Production of the effector cytokine interleukin-17, rather than interferon-γ, is more strongly associated with autoimmune hemolytic anemia

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ABSTRACT

Background
Interleukin-17A is the signature cytokine of the Th17 subset and drives inflammatory pathology, but its relevance to autoantibody-mediated diseases is unclear. Th1 cells secreting interferon-γ have been implicated in autoimmune hemolytic anemia, so the aim was to determine which cytokine is more closely associated with disease severity.

Design and Methods
Interferon-γ and interleukin-17A were measured in the sera of patients with autoimmune hemolytic anemia and healthy donors, and in peripheral blood mononuclear cell cultures stimulated with autologous red blood cells, or a panel of peptides spanning red blood cell autoantigen.

Results
Serum interleukin-17A, but not interferon-γ, was significantly raised in patients with autoimmune hemolytic anemia ($P<0.001$), and correlated with the degree of anemia. Interleukin-17A was also more prominent in the responses of peripheral blood mononuclear cells from patients with autoimmune hemolytic anemia to red blood cells, and, again unlike interferon-γ, significantly associated with more severe anemia ($P<0.005$). There were no interleukin-17A responses to red blood cells by peripheral blood mononuclear cells from healthy donors. Specific autoantigenic peptides were identified that elicit patients' interleukin-17A responses.

Conclusions
Interleukin-17A makes a previously unrecognized contribution to the autoimmune response in autoimmune hemolytic anemia, challenging the model that the disease is driven primarily by Th1 cells. This raises the possibility that Th17, rather than Th1, cells should be the target for therapy.

Key words: autoimmune hemolytic anemia, interleukin-17, interferon-γ, T-lymphocyte, autoimmunity.

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Introduction

Conventional treatments for autoimmune diseases are unsatisfactory, and the design of more targeted, effective approaches will depend on a better understanding of the pathogenic immune responses. Identification of key effector cytokines and blockade of their activity using biological therapies has proven to be an attractive strategy in cell-mediated inflammatory conditions,[1,2] but the cytokine response in many diseases that are driven by autoantibody remains poorly characterized.

Autoimmune hemolytic anemia (AIHA) provides a classic example of autoantibody-mediated pathology.[3,4] The autoantibody in most patients is 'warm reactive' with optimal affinity for red blood cells (RBC) at 37°C, predominantly of the IgG class, and mediates clearance by macrophages.[4,5] IgG antibody responses are typically T-cell-dependent, since B cells require help to switch class from IgM production, form germinal centers and undergo somatic mutation.[3,6,7] A substantial body of data demonstrates that T helper (Th) cells may be a therapeutic target in AIHA.[5,8] Valuable insights have been obtained from studies of both murine AIHA, particularly the spontaneous form affecting New Zealand Black (NZB) mice, and human patients. NZB IgG autoantibody production *in vivo* is retarded by treatment with anti-CD4 monoclonal antibody,[9] or by *CD4* gene deletion,[10] with disease being modulated by the administration of peptides bearing the dominant autoreactive Th cell epitope.[11] Findings in human AIHA are also consistent with the need for T-cell help. In most patients, RBC autoantibodies target the Rh proteins,[12,13] and these autoantigens are also recognized by effector Th cells that have been activated *in vivo*.[14] Th cells with similar specificities can be detected in healthy donors, but typically retain a naïve phenotype.[15,16] Many factors contribute to the stimulation of autoreactive Th cells, including the presence of inappropriately activated[16] or aberrant antigen-presenting cells.[17,18] Particular Th cytokines have been implicated in the pathogenesis or regulation of AIHA,[11,19,20] but their roles need to be re-evaluated in the light of recent progress in identifying novel Th subsets and mediators.

