

T Cell Response to Epstein-Barr Virus Transactivators in Chronic Rheumatoid Arthritis

By Emmanuel Scotet,* Jacques David-Ameline,* Marie-Alix Peyrat,* Agnès Moreau-Aubry,* Delphine Pinczon,* Annick Lim,† Jos Even,‡ Gilbert Semana,§ Jean-Marie Berthelot,|| Richard Breathnach,* Marc Bonneville,* and Elisabeth Houssaint*

From *Institut National de la Santé et de la Recherche Médicale U211, Institut de Biologie, 44035 Nantes Cedex 01; †Institut Pasteur, 75024 Paris; ‡Centre Régional de Transfusion Sanguine, 35000 Rennes; and §Service de Rhumatologie, CHU, 44035 Nantes Cedex 01, France.

Summary

Rheumatoid arthritis is a multistep disorder associated with autoimmune features of yet unknown etiology. Implication of viruses such as Epstein-Barr virus (EBV) in rheumatoid arthritis pathogenesis has been suspected on the basis of several indirect observations, but thus far, a direct link between EBV and rheumatoid arthritis has not been provided. Here we show that a large fraction of T cells infiltrating affected joints from a patient with chronic rheumatoid arthritis recognizes two EBV transactivators (BZLF1 and BMLF1) in a major histocompatibility complex-restricted fashion. Responses to these EBV antigens by synovial lymphocytes from several other chronic rheumatoid arthritis patients were readily detectable. Thus these results suggest a direct contribution of EBV to chronic rheumatoid arthritis pathogenesis. They also demonstrate for the first time the occurrence of T cell responses against EBV transactivating factors, which might be central in the control of virus reactivation.

Rheumatoid arthritis (RA)¹ is an incapacitating disease characterized by chronic joint inflammation and progressive destruction of articular structures, which affects about 1% of the population worldwide (1). Its etiology remains unresolved, but there is ample evidence that T cells contribute directly to RA pathogenesis. This is supported in particular by (a) the widespread T cell infiltration of affected synovial membranes from RA patients (2), (b) the genetic predisposition of RA, which is associated with certain MHC class II haplotypes (3–7), and (c) the beneficial effects of T cell-depleting treatments in RA patients (8, 9). Despite this, the specificity of T cells infiltrating RA lesions and their pathogenicity at late stages of the disease remain obscure. With respect to the first issue, several candidate antigens recognized by synovial T cells have been proposed. For instance, a pathogenic role of T cell-mediated immune responses against connective tissue proteins such as type II collagen is supported by the occurrence of increased proliferative responses to these Ag of synovial T cells as compared to PBL in RA patients (10, 11). Besides endogenous compounds, several arthritogenic Ag of mycobacterial

origin, such as stress proteins, have been shown to elicit synovial T cell responses in some RA patients, although the importance of stress proteins in synovial T cell-mediated responses against mycobacteria still remains controversial (12–15). Furthermore, a variety of viruses have been proposed as RA causative agents (1). In particular, EBV has long been linked to RA on theoretical grounds (e.g., because of the existence of shared epitopes between EBV proteins and RA autoantigens) and from clinical studies suggesting in particular increased anti-EBV serological responses in a fraction of RA patients (16, 17). However the physiopathological relevance of these observations is still unclear, particularly because no evidence for anti-EBV responses within RA lesions have been provided thus far. The pathogenicity of T cells at late stages of RA is also a controversial issue. On the one hand, both the extensive diversity of the TCRs expressed by synovial lymphocytes isolated from most chronic RA patients (18) and the evidence for joint destruction and/or active synovitis in the absence of local lymphoid infiltrate in animal models and in humans (19) argues against a major role of T cells in the perpetuation of RA. On the other hand, a specific Ag-driven T cell recruitment to the joints at late stages of RA is indirectly supported by studies demonstrating oligoclonal T cell expansions within the affected joints of several chronic RA patients (18).

¹Abbreviations used in this paper: BLC, B lymphoblastoid cells; RA, rheumatoid arthritis; SFL, synovial fluid lymphocytes; SML, synovial membrane-derived lymphocytes.

