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ABSTRACT

Objective: Type I interferons and apoptotic particles contribute to antinuclear autoimmunity in experimental models. This study assessed whether similar mechanisms contribute to break peripheral B-cell tolerance in humans by studying the induction of antinuclear antibodies by tumour necrosis factor blockade in spondyloarthritides.

Methods: 40 spondyloarthritis patients treated with infliximab or etanercept and 20 renal cell carcinoma patients treated with sorafenib were studied. Serum antinucleosome IgM and nucleosomes were measured by ELISA. Type I interferon serum activity was measured using a functional reporter cell assay. Synovial apoptosis was assessed by terminal transferase nick end-labelling (TUNEL) assay and anti-active caspase-3 immunostaining. Complement was measured by nephelometry.

Results: Despite a similar clinical improvement and reduction of synovial inflammation, antinucleosome IgM were induced by infliximab but not etanercept. This induction did not correlate with type I interferon activity, which was transiently downmodulated by infliximab but persistently upregulated by etanercept. In contrast, antinucleosome IgM levels did correlate with serum nucleosome levels, which were significantly upregulated by infliximab but not by etanercept treatment. This increase in serum nucleosome levels was not directly related to massive cell death, but rather to a decrease of complement 3 and 4 serum levels during infliximab treatment.

Conclusion: Infliximab and etanercept have a differential effect on both type I interferon activity and nucleosome levels. Only elevated serum nucleosomes relate to the induction of antinucleosome antibodies after infliximab treatment.

Random joining of immunoglobulin gene segments during V(D)J recombination generates multiple poly and autoreactive specificities in the early stages of human B-cell development. In normal individuals, discrete checkpoints in the bone marrow remove most of the self-reactive cells from the repertoire by deletion and/or receptor editing. Nevertheless, up to 20% of the antibodies expressed by mature naive B cells in healthy individuals appear to be autoreactive, indicating that these mechanisms of central tolerance are partly leaky. An additional peripheral checkpoint at the transition between the mature naive B-cell pool and antigen-experienced, IgM memory B cells removes these cells from the repertoire and thereby avoids overt autoimmunity.

In humoral autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), the increased self-reactivity in the mature naive B-cell pool even before the onset of an active immune response indicates that the central tolerance mechanisms are impaired. This increase of auto-reactivity in the naive pool is not expected to lead to overt autoimmunity as long as the peripheral tolerance mechanisms are intact. Therefore, additional mechanisms contribute to break peripheral tolerance in these diseases in order to allow the appearance of auto-reactivity in the memory pool. Data from experimental models suggest a potential contribution of an overload of nuclear antigens stimulating the B-cell receptor and Toll-like receptors, as well as of B-cell receptor-independent stimuli such as type I interferons. How far similar mechanisms contribute to break peripheral B-cell tolerance in humans remains to be investigated.

In contrast to SLE and RA, spondyloarthritides is a chronic inflammatory arthritis in which the absence of known autoantibodies suggests intact B-cell tolerance checkpoints. We recently described that even in this condition tumour necrosis factor (TNF) blockade leads to the induction of antinuclear antibodies (ANA). Such novel auto-reactivities can either result from a break in peripheral tolerance or be generated de novo by somatic hypermutation as demonstrated in healthy, non-autoimmune-prone individuals. The fact that the TNF blockade-induced ANA were largely restricted to the IgM isotype without corresponding IgG reactivities argues against the latter possibility. As such, TNF blockade in spondyloarthritides provides us with a unique human model to study the regulation of peripheral B-cell tolerance in humans. Taking advantage of the differential effect of the monoclonal anti-TNF antibody, infliximab, and the soluble TNF receptor, etanercept, on autoantibody induction, we investigated which of the mechanisms described in experimental SLE models could contribute to the induction of IgM ANA in spondyloarthritides. Based on the published data on the induction of apoptosis in lymphocytes and monocytes by infliximab but not etanercept, the hypothesis investigated here is that the differential induction of ANA by TNF blockers could relate to the fact that infliximab treatment can induce cell death and a subsequent release of nucleosomes. As it has been shown that type I interferons are central players in the response to these autoantigens, the difference in the induction of ANA by infliximab and etanercept could be related to the difference in type I interferon activity.
we additionally investigated the involvement of this cytokine in autoantibody induction.