It was recognized many years ago that Th cells can be classified into different functional subsets based on their cytokine secretion profile, with Th1 and Th2 populations being characterized by production of the respective signature cytokines interferon-γ (IFN-γ) and interleukin-4 (IL-4).[21] Early studies appeared to show that the pathogenicity of autoimmune responses could be determined by mutual antagonism between these helper subpopulations.[7,8,16,19] Evidence accumulated that many models of autoimmune disease, including those that are antibody-mediated, are driven by helper responses that are strongly dominated by the Th1 subset, and that inducing a corresponding Th2 bias can protect against pathology.[3,8,16,22] For example, we reported that the $CD4^+$ Th response to RBC autoantigen in patients with AIHA[19] or in NZB mice[20] was associated with IFN-γ, but not IL-4, production, and that deviating the murine response towards IL-4 ameliorated disease.[11] However, additional effector subsets have since been described, with Th17 cells that characteristically secrete the inflammatory mediator IL-17A attaining particular prominence.[23,24] IL-17A exerts potent pro-inflammatory properties via effects on a broad range of cells including epithelia and endothelia, fibroblasts, neutrophils, osteoblasts and monocytes/macrophages.[23] Following seminal reports that IL-17A in the absence of IFN-γ can drive pathology in rodent models such as experimental allergic encephalomyelitis,[25] it is now widely accepted that the Th17 subset is responsible for, or contributes to, inflammation previously attributed to Th1 cells in many autoimmune diseases.[26,27]

While the effects of Th17 cells and IL-17A have been shown to be relevant to many inflammatory pathologies, it remains far less clear what role, if any, they play in autoimmune diseases that are purely antibody-mediated. AIHA provides an opportunity to address this question. Until now, the Th1 IFN-γ response was believed to be important in the pathogenesis of AIHA and a key therapeutic target.[5,8,16,19,20] The purpose of the current study was to determine whether there is any additional contribution of Th17 cytokine to the autoimmune response in patients with AIHA and, in particular, whether IL-17A or IFN-γ production is more closely associated with the disease.

Design and Methods

Patients and control donors

AIHA was diagnosed in patients attending the Aberdeen Royal Infirmary on the basis of clinical and hematologic investigations, including a positive Coombs' test. The Grampian Health Board and the University of Aberdeen Joint Ethical Committee approved the protocol for investigation, and all patients gave informed consent. The group comprised 13 male and 20 female patients with a median age of 59 at presentation. Patients were only included in the series if they were considered to have warm-type primary AIHA, with no evidence of underlying disease. Samples for separation of serum and peripheral blood mononuclear cells (PMBC) were taken by venepuncture into plain and lithium heparin Vacutainers, respectively (Becton Dickinson, Oxford, UK). Blood was obtained pre-treatment at diagnosis or when patients were receiving only low to moderate doses of corticosteroids, with collections during any periods of aggressive immune suppressive treatment excluded from the study. Control blood samples were taken from healthy human volunteers (n=20, median age 52) with no serological evidence of warm RBC autoantibodies or anti-D alloantibodies.

Measurement of serum cytokine levels

Enzyme-linked immunosorbent assays (ELISA) for IFN-γ (Pharmingen, Oxford, UK) and IL-17A (e-Bioscience, Hatfield, UK) were performed using commercially available antibody pairs and cytokine standards according to the manufacturers' instructions. Briefly, serum samples were transferred into triplicate wells in microtiter plates (Maxisorp; Nunc Roskilde, Denmark) coated with monoclonal anti-cytokine capture antibody at the recommended concentrations. After incubation of sera for 12 h at 4°C, the plates were developed with the appropriate biotinylated monoclonal detection antibody, ExtraAvidin-alkaline phosphatase conjugate (Sigma, Dorset, UK) and p-nitrophenyl phosphate substrate (Sigma). The absorbance at 405 and 490nm was measured using a Multiscan MS plate reader (Labsystems, Basingstoke, UK). Cytokine levels were calculated by interpolation from a standard curve generated by incubating duplicate wells with doubling dilutions of recombinant human IFN-γ or IL-17. Results are presented as the mean cytokine concentration in triplicate wells.

Antigens and mitogens

A complete panel of 42 15-mer peptides, with five amino acid overlaps, was synthesized[14] (Pepceuticals Limited, Nottingham UK), corresponding to the sequence of the 30 kD Rh protein associated with expression of the D blood group antigen.[28,29] Peptides were checked for purity by mass spectrometry and used to stimu-

late PBMC cultures at a final concentration of 20 μg/mL.

