compared with IFN-γ the difference was less pronounced (p < 0.05). However, with respect to IL-2, no difference between the two stimulants was seen in RA patients, whereas in controls TT induced a stronger IL-2 response than A2/RA33 (p < 0.01). It is noteworthy that this response was comparable to the IL-2 response induced in RA patients by either A2/RA33 or TT, respectively.

Taken together, these data suggested that A2/RA33-responsive T cells belong predominantly to the Th1 subset in both RA patients and controls. However, in RA patients these cells secreted IL-2 and appeared to be expanded as indicated by increased IFN-γ production upon A2/RA33 stimulation relative to TT-induced stimulation.

MHC restriction of the A2/RA33-induced T cell response
To investigate whether A2/RA33-induced T cell activation was dependent on Ag presentation by MHC class II molecules, two mAbs directed to HLA-DR or to HLA-DR/DP/DQ, respectively, were used in inhibition experiments performed with PBMC of eight responsive patients and three responsive controls (Fig. 4). As shown in Fig. 4A, addition of the anti-HLA-DR Ab led to a marked reduction of IFN-γ secretion in all patients and controls (43–90% decrease, p < 0.02). Proliferation was inhibited to an even higher degree (63–94% decrease, p < 0.0003) (Fig. 4B). A similar result was obtained with the second anti-HLA class II mAb (data not shown). In contrast, an isotype-matched control Ab had no significant effect (Fig. 4, C and D).

T cell reactivity to Fil
The proliferative responses to both Fil and cFil were investigated in PBMC of 19 RA patients and 20 healthy controls. As shown in Fig. 5, proliferative responses to either form of Fil were observed in only four RA patients and two controls: three patients responded to both forms and the fourth patient recognized only Fil. Interestingly, cFil always elicited a lower response than Fil, indicating that arginine deamination may affect T cell recognition. Of the two controls, one responded to Fil and the other one responded to cFil. Mean SI of PBMC of RA patients was 1.7 ± 1.4 for Fil and 1.1 ± 0.7 for cFil, respectively, which did not significantly differ from the SI values obtained in controls (1.1 ± 0.6 for Fil and 1.3 ± 0.6 for cFil, respectively). In contrast, the proliferative responses to A2/RA33 of these 19 RA patients were significantly higher, with a mean SI of 4.1 ± 3.5 (p < 0.02 vs Fil and p < 0.002 vs cFil, respectively).

To learn whether citrullinated peptides could elicit a better T cell response, two synthetic cFil-derived peptides harboring major B cell epitopes (46) as well as their corresponding unmodified isofoms were used in proliferation assays involving PBMC from seven RA patients, three of whom had responded to the whole
molecule, and 13 controls (Table I). Among the three responsive patients, only patient A responded selectively to the citrullinated form of peptide 1 (and to both forms of peptide 2), whereas patients B and C responded only to the unmodified peptides. One of the four nonresponders (patient D) was reactive with both forms of peptide 2 and one of the 13 controls responded to unmodified peptide 1. Thus, these data confirmed that deimination can affect T cell recognition.

**A2/RA33-reactive TCC**

T cell lines specific for A2/RA33 were established from the peripheral blood of six RA patients and three healthy controls. Using the limiting dilution technique, between one and six Ag-specific TCC per individual could be generated (Table II). Sixteen TCC were derived from RA patients, the majority of which (i.e., 13 of the 14 TCC analyzed) were CD4 CD8+ and thus belonged to the Th cell subset. The TCC showed high variability in their proliferative responses, with SI ranging from 2.2 to 47 and a mean SI of 7.9 ± 12.3. Analysis of the cytokine secretion pattern, in contrast, revealed high IFN-γ production (i.e., >100 pg/ml) by the majority of the clones with levels in the SN exceeding 1 ng/ml in 10 of them, while IL-4 was generally not detectable. Cytokine production did not correlate with proliferation because even poorly proliferating TCC secreted large amounts of IFN-γ.

Proliferation of the 12 TCC derived from control individuals was comparable to patient-derived clones with SI ranging from 2.1 to 37 with a mean SI of 7.4 ± 10.4. In contrast, phenotyping revealed some differences because only seven TCC were CD4 CD8−, while two clones were CD4 CD8+ and three clones were CD4 CD8+ (all from donor RM). Although all TCC produced IFN-γ (and no IL-4), only five of them secreted high amounts (>1 ng/ml), which, interestingly, were all derived from donor RM. However, only one of these high producers (RMHC.200) showed a Th1 phenotype (CD4 CD8+), while the remaining four clones were either CD4 CD8+ or double positive. Among the six remaining CD4 CD8− clones only two secreted IFN-γ >100 pg/ml (RMHC.80 and WiWiHC.11) showed a pronounced Th1 phenotype (IFN-γ >100 pg/ml).