MATERIALS AND METHODS

Patients

We included 40 spondyloarthritis patients who fulfilled the European Spondyloarthritis Study Group criteria and had active disease defined as the presence of at least one swollen joint and/or inflammatory spinal pain. Twenty of these patients were treated with infliximab 5 mg/kg intravenously at weeks 0, 2 and 6 followed by 10 mg/kg every 14 weeks. The other group of 20 patients was treated with etanercept 25 mg subcutaneously twice a week. None of the patients received disease-modifying antirheumatic drugs and/or systemic or local corticosteroids. The median age was 49 years (38–66) and the median disease duration was 18.5 years (1–43) in the infliximab cohort. In the etanercept group, the median age was 38 years (20–71) and the median disease duration was 10 years (1–41). In addition, we included 20 patients with renal cell carcinoma who were treated with sorafenib at weeks 0, 8 and 16. The median age of these patients was 60 years (56–67). These patients were treated with sorafenib at weeks 0, 8 and 16. The median age was 49 years (38–66) and the median disease duration was 18.5 years (1–43) in the infliximab cohort. In the etanercept group, the median age was 38 years (20–71) and the median disease duration was 10 years (1–41). In addition, we included 20 patients with renal cell carcinoma who were treated with sorafenib at weeks 0, 8 and 16. The median age of these patients was 60 years (56–67). These patients responded to therapy; the time to regression was 181 days (116–213). All patients gave written informed consent to participate in the study as approved by the local Medical Ethics Committee.

Samples

Serum samples were obtained at weeks 0, 2, 6, 12 and 34 in the infliximab-treated patients, at weeks 0, 4, 8, 12 and 32 in the etanercept-treated patients and at weeks 0, 2, 8 and 16 in the renal cell carcinoma patients. Synovial biopsies were obtained from a clinically swollen knee joint by needle arthroscopy before and after 12 weeks of TNF blockade in all spondyloarthritis patients. Additional biopsies were also obtained after 1 week of treatment (n = 12 infliximab-treated patients). Eight biopsies per patient were snap frozen for immunohistochemistry.

Synovial histopathology

Immunohistochemistry was performed with the following antibodies: anti-CD4 (clone MT310; DakoCytomation, Glostrup, Denmark), anti-CD8 (clone DK25; DakoCytomation), anti-CD20 (clone L26; DakoCytomation) and anti-CD68 (clone EBM11; DakoCytomation). Concentration and isotype-matched negative controls were included. Cells were visualised using the LSAB+ kit and AEC substrate (DakoCytomation). Sections were blinded and scored on a semiquantitative four-point scale by two independent observers.

Serum measurements

Serum antinucleosome antibodies were detected by Anti-Nucleo ELISA (GA Generic Assays, Dahlewitz, Germany). The supplied anti-human IgG conjugate was replaced by a specific anti-human IgM (rabbit) horseradish peroxidase (Abcam, Cambridge, UK). We previously validated the specificity of this secondary antibody. Serum nucleosomes were detected with the cell death detection ELISA kit (Roche Diagnostics, Penzberg, Germany). Serum levels of complement C3 and C4 were assessed by nephelometry. Rheumatoid factor may interfere with these assays, but all patients were rheumatoid factor negative.

Type I interferon serum activity

Type I interferon serum activity was measured as previously described. Briefly, WISH epithelial cell line cells were cultured in the presence of 50% serum for 6 h. RNA was extracted using the RNeasy mini kit (Qiagen, Chatsworth, California, USA) and reverse transcribed to complementary DNA (SuperScript III RNAse H reverse transcriptase; Invitrogen, Carlsbad, California, USA). A real-time quantitative PCR reaction was performed for the following interferon inducible genes: IFIT-1, PRKR and MX-1. HPRT-1 was used as a housekeeping gene control.

Detection of synovial apoptosis

Apoptotic cells were detected by both terminal transferase nick end-labelling (TUNEL) and anti-active caspase 3 staining. The in-situ cell death detection kit (Roche Applied Science, Indianapolis, Indiana, USA) was used to visualise DNA strand breaks. Positive and negative controls were included. Active caspase 3 was detected using a polyclonal rabbit antibody (R&D Systems, Abingdon, UK). A concentration and isotype-matched negative control was included. Staining was visualised using the LSAB+ kit and AEC substrate (DakoCytomation).