Fresh, washed RBC were added to PBMC cultures to stimulate T-cell responses at the previously determined optimum concentration of 5×10^6 RBC/mL.[30-32]

The nominal antigen *Mycobacterium tuberculosis* purified protein derivative (PPD; Statens Seruminstitut, Denmark) was used as a positive control T-cell stimulus and added to cultures at a final concentration of 10 μg/mL. PPD readily invokes recall T-cell responses in a high proportion of UK citizens[15] as most have been immunized with the Bacilli Calmette-Guérin vaccine.

Isolation and culture of peripheral blood mononuclear cells

PBMC were isolated from fresh whole blood by density gradient centrifugation (Lymphoprep 1077; Nycomed Denmark). The viability of the PBMC was greater than 90%, as confirmed by trypan blue staining. As previously described,[14,19] PBMC were cultured at a final concentration of 1.25×10^6 cells/mL in the alpha modification of Eagles medium (α-MEM; Gibco/Invitrogen, Paisley, UK) supplemented with 1% 2 mM L-glutamine (Invitrogen), 2% 20 mM HEPES buffer (Sigma), 2% penicillin streptomycin (Invitrogen) and 5% autologous serum. PBMC were incubated with peptides or control stimuli for 5 days at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Measurement of helper T-cell cytokine production

The production of IFN-γ and IL-17A in cultures was measured by a highly sensitive cellular ELISA[19,33] using the same antibody pairs and standards as those used for determining serum levels (Pharmingen and eBioscience). Briefly, 5 days after stimulation with antigen, PBMC cultures were transferred into duplicate wells in microtiter plates (Nunc) coated with the respective monoclonal anti-cytokine capture antibody. After incubation with the paired detection antibody, wells were developed and concentrations determined as described for serum ELISA. Results are presented as the mean cytokine concentration in duplicate wells, or as stimulation index (SI), expressing the ratio of mean concentration in stimulated *versus* unstimulated control cultures. An SI>2.0 is interpreted as representing a significant positive response.[19,33]

Statistical analysis

The non-parametric Mann-Whitney U test and Spearman's rank correlation were performed utilizing Minitab 15 software (Minitab Ltd., Coventry, UK), with the level for statistical significance taken as $P<0.05$ (two-tailed).

Results

Interferon-γ and interleukin-17 levels in sera of patients with autoimmune hemolytic anemia and healthy control donors

The first step towards determining whether it is Th1 or Th17 responsiveness that is more strongly associated with AIHA was to compare the levels of the respective signature cytokines, IFN-γ and IL-17A, in the sera from patients with AIHA and from matched healthy donors. Sera were obtained at diagnosis before treatment, or when receiving only low to moderate doses of corticosteroids. It can be seen from Figure 1 that, while there is a non-significant ($P=0.17$; Mann-Whitney U test) trend for higher levels of IFN-γ in the sera of patients than in healthy donors, elevated IL-17A concentrations are very strongly associated with the disease ($P<0.001$). Many factors determine the severity of anemia in AIHA,[4,34-36] but it was also asked whether the levels of either cytokine in the serum of patients were related to their blood hemoglobin levels. Figure 2 demonstrates that there was no clear relationship between concentrations of IFN-γ and hemoglobin, but that IL-17A levels were significantly higher in the more anemic patients, exhibiting a significant inverse correlation with hemoglobin (Rs=-0.49; $P=0.034$).

Interferon-γ and interleukin-17 production by red blood cell-reactive T cells from patients with autoimmune hemolytic anemia and healthy control donors

The next question addressed was whether RBC-reactive T cells contributed to the production of IFN-γ or IL-17A in AIHA patients. The initial approach was to test the ability of PBMC from patients or healthy control donors to produce each cytokine in response to stimulation with autologous RBC. Intact RBC have previously been demonstrated to act as a very effective source of antigen for stimulating T-cell responses *in vitro*.[30-32] Representative results from three AIHA patients are illustrated in Figure 3A and demonstrate that, typically, IL-17A predominated over IFN-γ in these autoimmune Th responses. The data obtained by stimulating PBMC with autologous RBC are summarized in Figure 3B, which compares IFN-γ and IL-17A responses in patients with active (hemoglobin <120 g/L) or low-grade (hemoglobin >120 g/L) hemolysis and from healthy control donors. Although positive IFN-γ responses (SI>2) to RBC autoantigen were commoner in patients with more severe anemia (n=7) than in those with low-grade hemolysis (n=7) or in healthy donors (n=7), the differences in cytokine production