Table 1. TCR Features, Frequency, and HLA Restriction of Autologous BLC-reactive Synovial T Cell Clones Derived from Chronic RA Patients 1 and 19

T cell clone	TCR BVBJ composition	CDR3 β length (aa)	Percentage of SFL*	Percentage of PBL*	HLA restriction
Patient 1					
A2.10	BV2BJ1S2	8	5.6	<0.2	A*0201
A2.19	BV2BJ2S3	8	1.5	<0.2	B*4002
A2.3	BV2BJ2S7	8	4.0	<0.2	B*4002
A2.8	BV2BJ2S7	8	4.0	<0.2	B*4002
A2.1	BV2BJ2S7	8	4.0	<0.2	B*4002
A2.21	BV2BJ1S3	9	<0.2	<0.2	B*4002
A2.4	BV2BJ2S5	10	2.0	<0.2	B*4002
A14.11	BV14BJ2S7	9	<0.2	<0.2	B*4002
A14.7	BV14BJ2S3	11	8.8	<0.2	B*4002
A22.28	BV22BJ2S1	8	1.5	<0.2	B*4002
A22.19	BV22BJ2S2	8	1.3	<0.2	B*4002
A22.13	BV22BJ2S7	8	1.0	<0.2	B*4002
A22.32	BV22BJ2S7	9	<0.2	<0.2	B*4002
A22.34	BV22BJ2S2	12	<0.2	<0.2	B*4002
A22.18	BV22BJ2S3	12	<0.2	<0.2	B*4002
A17.10	BV17BJ2S3	8	0.3	<0.2	Cw*0102
A17.11	BV17BJ2S4	8	0.4	<0.2	C*0102
Patient 19					
B1	ND	ND	ND	ND	A*2401
B2	ND	ND	ND	ND	B*3501
B3	ND	ND	ND	ND	B*3501
B4	ND	ND	ND	ND	B*3501

All T cell clones listed in this table expressed distinct TCRs as demonstrated by junctional sequence analysis (20).

The T cell clones listed here represented 19 of 20 $V\beta 2^+$, 5 of 10 $V\beta 14^+$, 4 of 7 $V\beta 17^+$, and 14 of 14 $V\beta 22^+$ $CD8^+$ T cell colonies isolated from patient 1 SFL and 4 of 12 $CD8^+$ T cell colonies isolated from patient 19 (20).

*Percentage of SFL or PBL expressing TCR- β chains with the same VJ combination and the same length as that of the reactive T cell clone, estimated by semiquantitative PCR analysis (20).

In an attempt to evaluate the repertoire diversity and specificity of T cells activated during chronic RA, we recently studied the specificity of synovial T lymphocytes derived from patients with typical long lasting chronic RA. Because in several patients the majority of synovial T cells were $CD8^+$, both $CD4^+$ cells, which presumably comprise T cells reactive against RA susceptibility HLA class II alleles, and $CD8^+$ cells were analyzed. $CD4^+$ and/or $CD8^+$ synovial T cell responses towards autologous and/or allogeneic B lymphoblastoid cells (BLC) expressing one or several autologous HLA alleles were detected in four of six patients studied (David-Ameline, J., M.A. Peyrat, and M. Bonneville, unpublished results). Furthermore, in two patients, an enrichment for autologous BLC-reactive cells among synovial T cells was evidenced by comparative analyses of patients' PBL and synovial T cells (20; results summarized in Table 1). Together, these results suggested frequent expansions of cells reactive against autologous BLC during chronic RA. Here we make use of this material to characterize by

an expression cloning approach the antigens recognized by synovial T cells derived from these patients. A dominant response of synovial T cells to two EBV transactivators is demonstrated, thus suggesting an Ag-driven recruitment of T cells to the synovium in these patients and a possible role of these EBV-specific T cells in chronic RA pathogenesis. More generally, this study provides the first clear-cut evidence for T cell responses against EBV proteins playing a key function during virus replication. Such responses might have a central role in the control of virus spreading under physiological and pathological situations.

Materials and Methods

Patients

Patient 1 (HLA-A*0201/02, -B*2705/4002, -Cw*0102/15, DRB1*0101/0401, DQB1*0301/0501) had a typical erosive RA lasting for 6 yr at the time of the first sample collection. He fulfilled six of seven criteria for RA defined by Arnett et al. (21).