Statistical analysis

All data were non-parametric, they are represented as medians (interquartile range). The Mann–Whitney U-test and the Wilcoxon signed rank test were performed. Correlations were calculated with Spearman’s rho correlation coefficient. p Values of 0.05 or less were considered significant.
RESULTS

Infliximab, but not etanercept, induces antinucleosome IgM

The global efficacy of infliximab and etanercept treatment in spondyloarthritis has been described previously. Both TNF blockers led to clinical improvement (table 1) and induced a decrease of the global inflammatory infiltration as well as the number of T lymphocytes and macrophages in the synovial sublining (table 1). Titres of antinucleosome IgM antibodies were not different between healthy controls and spondyloarthritics at baseline (fig 1A). Infliximab treatment induced a median increase in the antinucleosome IgM levels of 94% (-22–648%) at week 12 (p < 0.001) and 123.5% (7–453%) at week 34 (p < 0.001; fig 1B). In contrast, there was no increase in the antinucleosome IgM levels in the etanercept-treated patients, with median changes compared with baseline of 6% (-25–280%) at week 12 and 1% (-29–218%) at week 32 (fig 1C). As previously reported, the induction of antinucleosome antibodies corresponded well with the ANA and antinucleosome (fig 1C). As previously reported, the induction of antinucleosome IgM by infliximab but not etanercept thus can not be explained by increased type I interferon activity during treatment.

Serum nucleosome levels increase during infliximab but not etanercept treatment and correlate with the induction of antinucleosome IgM

As humoral autoimmunity in experimental SLE models can result from excessive exposure of the immune system to nuclear antigens, we investigated whether type I interferon contributes to the induction of antinucleosome IgM during TNF blockade in spondyloarthritis. At baseline, type I interferon serum activity in spondyloarthritis patients was not different from healthy controls (1.81 (1.45–2.15) versus 1.85 (1.25–2.21)). During infliximab treatment, type I interferon serum activity was downmodulated at week 2 (p = 0.005) but returned to baseline at week 12 (fig 2A). On the contrary, etanercept induced a persistent upregulation of type I interferon serum activity (p < 0.03 from weeks 4 to 12; fig 2B). The induction of antinucleosome IgM by infliximab but not etanercept thus can not be explained by increased type I interferon activity during treatment.

The differential effect of infliximab and etanercept on type I interferon serum activity does not correlate with the induction of antinucleosome IgM

Type I interferons are a group of cytokines promoting humoral immunity. They play a crucial role in autoimmune pathology such as SLE and Sjögren’s syndrome. As TNFα suppresses IFNα production by plasmacytoid dendritic cells in vitro and the blockade of TNFα in vivo leads to an increased activation of the type I interferon pathways in juvenile chronic arthritis and Sjögren’s syndrome, we investigated whether type I interferon contributes to the induction of antinucleosome IgM during TNF blockade in spondyloarthritis. At baseline, type I interferon serum activity in spondyloarthritis patients was not different from healthy controls (1.81 (1.45–2.15) versus 1.85 (1.25–2.21)). During infliximab treatment, type I interferon serum activity was downmodulated at week 2 (p = 0.005) but returned to baseline at week 12 (fig 2A). On the contrary, etanercept induced a persistent upregulation of type I interferon serum activity (p < 0.03 from weeks 4 to 12; fig 2B). The induction of antinucleosome IgM by infliximab but not etanercept thus can not be explained by increased type I interferon activity during treatment.