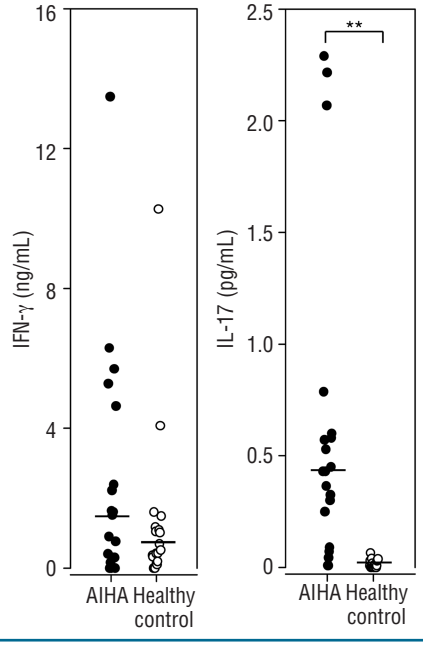

Figure 1. Serum IFN-γ and IL-17 levels in patients with AIHA compared to those in healthy donors Levels of IFN-γ and IL-17A were measured in serum samples taken from patients with AIHA (n=20) and matched healthy control donors (n=19). Bars indicate median values. There is no significant difference between the levels of IFN-γ in the two groups, but there is a significant increase in serum IL-17A in AIHA patients compared to healthy donors (**Mann-Whitney U test $P<0.001$). Where more than one serum sample was taken on different occasions from patients or donors, mean values are used.

were not statistically significant (P=0.17 *versus* patients with low-grade hemolysis; P=0.26 *versus* healthy controls; Mann-Whitney U test). In contrast, PBMC from all patients with active hemolysis exhibited strong IL-17A responses, which were significantly higher than those in the other groups (P=0.017 *versus* patients with low-grade hemolysis; P<0.001 *versus* healthy controls; Mann-Whitney U test). There were no positive (SI>2) IL-17A responses by PBMC to RBC in any of the healthy donors.

Interferon-γ and interleukin-17 production by red blood cell autoantigen-specific T cells from patients with autoimmune hemolytic anemia

The Rh proteins are major targets for autoantibodies and autoreactive Th cells in most cases of AIHA.[12-14,19] The final set of experiments determined whether the responses of RhD protein-specific autoreactive T cells from patients commonly include production of IL-17A, in addition to the Th1 cytokine IFN-γ as previously reported.[19] When PBMC were stimulated with a panel of peptides spanning the RhD protein, one or more sequences elicited IL-17A secretion in nine out of 18 AIHA patients tested. There was no such prominent IL-17A production by patients' PBMC responding to the control recall antigen mycobacterial PPD. Consistent with earlier studies, IFN-γ responses to the panel were also frequent (in 11 of 18 patients), but were not necessarily stimulated by the same peptides as those that induced IL-17A. Representative positive results are illustrated in Figure 4 A-B and all the data are summarized in Figure 4C, which depicts the proportions of the patients whose PBMC responded to each of the RhD peptides by producing IFN-γ or IL-17A. Typically, multiple sequences elicit each type of response,