**Expression of A2/RA33 in synovial tissue**

Expression at the protein level was studied in synovial tissue from five RA and three OA patients using a mAb against A2/RA33 (Fig. 6). These investigations revealed the autoantigen to be abundantly expressed in RA synovial tissue, particularly in the lining layer, where macrophage-like type A synoviocytes are the predominating cell population, and in the sublining area (Fig. 6A). Double staining experiments confirmed abundant expression by CD68-positive macrophages (Fig. 6, B and C) and CD68-negative fibroblast-like synoviocytes, as well as by endothelial cells, whereas A2/RA33 expression was scarcely detectable in T and B cells (data not shown). Remarkably, A2/RA33, which in cultured cells shows a predominant nuclear localization (51), was detected not only in the

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**Table I.** Proliferative responses of PBMC from RA patients to Fil-derived peptides

<table>
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<tr>
<th>Patient</th>
<th>cFil</th>
<th>Fil</th>
<th>cfilpep</th>
<th>filpep 1</th>
<th>filpep 2</th>
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<tbody>
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<td>1.8</td>
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<td>2.2</td>
</tr>
<tr>
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<td>1</td>
<td>1.9</td>
<td>1.4</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td>D</td>
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<td>1.9</td>
<td>1.2</td>
<td>1.4</td>
<td>3.2</td>
<td>3.6</td>
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</table>

* Two synthetic peptides (for sequences refer to Materials and Methods) containing dominant B cell epitopes were employed in citrullinated (cFilpep) and unmodified (filpep) forms. SI ≥ 2 are underlined.

**Table II.** Phenotype, proliferation, and cytokine production of A2/RA33-reactive TCC derived from RA patients and healthy controls

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype</th>
<th>SI</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
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<td></td>
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<td>2.2</td>
<td>&gt;2000</td>
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<tr>
<td></td>
<td>KBR.2 CD4 CD8+</td>
<td>2.8</td>
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* Sixteen TCC were derived from six RA patients. Mean SI was 7.9 ± 12.3; median SI was 3.4. All but one clone showed a CD4 CD8+ phenotype (clone HWRA.151 was CD4 CD8−). The majority of clones showed a pronounced Th1 phenotype (IFN-γ ≥100 pg/ml) and secreted significantly more IFN-γ than CD4 CD8− control clones (p < 0.04).

<sup>b</sup> Twelve TCC were derived from three healthy controls. Mean SI was 7.4 ± 10.4; median SI was 3.2. Seven TCC showed a CD4 CD8+ phenotype, two clones were CD4 CD8− (RMHC.87 and BSHC.16), and three clones were double positive (RMHC.126, 140, and 116). Only three CD4 CD8− clones (RMHC.200, RMHC.80, and WiWiHC.11) showed a pronounced Th1 phenotype (IFN-γ ≥100 pg/ml).
nucleus but also in the cytoplasm of RA synovial cells (Fig. 6, A and C). In contrast, in OA synovial tissue only few cells stained positive and the degree of cellular expression appeared to be generally weaker (Fig. 6, D and E).

Discussion
The two Ags investigated in this study, A2/RA33 and cFil, represent well-characterized target structures of the humoral autoimmune response of RA patients. Because the factors driving the Aab response to these two proteins are unknown, we were interested to search for the presence of autoreactive T cells in blood and synovial fluid of RA patients, assuming that autoreactive T cells might have more pathogenetic relevance than Aab, whose contribution to the pathophysiology of RA is still unclear. The data obtained in the course of this study seem to support such an assumption, because T cell responses to A2/RA33 were observed in peripheral blood of ~60% of the RA patients and in all synovial fluids investigated and thus occurred more frequently than anti-A2/RA33 Aab. Furthermore, cellular responses to A2/RA33 were seen in only 20% of the controls and were significantly weaker than in RA patients, even in patients suffering from PSA, an inflammatory and destructive arthropathy showing some similarities with RA, particularly with respect to TNF-α production (52).