Polyarthritis remained active during the whole follow-up period. This patient received low-dose prednisone (7–12 mg/d) throughout. Synovial fluid lymphocytes (SFL) were obtained during synovial fluid analysis before steroid infiltrations. Synovial membrane lymphocytes (SML) were obtained during surgery, performed 27 mo after collection of SFL. All other patients fulfilled at least four out of seven criteria for RA (21). Anti-EBV serology was studied in patients 1, 5, and 6. All had serum antibodies against at least one of the following EBV proteins: RA nuclear antigen, Epstein-Barr nuclear antigen 1, early antigens, and viral capsid antigens. These patients showed dramatically elevated titers of serum antibodies against early antigens (range 160 to >640).

Cells

PBL and SFL were maintained in RPMI 1640 supplemented with 10% human serum, 1 mM L-glutamine (hereafter referred to as culture medium), and recombinant IL-2 (100 IU/ml). T cell clones were isolated and restimulated once a month as follows. Synovial T cells (either total or sorted by means of TCR BV region-specific mAb) were seeded at 0.3 cells/well and cultured in culture medium supplemented with IL-2 (100 IU/ml), purified PHA (leucoagglutinin, 0.5 µg/ml) and irradiated (30 Gy) allogeneic feeder cells. Such culture conditions allow the growth of virtually all T cells and do not introduce any bias in the T cell repertoire, as demonstrated by flow cytometry analysis using TCR BV region-specific mAb and by analysis of TCR CDR3 length distribution (22; Lim, A., M.A. Peyrat, and M. Bonneville, unpublished data). Coreceptor phenotype, TCR features, and HLA restriction of T cell clones are detailed elsewhere (20) and summarized in Table 1.

Expression Cloning of T Cell Antigens

Construction of the cDNA Library. Poly(A)⁺ RNA was prepared from patient 1 BLC using an mRNA purification kit (Pharmacia Fine Chemicals, Piscataway, NJ). cDNAs were synthesized using a Zap-cDNA synthesis kit (Stratagene Inc., La Jolla, CA). BstXI adaptors (Invitrogen, San Diego, CA) were ligated to the cDNAs. After size fractionation, cDNAs were cloned into pcDNA3 (Invitrogen) digested with BstXI. Recombinant plasmids were electroporated into *Escherichia coli* XI-1 and selected with ampicillin (50 µg/ml). The library was divided into 288 pools of 200 cDNA clones. Each pool was amplified to saturation, and plasmid DNA was extracted by the alkaline lysis method.

Transfection of COS Cells. Transfection was performed by the DEAE-dextran-chloroquine method as described (23, 24). In brief, 1.5×10^4 COS cells were transfected with 100 ng of plasmid containing the relevant HLA DNA and 100 ng of a pool of the cDNA library. The genomic HLA-A*0101, which was kindly provided by Dr. J. Girdlestone (Medical Research Council, Cambridge, UK), was cloned into pcDNA3. Genomic HLA-A*0201 DNA, HLA-A*2401 cDNA, and HLA-A*0301 were kindly provided by T. Boon (Ludwig Institute, Brussels, Belgium). HLA-A*0201 was cloned into pSV2 vector, HLA-A*2401 and A*0301 were in pcDNA3. The cDNA HLA-B*3501, which was kindly provided by Dr. L. Satz (Laboratory of Immunogenetics, Hospital de Clinicas Jose de San Martin, Buenos Aires, Argentina), was cloned into pcDNA3. HLA-B*4002 and Cw*0102 were cloned from the B cell cDNA library. Transfectants were then tested for their ability to stimulate TNF production by T cells (see below). Each positive pool was subcloned into 800–1,600 bacteria, which were each tested for their ability to trigger T cell clone TNF release after expression in COS cells cotransfected with relevant

HLA DNA. Positive clones were sequenced by the dideoxy chain termination method.

T Cell Stimulation Assay. T cells were added to COS cells 24–48 h after transfection, and culture supernatants were harvested 6–18 h later and tested for TNF content by measuring culture supernatant cytotoxicity to WEHI164 clone 13 in a colorimetric assay (25).

Production of Truncated BMLF1 or BZLF1 cDNA. Truncated BMLF1 cDNA fragments, generated by partial digests, were cloned into pcDNA3. pcDNA3 vector was also used to clone a 140-bp fragment after PCR amplification of the Δ SacII BMLF1 fragment with oligonucleotides BM1 (5'-GCGGATCCGCCACCATG-3'), introducing a BamHI site for cloning and an internal ATG, and SP6 (5'-ATTTAGGTGACACTATAG-3'). Truncated BZLF1 cDNA fragments, generated by partial digests, were cloned into pcDNA3.