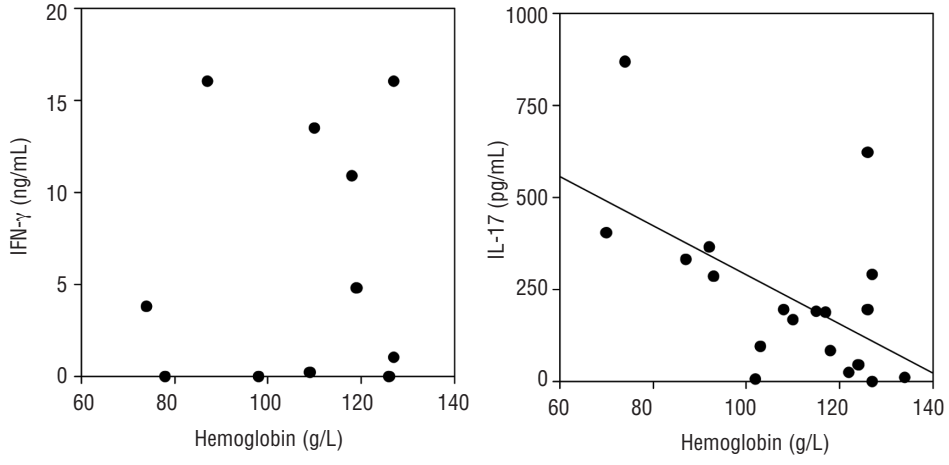

Figure 2. Relationships between degree of anemia and serum IFN-γ and IL-17 concentrations in patients with AIHA. The concentrations of serum IFN-γ (n=11) and IL-17A (n=19) in patients with AIHA were compared to hemoglobin levels at the time of blood donation as an indicator of the degree of anemia. There is no significant association between the concentrations of hemoglobin and IFN-γ (Rs=0.19; P=0.55) but hemoglobin and IL-17A levels demonstrate a significant inverse correlation (Rs= -0.49; P=0.034).