Table 1

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Infliximab Baseline</th>
<th>Infliximab Week 12</th>
<th>Etanercept Baseline</th>
<th>Etanercept Week 12</th>
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<tr>
<td>Patient pain (VAS)</td>
<td>69 (14–100)</td>
<td>16 (1–86)**</td>
<td>69 (17–99)</td>
<td>12 (0–80)**</td>
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<tr>
<td>Patient global (VAS)</td>
<td>69 (17–100)</td>
<td>15 (0–73)**</td>
<td>73 (18–100)</td>
<td>14 (0–69)**</td>
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<td>Physician global (VAS)</td>
<td>64 (35–89)</td>
<td>15 (8–75)**</td>
<td>54 (36–89)</td>
<td>14.5 (1–59)**</td>
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<td>Duration of morning stiffness (minutes)</td>
<td>133 (0–300)</td>
<td>10 (1–90)**</td>
<td>75 (18–100)</td>
<td>12 (0–74)**</td>
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<tr>
<td>C-reactive protein (mg/l)</td>
<td>17 (10–29)</td>
<td>3 (0–79)**</td>
<td>10 (0–150)</td>
<td>3 (0–34)**</td>
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<tr>
<td>Erythrocyte sedimentation rate (mm/h)</td>
<td>28 (11–101)</td>
<td>7 (1–59)**</td>
<td>16 (1–86)</td>
<td>8 (1–39)**</td>
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<tr>
<td>Swollen joint count (number)</td>
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<td>1 (0–7)**</td>
<td>3 (1–19)</td>
<td>0.5 (0–10)**</td>
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<tr>
<td>Tender joint count (number)</td>
<td>10 (1–20)</td>
<td>0 (0–6)**</td>
<td>5 (1–23)</td>
<td>1 (0–13)**</td>
</tr>
</tbody>
</table>

Histological parameters were scored on a four-point semiquantitative scale. Data are represented as median (range). *p < 0.05; **p < 0.001. TUNEL, terminal transferase nick end-labelling.

The patients were evaluated at baseline and after 12 weeks for clinical and histological response. The patient’s assessment of pain and the patient’s and physician’s assessment of global disease activity were scored on a 100 mm visual analogue scale (VAS). Histological parameters were scored on a four-point semiquantitative scale. Data are represented as median (range). *p < 0.05; **p < 0.001. TUNEL, terminal transferase nick end-labelling.
Infliximab and etanercept had a differential effect on type I interferon (IFN) serum activity (A and B) and serum nucleosome levels (C and D). The data are represented as median and interquartile ranges compared with baseline values (*p < 0.05; **p < 0.01). The early changes in serum nucleosome levels (between baseline and week 2 for infliximab-treated patients and week 4 for etanercept-treated patients) correlated with the changes in serum antinucleosome IgM antibodies between baseline and week 12 (r = 0.383; p = 0.008) (E) and between baseline and week 34 (r = 0.453; p = 0.014) (F).

Neither infliximab nor etanercept increase synovial apoptosis

Considering the suggested induction of apoptosis of activated lymphocytes and monocytes by infliximab but not etanercept in vitro and in Crohn’s disease in vivo, we investigated whether a similar mechanism contributes to the rise in serum nucleosome levels. At baseline, TUNEL staining of synovial biopsies showed the presence of distinct cells exhibiting DNA damage in both the intimal synovial lining layer and the synovial sublining (fig 3A and C). However, neither infliximab nor etanercept significantly increased the number of TUNEL-positive cells at week 12 compared with baseline (fig 3B and D and table 1). Similar results were obtained analysing anti-active caspase-3 staining (table 1). An additional analysis of spondyloarthristis biopsies obtained after 1 week of treatment with infliximab also failed to show increased apoptosis (data not shown). Whereas these data show that DNA fragmentation is observed in inflamed synovium, they do not provide evidence for apoptosis induction after infliximab or etanercept treatment in vivo.

Induction of massive cell death in vivo is not sufficient to increase serum nucleosome levels

As this synovial analysis in spondyloarthritis cannot exclude the possibility of apoptosis induction at sites other than the inflamed joint, we used a different type of targeted treatment to investigate the relationship between the induction of cell death and the elevation of serum nucleosomes and antinucleosome antibodies. Sorafenib, a tyrosine kinase inhibitor used in renal cell carcinoma, is known to induce acute and massive renal cell death. In our cohort of 20 renal cell carcinoma patients, sorafenib treatment at week 12 induced marked necrosis of the tumour (fig 4E and F). However, the serum levels of nucleosomes did not increase during treatment up to week 16 (data not shown), indicating that the induction of massive cell death is not sufficient to increase serum nucleosome levels. Accordingly, there was no induction of antinucleosome IgM in these patients (data not shown).
Infliximab but not etanercept decreases serum complement levels

As the increase in serum nucleosome levels could not be explained solely by enhanced release during massive cell death, we investigated whether TNF blockade with infliximab affected the clearance of apoptotic material. C-reactive protein (CRP) is an important acute phase protein implicated in clearing mechanisms. We observed a similar decrease of CRP in both infliximab and etanercept-treated patients (table 1). In accordance, we did not observe any correlation between CRP levels or

Figure 3  Representative pictures of terminal transferase nick end-labelling (TUNEL) staining in spondyloarthritis synovium before and after 12 weeks of tumour necrosis factor blockade with either infliximab or etanercept (A–D) (magnification ×320). Representative computed tomography scans of the kidneys of a patient treated with sorafenib, at baseline (E) and after 8 weeks of treatment (F), showing massive necrosis of the renal cells.