Peptide Assays

Lyophilized peptides (Chiron Mimotopes Corp., Victoria, Australia) were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid, and stored at -80°C . In cytotoxicity assays, 2.5×10^4 ⁵¹Cr-labeled A2.10 T cells were incubated in the presence of peptides at various concentrations, and ⁵¹Cr release was measured after 3 h at 37°C. Percentage of specific lysis was calculated as described in (22). In TNF release assays, 2.5×10^4 T cells were incubated for 5 h with the peptides at various concentrations, and the amount of TNF released in the supernatant was estimated by the WEHI 164 cytotoxicity assay.

T Cell Cytotoxicity and Proliferation Assays

Both kinds of assays were performed as previously described (22). In brief, cytotoxic activity of T cell clones toward BLC was measured by a standard 4-h ⁵¹Cr-release assay at two E/T ratios. T cell clone cytolytic potential was estimated by lectin-dependent killing of target cells (i.e., by evaluating T cell cytotoxic activity in the culture medium supplemented with 0.5 µg/ml of purified PHA [leucoagglutinin]). Proliferative activity of responder T cells was estimated after a 48-h culture of 10^4 responder cells with 2.5×10^4 irradiated BLC in 100 µl of culture medium supplemented with rIL-2, followed by an overnight pulse with 0.5 µCi of tritiated thymidine.

COS Transfection Assays Using Polydonal T Cell Lines

Patients' PBL, SFL, or SML were cultured for 15 d in culture medium supplemented with rIL-2 and leucoagglutinin. Under such culture conditions, no TCR repertoire skewings were introduced, as suggested by comparative analysis of TCR BV CDR3 length distribution of some synovial samples before and after culture (Lim, A., M.A. Peyrat, and M. Bonneville, unpublished observations). After washing, cells were maintained for 7 d in culture medium with rIL-2 but without lectin, to decrease spontaneous TNF release. TNF secretion in culture supernatant was then estimated as described above, after incubating for 6 h varying numbers of responder T cells (from 10^3 to 10^5) together with confluent COS cells transfected 24 h earlier with HLA and/or EBV cDNAs.

Results

We recently showed that a large fraction of synovial T cell clones derived from two patients (hereafter referred to as patients 1 and 19) proliferated in vitro when exposed to au-