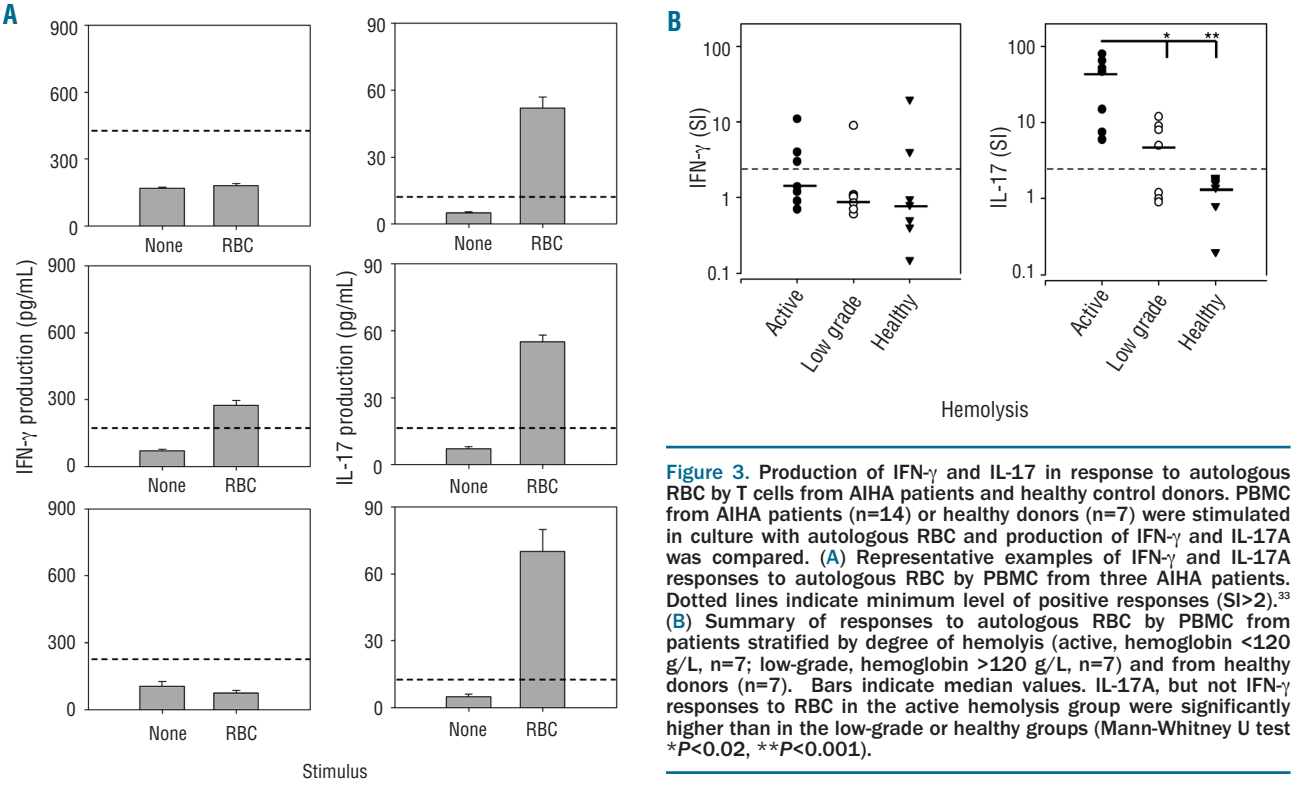

Figure 3. Production of IFN-γ and IL-17 in response to autologous RBC by T cells from AIHA patients and healthy control donors. PBMC from AIHA patients (n=14) or healthy donors (n=7) were stimulated in culture with autologous RBC and production of IFN-γ and IL-17A was compared. (A) Representative examples of IFN-γ and IL-17A responses to autologous RBC by PBMC from three AIHA patients. Dotted lines indicate minimum level of positive responses (SI>2).[33] (B) Summary of responses to autologous RBC by PBMC from patients stratified by degree of hemolyis (active, hemoglobin <120 g/L, n=7; low-grade, hemoglobin >120 g/L, n=7) and from healthy donors (n=7). Bars indicate median values. IL-17A, but not IFN-γ responses to RBC in the active hemolysis group were significantly higher than in the low-grade or healthy groups (Mann-Whitney U test *P<0.02, **P<0.001).