Figure 4  Serum levels of complement C3 were not different at baseline between both groups of spondyloarthritis patients (A and B, left dataset). Infliximab (A) but not etanercept (B) decreased the serum complement C3 levels after 12 weeks of treatment. **p<0.01.
changes in CRP levels and antinucleosome levels. Beside CRP, the complement factors C3 and C4 have also been reported to play an important role in the clearance of apoptotic cells.43–45 Baseline levels of serum C3 and C4 were comparable with healthy controls (data not shown). C3 levels dropped significantly during infliximab treatment (from 1.5 g/l (1.2–1.4) to 1.0 g/l (0.9–1.2); p<0.001; fig 4A). In contrast, C3 levels remained stable during etanercept treatment over 12 weeks (from 1.5 g/l (1.1–1.5) to 1.2 g/l (1.0–1.3); fig 4B). Similarly, C4 remained stable during 12 weeks of treatment with etanercept, but dropped significantly during infliximab treatment (from 0.26 g/l (0.23–0.32) to 0.20 g/l (0.14–0.22); p = 0.001). Taken together, these data indicate that infliximab but not etanercept treatment is associated with a significant drop in serum C3 and C4 levels.

**DISCUSSION**

Whereas the absence of known autoantibodies suggests normal, functional central and peripheral tolerance of B-cell checkpoints in spondyloarthritis, we previously reported the induction of IgM ANA by TNF blockade in these patients.11 As there is a differential induction by the monoclonal anti-TNF antibody, infliximab, and the soluble TNF receptor, etanercept,12 we investigated which of the mechanisms described in experimental SLE models could contribute to break peripheral B-cell tolerance in this unique human model of antinuclear antigen autoimmunity.

We observed a clear modulation of type I interferon serum activity. Type I interferons are a group of cytokines reported to play a crucial role in autoimmune pathology such as SLE and Sjögren’s syndrome.7–10 They are central players in a positive feedback loop generating more nuclear material and ANA.17–19 TNF blockade sustains type I interferon production in vitro.20 In agreement with these experimental data, we reported previously that etanercept treatment induces a persistent upregulation of type I interferon in Sjögren’s syndrome.29 We confirmed this observation in spondyloarthritis patients treated with etanercept. As to infliximab, a cross-sectional analysis of five patients with active systemic onset juvenile chronic arthritis despite anti-TNF treatment for variable periods showed an increase in both interferon type I regulated genes in peripheral blood mononuclear cells and anti-dsDNA antibody levels.33 In contrast, our prospective, longitudinal data in 20 spondyloarthritics patients with a good clinical response to infliximab showed that type I interferon serum activity was transiently downmodulated before returning to normal levels. Neither baseline levels nor changes of type I interferon activity correlated with the induction of antinucleosome IgM in spondyloarthritis. This is consistent with the observation that type I interferon changes during etanercept treatment in Sjögren’s syndrome did not affect specific autoantibody levels.30 Although the reasons for the differential effect of infliximab and etanercept on type I interferon serum activity remain speculative, these data emphasise the complexity of the cross-regulation between TNF and type I interferon in vivo and indicate that changes in type I interferon are not directly related to the break of B-cell tolerance in our spondyloarthritis model.

Beside factors affecting autoreactive B cells, the antigen itself can also determine the outcome of the B-cell response. Apoptotic material represents an important source of autoantigens as intracellular and nuclear antigens are modified during the apoptotic process and are translocated to the cell membrane.7–10 Under normal physiological conditions, apoptotic cell blebs are rapidly cleared in a non-immunogenic process. However, overload with apoptotic bodies due to increased production or impaired clearance can produce the induction of ANA.30 A link between infliximab treatment and an increase in serum nucleosomes was reported in a previous study in 11 RA patients,30 although it is probably not a very early event as we did not observe it within the first 24 h of infliximab treatment.46 In the present study, we confirm a significant increase in serum nucleosome levels after 2 weeks of infliximab treatment. We demonstrate that this is specific for infliximab and not for etanercept and that this phenomenon relates directly to the subsequent appearance of antinucleosome IgM antibodies. As such, these data identify the elevated levels of serum nucleosomes as an important factor contributing to the break of B-cell tolerance during TNF blockade.