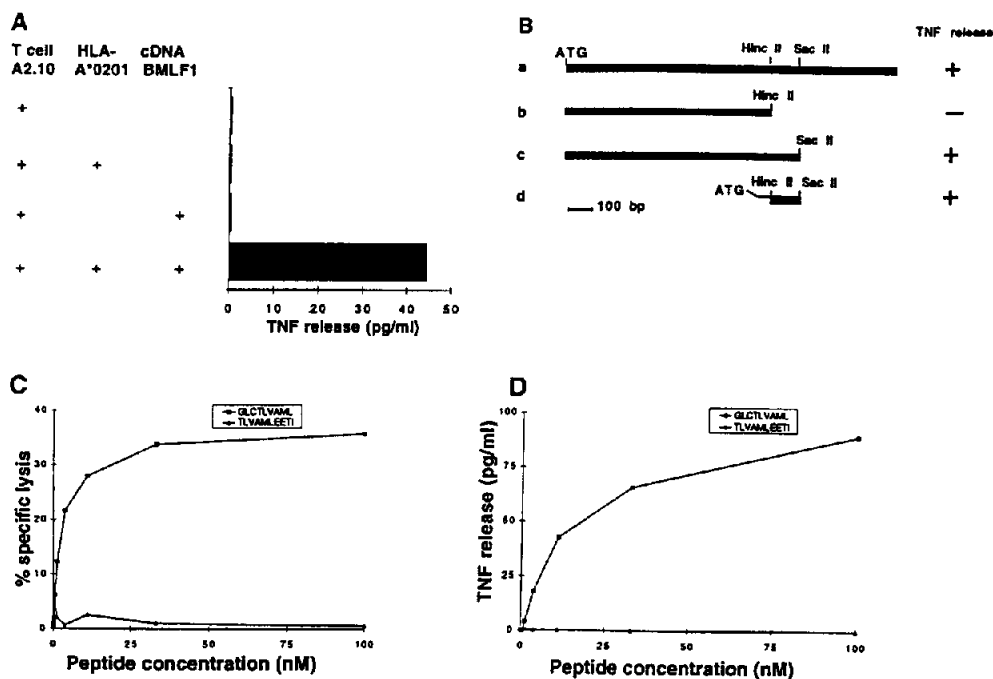


Figure 1. Identification of the antigenic peptide recognized by the synovial T cell clone A2.10. (A) BLMF1 recognition by the HLA-A*0201-restricted T cell clone A2.10. A2.10 T cells were added to COS cells transfected with BLMF1 cDNA and HLA-A*0201 DNA, or with each of these DNA. TNF production was assessed after a 6–18-h incubation as described (18). (B) Mapping of the BLMF1 sequence coding for the antigenic peptide recognized by clone A2.10. BLMF1 cDNA fragments were cloned into pcDNA3 and tested for their ability to induce A2.10 TNF production after transfection into COS cells together with HLA-A*0201. +, A2.10 TNF release >22 pg/ml. -, A2.10 TNF release <1 pg/ml. (C) Induction of A2.10 clone autotoxicity by synthetic BLMF1 peptides. 10 peptides (5 9-mers, 4 10-mers, and 1 11-

mer) encoded by the 140 bp fragment defined in B and containing consensus binding "anchor" residues to HLA-A*0201 were synthesized. Significant T cell clone lysis was observed with peptide GLCTLVAML but with none of the 9 other peptides tested. Data obtained with peptide TLVAMLEETI are shown as a negative example. (D) Induction of A2.10 clone TNF release by synthetic BLMF1 peptides. Peptide GLCTLVAML but none of the 9 other peptides tested stimulated production of TNF by clone A2.10. Peptide TLVAMLEETI is shown as a negative control.

tologous BLC (20). The fact that autologous BLC-reactive clones were found in both CD4⁺ and CD8⁺ T cell subsets suggested that this reactivity was a general feature of synovial T cells, regardless of their coreceptor phenotype (20). In an attempt to establish the fine specificity of these cells, cDNAs from a patient 1 BLC library were screened for their ability to trigger TNF production by synovial T cell clones after transient cotransfection into COS cells together with DNAs encoding the appropriate MHC allele (23, 24). Because this approach is well suited for cloning MHC class I- but not class II-restricted Ags (23), this analysis focused on the characterization of Ag recognized by CD8⁺ synovial T cell clones.

Recognition of the EBV Transactivator BLMF1 by the Synovial T Cell Clone A2.10. A T cell clone from patient 1, A2.10, recognizing autologous BLC in an HLA-A*0201-restricted fashion (Table 1), was analyzed first. Using the COS transfection approach (23, 24), we identified 2 out of 270 cDNA pools that induced TNF production by clone A2.10 after cotransfection with HLA-A*0201 DNA. Out of 800 clones derived from one positive pool, one 1,591-bp cDNA induced TNF production by A2.10 cells when cotransfected with HLA-A*0201 DNA (Fig. 1 A). Its sequence was identical to that of an EBV gene, BLMF1, encoding a 439-amino acid protein expressed during the early stage of the virus replicative cycle (26, 27). Because of the transactivating properties of the BLMF1 product (26), this protein could upregulate the expression of endogenous Ag recognized in turn by the synovial T cell clone rather than being

recognized itself. To address this question, truncated BLMF1 cDNAs were tested for their ability to activate A2.10 TNF release after cotransfection with HLA-A*0201 DNA. A BLMF1 region encoded by a HincII/SacII 140-bp sequence was shown to be stimulatory (Fig. 1 B). Of several peptides located in this region and synthesized on the basis of their consensus binding capacities to HLA-A*0201 (28), only the nonapeptide GLCTLVAML (amino acids 259–267) induced autotoxicity and TNF release by A2.10 cells (Fig. 1, C and D). This formally demonstrates a classic MHC-restricted recognition of the BLMF1 protein.

Recognition of the EBV Transactivator BZLF1 by the Synovial T Cell Clone A17.11. The antigenic specificity of another synovial T cell clone from patient 1, A17.11, which recognized autologous BLC in an HLA-Cw*0102-restricted fashion (Table 1), was then studied. Because this clone did not react with COS cells cotransfected with BLMF1 and HLA-Cw*0102 cDNAs, the cloning strategy described above was applied once again (23). One cDNA, which conferred recognition of COS cells by A17.11 cells after cotransfection with HLA-Cw*0102 DNA (Fig. 2), contained a 753-bp cDNA insert encoding BZLF1, another early EBV protein with transactivating properties (29).