Although no significant correlation was found between the A2/RA33 response and the MHC susceptibility alleles HLA-DR4 and DR1, it should be noted that >70% of the reactive patients carried the shared epitope. Importantly, this autoimmune response appeared to be largely HLA-DR restricted as demonstrated by inhibition experiments with mAb against MHC class II molecules. Studies are currently in progress to investigate interaction of A2/RA33 peptides with RA-associated HLA-DR molecules.

Cytokine analysis revealed abundant IFN-γ secretion in response to A2/RA33 by the majority of patient and control PBMC, whereas production of IL-4 was considerably lower or undetectable. The data obtained with A2/RA33-specific TCC bolster these observations because almost all TCC derived from RA patients showed a strong Th1 phenotype that was more pronounced than that of control-derived Th1 clones. Taken together, these data indicate a strong preponderance of Th1 cells among A2/RA33-reactive T cells, which is in agreement with previous observations on Th1:Th2 ratios in RA patients (9–12, 53). Remarkably, IFN-γ production of PBMC of RA patients was much higher in response to A2/RA33 than to TT, whereas PBMC from control subjects responded to both Ags in a comparable manner. Therefore, in RA patients A2/RA33-reactive T cells appeared to be more prevalent in relation to TT-responsive T cells, suggesting Ag-driven expansion. This conclusion is supported by the observation that A2/RA33-induced IL-2 production appeared to be largely restricted to PBMC of RA patients.

The presence of autoreactive T cells to A2/RA33 in some healthy persons was not unexpected and is consistent with observations repeatedly made by other investigators in both humans and animal models (54–60). Under normal physiological conditions autoreactive T cells are under tight control and may even play beneficial roles (61, 62). The lack of significant IL-2 secretion as well as the low proliferative capacity of control PBMC in response

FIGURE 6. Immunohistochemical analysis of A2/RA33 expression in synovial tissue. Cryosections from a patient with RA (A–C and F) and a patient with OA (D and E) were stained with a mAb against A2/RA33 (A–E) or an isotype-matched control mAb (F). Cells were counterstained with either hematoxylin (A, D, and F) or an anti-CD68 mAb (B, C, and E) to identify synovial macrophages. Cells expressing A2/RA33 are stained brown; CD68-bearing cells are blue. Pronounced expression of A2/RA33 is visible in the lining layer and in the sublining areas of RA tissue (A–C), whereas in OA tissue many fewer cells are stained (D and E). Note both nuclear and cytoplasmic localization of A2/RA33 in RA synovial cells. Magnification: 100-fold (B, E, and F), 200-fold (A and D), and 400-fold (E).
to A2/RA33 may indicate an anergic state preventing these cells from becoming expanded. Furthermore, TCC derived from controls were phenotypically more heterogeneous, with some of them being CD4^+CD8^-, indicating the existence of immature subpopulations of A2/RA33-reactive cells in the blood of healthy persons. Thus, it will be particularly interesting to see whether there exist differences in epitope recognition between RA patients and healthy subjects as has been observed in organ-specific autoimmune disease (63).

The pronounced overexpression and cytoplasmic localization of A2/RA33 observed in synovial membranes of RA patients was striking and was not seen in tissue of OA patients, who also did not show a significant autoimmune response to A2/RA33. Remarkably, abundant cytoplasmic expression was particularly seen in synovial macrophages and may form a prerequisite for presentation of this autoantigen to autoreactive T cells. It is well known that the stressful conditions in inflamed tissues lead to activation, overexpression, aberrant localization, or deposition of numerous molecules, some of which may function as autoantigens (64). At present, the molecular mechanisms leading to aberrant localization of A2/RA33 are unknown, but, interestingly, a similar observation has recently been made in pathological tissue of lung cancer patients (65). Thus, under conditions of chronic cellular stress translocation of A2/RA33 to the cytoplasm seems to occur in which this protein may exert a hitherto unknown function. A2/RA33 is a shutting protein involved in transport of certain mRNAs and possibly some of which may function as autoantigens in RA patients (66). At present the molecular mechanisms leading to aberrant localization of A2/RA33 are unknown, but, interestingly, a similar observation has recently been made in pathological tissue of lung cancer patients (65). Thus, under conditions of chronic cellular stress translocation of A2/RA33 to the cytoplasm seems to occur in which this protein may exert a hitherto unknown function. A2/RA33 is a shutting protein involved in transport of certain mRNAs and possibly also in regulation of their translation as suggested for myelin basic protein or glucose transporter 1 (66, 67). Like other shutting proteins, A2/RA33 is known to undergo posttranslational modifications such as phosphorylation or methylation (68, 69). Therefore, it is conceivable that the cytoplasmic form differs from the nuclear one and might in fact be more immunogenic: taking into consideration that posttranslational modifications may lead to the formation of neoeptopes and render a protein autoantigenic (70). Thus, it may well be possible that the Aab are primarily directed to posttranslationally modified (i.e., the cytoplasmic) forms of A2/RA33 and escape detection in our assays where Ag is used that is either bacterially expressed or derived from cultured cells in which expression is largely restricted to the nucleus (51). To address this issue, A2/RA33 must be isolated from human synovial tissue and subjected to a detailed biochemical and immunological analysis.