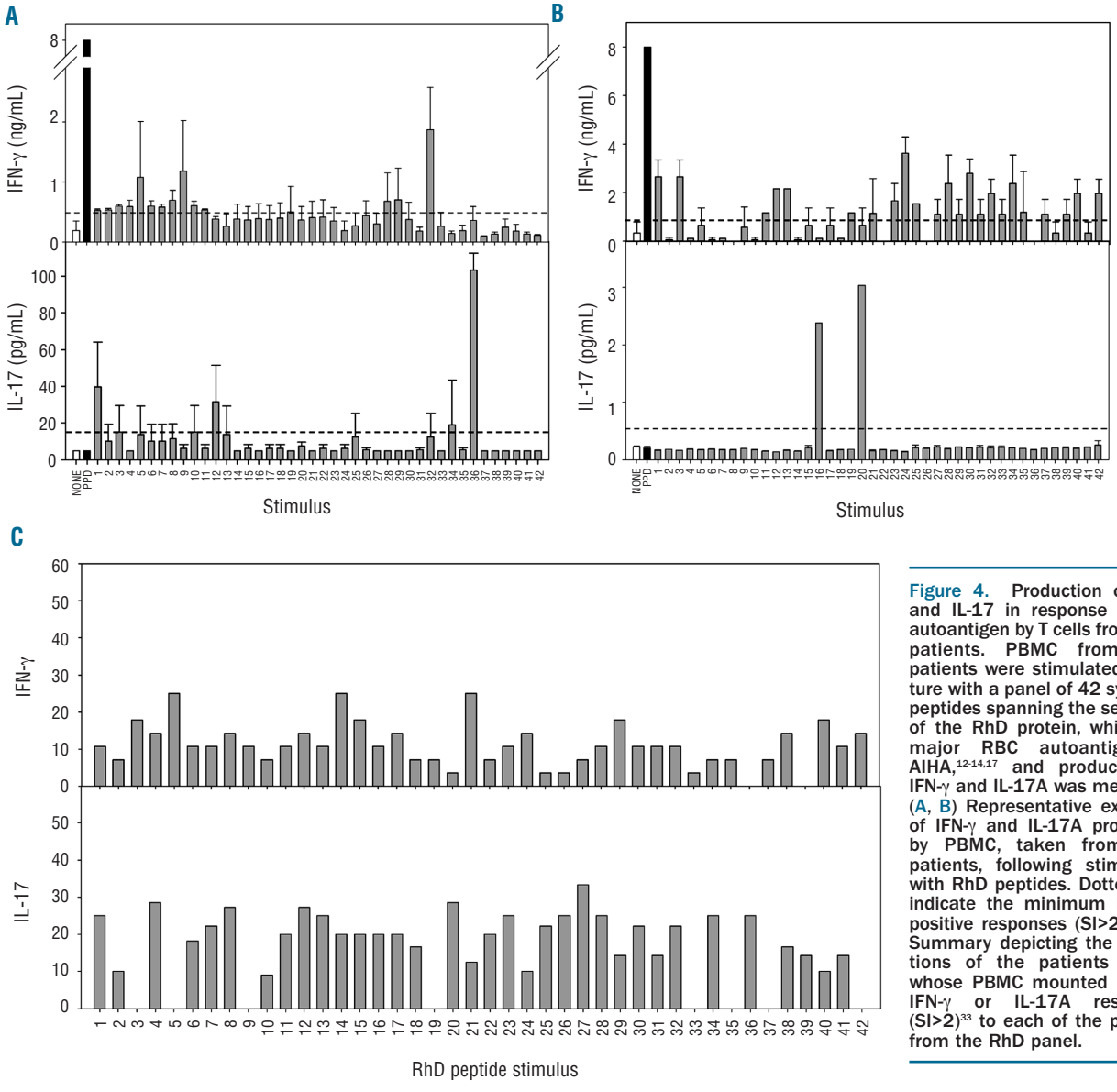

Figure 4. Production of IFN-γ and IL-17 in response to RhD autoantigen by T cells from AIHA patients. PBMC from AIHA patients were stimulated in culture with a panel of 42 synthetic peptides spanning the sequence of the RhD protein, which is a major RBC autoantigen in AIHA,[12-14,17] and production of IFN-γ and IL-17A was measured. (A, B) Representative examples of IFN-γ and IL-17A production by PBMC, taken from AIHA patients, following stimulation with RhD peptides. Dotted lines indicate the minimum level of positive responses (SI>2).[30] (C) Summary depicting the proportions of the patients (n=13) whose PBMC mounted positive IFN-γ or IL-17A responses (SI>2)[33] to each of the peptides from the RhD panel.

and, although their identities can vary widely between individuals, particular peptides are stimulatory in up to one third of patients.

Discussion

The main finding reported here is that production of the signature Th17 cytokine IL-17A is more strongly associated with AIHA than are Th1 IFN-γ responses, which have previously been described in the disease. Not only were the levels of IL-17A, but not IFN-γ, significantly increased in the serum of patients *versus* healthy control donors, but IL-17A was the more prominent cytokine in the responses of patients' T cells to autologous RBC used as a source of antigen. It was also confirmed that a specific autoantigen, the RhD protein,[12-14,19] contains epitopes that elicit such IL-17A responses. These results raise the possibility that autoreactive Th17 cells contribute to pathogenic helper T-cell activity in AIHA, and are responsible for at least some of the effects previously attributed to the Th1 subset.

Following the recognition that Th17 cells constitute a distinct helper subpopulation, the pathogenesis of many inflammatory immune diseases has been re-examined and it has become clear that they, rather than Th1 cells, can be the major drivers of pathology.[26,27] However, until now it has remained open as to whether they can also play a direct role in autoimmune diseases, such as AIHA, which are purely antibody-mediated. In AIHA, the focus has been on the respective pathogenic and protective properties of the Th1 and Th2 cytokines IFN-γ and IL-4, mediated via differential effects on antibody class switching.[5,8,11] By contrast, the inflammatory Th17 cytokine IL-17A was thought to be irrelevant for stimulation of B-cell responses. However, recent studies in mice have demonstrated that Th17 cells can indeed function as helpers for antibody responses, able to stimulate B cells to proliferate, switch class to IgG production and form germinal centers.[37] Although some of these properties could be partially attributed to co-production of additional Th17 cytokines such as IL-21, they were also shown to be highly dependent on IL-17 itself. In particular, IL-17, like IFN-γ, was found to be responsible for switching to IgG_{2a}[37] which may be the most potent murine isotype in