The appearance of elevated levels of serum nucleosomes can be due to high levels of release during cell death and/or disruption of the normal pathways of clearance of the cell debris.27 In mice, increased in-vivo cell death induced by lipopolysaccharide or anti-Fas resulted in a rise in plasma nucleosomes in a dose-dependent fashion.47 As infliximab has been suggested to induce apoptosis of activated leucocytes in vitro,28 37 39 this mechanism could contribute to the subsequent release of nucleosomes in the circulation in vivo. However, there is as yet no apparent evidence for the specific induction of cell death by infliximab or etanercept in human arthritis. Several studies in RA and psoriatic arthritis demonstrated that infliximab does not induce apoptosis in the inflamed synovium despite a marked clinical and histological response to treatment.30 31 Confirming the former studies,30 31 we could not detect any increase in synovial apoptosis in spondyloarthritics treated with any of these TNF blockers. As the discrepancy between the increase in serum nucleosomes and the absence of synovial apoptosis may be due to infliximab-induced cell death at other sites, we turned to another model to assess the potential relationship between massive cell death and a rise in serum nucleosomes and, eventually, antinucleosome antibodies. In renal cell carcinoma, treatment with the tyrosine kinase inhibitor, sorafenib, leads to massive cell necrosis.48 Necrosis, as opposed to apoptosis, is not a strictly regulated physiological process; the release of inflammatory mediators and modified antigens evokes a strong inflammatory response and is therefore more likely to induce humoral autoimmunity against nuclear antigens. However, the massive cell death in this model did not lead to a rise in serum nucleosomes and, subsequently, antinucleosome antibodies. Taken together, these data consistently indicate that the rise in serum nucleosomes can not merely be explained by infliximab-induced cell death.

The serum nucleosome levels are not only regulated by release but also by adequate clearance. Factors such as mannose-binding lectin, serum amyloid P, CRP and complement prevent nuclear antigens from appearing in the circulation under normal conditions. Mice with deficiencies in some of these factors exhibit a delayed clearance of apoptotic cells, which is directly linked to the production of autoantibodies and the development of autoimmune disease.49–50 In our spondyloarthritics patients, we observed a similar decrease of CRP during infliximab and etanercept treatment, but only the former led to a highly significant decrease in C3 as well as C4 after 12 weeks of treatment. As both nucleosomes and antinucleosome antibodies are induced, it may be expected that they form immune-complexes that lead to complement consumption. On the other hand, infliximab does not induce complement activation46 51 suggesting that the decrease in C3 and C4 serum levels might be...
a direct effect rather than due to consumption, but this hypothesis needs to be formally confirmed.

In this context, it is also noteworthy that the increase in nucleosomes was transient and disappeared after 12 weeks despite the low complement levels. On the other hand, the ANA themselves persist over more than 2 years, but disappear after the interruption of infliximab treatment. This suggests that antinucleosome IgM antibodies contribute to clear these nucleosomes and disappear when the triggering mechanism has been relieved. Although clearly different from preimmune natural IgM antibodies, the ANA induced by infliximab treatment are also restricted to the IgM isotype and could have natural IgM antibodies, the ANA induced by infliximab on nucleosomes and disappear when the triggering mechanism supported by a Vidi grant of the Netherlands Organization for Scientific Research.

In conclusion, in our human model of ANA induction we were able to demonstrate that the break in peripheral B-cell tolerance is not due to elevated levels of type I interferons, but can be attributed to an increase in the availability of the antigen. The increase in serum nucleosome levels is most probably due to defects in clearance mechanisms rather than an increased release of nuclear antigens.

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Ethics approval: This study was approved by the local Medical Ethics Committee.

Patient consent: Obtained.

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44. Flierman R, Daha MR. The clearance of apoptotic cells by complement. *Immunobiology* 2007;212:363–70.