Although Aab to the second Ag investigated, Fil, can be more frequently found in RA patients than anti-A2/RA33 Aab (26, 36, 39), cellular reactivity to Fil was seen in only few RA patients. Moreover, these responses were substantially weaker than those against A2/RA33, which could be detected in all patients reactive to Fil. Thus, these data suggest that Fil may not be the T cell Ag driving the humoral autoimmune response to citrullinated targets. Nevertheless, B cells secreting AFA have been detected in synovial tissue and fluid of RA patients (71, 72), and citrullinated proteins apparently different from Fil (which is not expressed in the joint) have been demonstrated to be present in the synovium of RA patients (41, 73). Remarkably, fibrin was recently identified as one of the major citrullinated proteins in the joint and, importantly, citrullinated fibrin was recognized by purified AFA (41). Thus, it is conceivable that T and B cells responding to fibrin-derived (citrullinated) epitopes are present in the synovial compartment and may be prominent players in the RA autoimmune orchestra. However, it should be borne in mind that T cells driving the anti-citrulline B cell response may not necessarily be directed to citrullinated epitopes or Ags. The observation that cFil generally induced a weaker response indicates that deimination may affect T cell recognition, and future studies will have to show the specificity of the T cells that actually drive the Aab production to citrullinated targets.

At the present time it is unclear whether any of the autoantigens recognized by RA patients or any of the Ags eliciting chronic arthritis in animal models (such as cartilage Ags or the mycobacterial 65-kDa heat shock protein) are of relevance in the pathogenesis of RA (23, 24, 74). This is particularly true for type II collagen, even though collagen-induced arthritis is the most widely used model of RA and even though autoimmunity to collagen II occurs early in the course of RA (75, 76). An interesting novel candidate Ag is the glycolytic enzyme glucose-6 phosphate isomerase, which has been identified as the disease-inducing autoantigen in the KRN × NOD model of RA (77, 78). This finding demonstrates once more the potential pathogenetic importance of autoimmune reactions to ubiquitously expressed targets that are not joint specific. Similar to the observations made for A2/RA33 or citrullinated fibrin, glucose-6 phosphate isomerase was recently described to be present in a high concentration in synovial tissue of RA patients who also had anti-glucose-6 phosphate isomerase Aab in their serum (79). This is suggestive of pathogenetic involvement also in human RA; however, this remains to be proven.

Taken together, the frequent presence of A2/RA33-reactive Th1-like cells in the blood of RA patients, their presence in the synovial compartment in conjunction with the observed aberrant expression of A2/RA33 in the inflamed synovium, and the occurrence of Aab to A2/RA33 early in the course of RA (42–44) suggest that this protein may be an important autoantigen in RA. Moreover, A2/RA33 Aab are among the earliest detectable Aab in MRL/lpr lupus mice (80), which, in addition to anti-DNA and other systemic lupus erythematosus-specific Aab, also produce RF and may develop erosive arthritis (81), and have recently been found to occur also in TNF-α transgenic mice (Ref. 82 and S. Hayer, B. Jahn-Schmid, D. Plows, K. Skriner, H. Erlandsson-Harris, S. Haralambous, F. Monnaeus, S. Trembleau, G. Schett, W. van Venrooij, et al., manuscript in preparation), which represent a well-established model of RA (83). Although the significance of these observations is not yet clear, they may be considered as additional indications for pathogenetic relevance of this autoimmune response. Further studies in patients and experimental animals will show whether A2/RA33 is indeed part of the inflammatory and deleterious cascade of events characteristic of RA.

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