mediating clearance of opsonized cells by macrophages.[5,11,38] Taken together with our human data, this work suggests a model whereby IL-17A from autoreactive Th17 responses can augment, or potentially substitute, the Th1 cytokine IFN-γ in providing help for B cells producing pathogenic autoantibody in AIHA. We measured only the signature cytokine IL-17A, so we cannot exclude that this is a marker for other helper mechanisms mediated by Th17 cells, rather than acting as a key mediator in its own right. In this regard, Th17 cells share several features with follicular helper T cells that can also drive germinal center formation and class switching, including expression of ICOS, IL-21 and the transcription factor c-Maf.[39] It also remains possible that the elevated levels of IL-17A in both sera and autoantigen-stimulated cultures, which were characteristic of AIHA patients, represent only markers of chronic immune activation and play no direct role in the disease. In this scenario, human patients would resemble IL-2 knockout mice that develop AIHA with an increase in IL-17-producing Th cells, but in which elimination of IL-17 does not confer protection.[40] However, the closer associations of IL-17A than IFN-γ with AIHA cannot easily be explained by generalized immune activation, and the potent ability of IL-17A to provide B-cell help in mice[37] provides the mechanism to contribute to pathogenesis. This argument is further strengthened by recent evidence that Th17 cells can provide B-cell help in murine autoantibody-induced arthritis[41] and that B-cell activity in patients with acute viral myocarditis correlated positively with IL-17, but not IFN-γ.[42] Increased levels of IL-17 and Th17 cells have also been reported in the blood of patients with autoimmune thrombocytopenia[43,44] supporting the view that the Th17 subset contributes to pathology in a variety of autoantibody-mediated diseases.

Multiple, complex factors influence the degree of anemia in AIHA, including activation state of the reticulo-endothelial system, titer, isotype and glycosylation of autoantibodies, total serum IgG levels and rate of erythropoiesis.[4,34-36,38,45] It was, therefore, noteworthy that the levels of a particular Th cytokine, IL-17A, and its production by autoreactive T cells, were not only associated with AIHA but correlated significantly with the severity of the disease. There are precedents for factors related to the T-cell response, which could be considered upstream of the production and hemolytic effects of antibodies, influencing the degree of anemia. For example, we have previously reported that low regulatory T-cell activity can be associated with more severe AIHA.[19] Further evidence for the effects of apparently subtle variations in the Th response on anti-RBC antibody production is provided by the correlation between the number of stimulatory helper epitopes and the titer of anti-D in alloimmunized D-negative donors.[46]

In AIHA, both the Th cytokines IL-17A and IFN-γ were produced in response to a range of different epitopes from the RhD autoantigen. Most, but not all, patients demonstrated these types of effector activity against RhD sequences: those who were unresponsive may either have been sampled during periods of the disease when autoaggressive Th cells were regulated,[19] or represent cases in which other autoantigens contribute to help.[14] The current study supports an emerging model whereby IL-17 is important in providing help for pathogenic B cells, but Th and autoantibody epitopes, including those from RBC, are not necessarily found on the same autoantigen.[14] Thus, RhD peptide-specific Th17 responses may not be required for production of autoantibody to RhD protein. Variation between patients in the identities of the autoantigenic peptides that elicit responses has been described previously.[14,19] Such differences have been attributed to variations in the HLA type of the individuals,[19] and reflect the ability of particular class II molecules to bind and present each peptide.[47] Conversely, the mapping of some peptides that can stimulate responses in several patients may be due to promiscuity for binding different class II molecules, and to processing mechanisms that can constrain the sequences available for presentation.[47,48] The observation that Th cells producing distinct cytokines, in this instance IFN-γ and IL-17A, may recognize different epitopes parallels earlier analyses of AIHA[19] and is supported by studies of T-cell responses to islet cell autoantigen in a murine model of non-obese diabetes.[49] The reasons why different epitopes tend to elicit particular types of response remain unclear but may be related to the affinities of the corresponding trimolecular interactions between antigenic peptide, MHC molecule and T-cell receptor.[50]

The evidence presented here for Th17 cells playing a pathogenic role in AIHA has important implications for therapy. First, it raises the possibility that blocking the activity of the Th17 pathway may be a complementary or more effective treatment strategy than inhibiting Th1 responses. Targeting the two subsets need not be mutually exclusive since, for example, the biologics ustekinumab and ABT-874 that block the p40 subunit shared by IL-12 and IL-23 are capable of interfering with the development of both Th1 and Th17 cells, and have been successful in treating inflammatory diseases such as psoriasis.[51,52] Alternatively, there is considerable interest in re-inducing tolerance to the specific autoantigenic peptides that drive pathogenic Th cell responses[8,53] and the current work indicates that therapies of this type for AIHA should include epitopes that induce Th17 cytokine as targets.

Authorship and Disclosures

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