Dominant Response of Synovial T Cells from Patient 1 to EBV Transactivators. We then tested the reactivity toward BLMF1- or BZLF1-transfected COS cells of the whole panel of BLC 1-reactive CD8⁺ synovial T cell clones with a known HLA restriction element (A*0201, B*4002, or Cw*0102) and expressing distinct T cell receptors (Table 1).

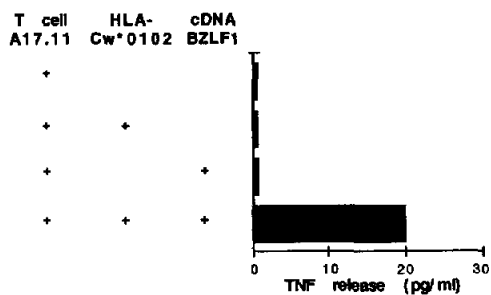


Figure 2. BZLF1 recognition by the HLA-Cw*0102-restricted T cell clone A17.11. TNF released by T cell clone A17.11 was measured after incubation with COS cells transfected with BZLF1 and HLA-Cw*0102 cDNA or with each of these cDNA. The experimental protocol is the same as described in the legend to Fig. 1.

Whereas one other clone reacted with BZLF1 in the context of HLA-Cw*0102 (clone A17.10, Table 2), most T cell clones recognized BZLF1 in the context of HLA-B*4002 (representative data shown in Fig. 3 A and summarized in Table 2). The characterization of T cell clones recognizing BZLF1 in the context of HLA-B*4002, for which peptide anchor motifs are well defined (28), allowed us to determine whether, similarly to BMLF1, this transactivator was recognized as a peptidic antigen presented by MHC products. In support of this, BZLF1 cDNA fragments able to trigger TNF production by T cell clone A14.7 after cotransfection with HLA-B*4002 DNA into COS cells could be defined (Fig. 3 B), and a synthetic peptide located in the stimulatory BZLF1 region was shown to trigger T cell clone autotoxicity and TNF release (Fig. 3, C and D).

That there is an enrichment for T cells reacting against BZLF1/BMLF1 antigens within the inflamed joints of patient 1 is supported by two lines of evidence. First, T cells expressing TCR- β chains with V β /J β combinations and lengths identical to those expressed by EBV-reactive T cell clones accounted for ~30% of synovial T cells, but only a minute fraction of peripheral blood T cells from patient 1 (Table 1). Second, TNF production by short-term cultured synovial fluid and peripheral blood T cells from patient 1 induced by COS cells transfected with either HLA-A*0201, B*4002, or Cw*0102 DNAs together with either BZLF1 or BMLF1 cDNAs differ dramatically: compare the amounts of TNF released by synovial as compared to peripheral blood T cells after exposure to COS cells transfected with BMLF1 and A*0201 DNAs (Fig. 4, upper left) or BZLF1 and B*4002 DNAs (Fig. 4, lower middle). Significantly, dominant responses to BZLF1/B*4002 and BMLF1/A*0201 were seen with synovial membrane-derived lymphocytes recovered 27 mo after the first sample (Fig. 4), indicating that these reactivities are common and long-lasting features of synovial lymphocytes derived from patient 1.

T Cell Responses to BMLF1/BZLF1 in Other Chronic RA Patients. An enrichment for autologous BLC-reactive T cells within the synovium in another chronic RA patient (patient 19) was suggested by the fact that unlike PBL clones, 4 of 12 randomly chosen CD8⁺ synovial clones derived

Table 2. TNF Responses of Synovial T Cell Clones from Patient 1 to BMLF1- or BZLF1-transfected COS Cells

T cell clone	TNF release		
	BMLF1 A*0201	BZLF1	
		B*4002	Cw*0102
			pg/ml
A2.10	44.5	—	—
A2.19	—	45.8	—
A2.3	—	37.4	—
A2.8	—	39.5	—
A2.1	—	18.6	—
A2.21	—	30.8	—
A2.4	—	1.2	—
A14.11	—	6.0	—
A14.7	—	39.3	—
A22.28	—	13.0	—
A22.19	—	37.5	—
A22.13	—	25.0	—
A22.32	—	25.0	—
A22.34	—	25.0	—
A22.18	—	20.0	—
A17.10	—	—	20.0
A17.11	—	—	13.6

TNF production by T cell clones was estimated after incubation with COS cells transfected with either BMLF1 or BZLF1 cDNAs and either HLA-A*0201, -B*4002, or -Cw*0102 DNAs. Shown are the results obtained with HLA/Ag combinations yielding significant TNF production (0.5 pg/ml) by any of the T cell clones tested. —, TNF secretion <0.5 pg/ml.

from this patient reacted against the autologous BLC (20). Because the HLA restriction of these clones was established (3 clones were HLA-B*35 restricted and 1 clone was HLA-A*24 restricted) (Table 1), we could evaluate their reactivity toward BZLF1 and BMLF1 after transfection of the corresponding cDNA into COS cells together with HLA-A*2401 or -B*3501 cDNAs. Whereas the HLA-A*24-restricted clone reacted against neither BMLF1 nor BZLF1 (data not shown), all the HLA-B*35-restricted T cell clones turned out to recognize BZLF1 (Table 3).

T cell responses to BMLF1 and BZLF1 were also studied at the polyclonal level in patient 19 and in several HLA-A*02⁺ chronic RA patients. In agreement with the above results, a clear TNF response was detected when incubating short-term cultured synovial T cells from patient 19 with HLA-B*3501/BZLF1-transfected COS cells (Fig. 5, right). Moreover, comparison of the amounts of TNF released by PBL and SFL under these assay conditions strongly suggested an enrichment within the synovium for T cells reactive against BZLF1 in patient 19 (Fig. 5). Significant TNF

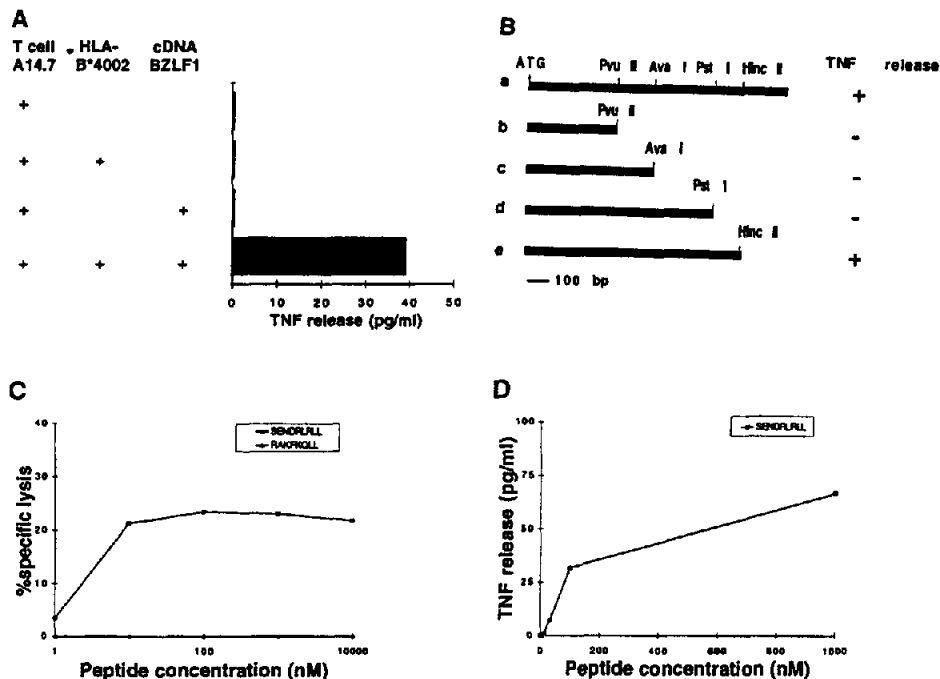


Figure 3. Identification of the BZLF1 peptide recognized by the T cell clone A14.7. (A) BZLF1 recognition by the HLA-B*4002-restricted T cell clone A14.7. TNF release by clone A14.7 was measured after incubation with COS cells transfected with BZLF1 and HLA-B*4002 cDNA or with each of these cDNA. (B) Mapping of the BZLF1 sequence coding for the antigenic peptide recognized by clone A14.7. For experimental procedures, see Materials and Methods and legend to Fig. 1. (C) Induction of A14.7 clone autotoxicity by synthetic BZLF1 peptides. One peptide (SENDRLRL) encoded by the 90-bp PstI/HincII fragment defined in B and containing consensus binding anchor residues to HLA-B*4002 (28) was synthesized. T cell clone cytotoxicity was estimated after incubation for 3 h at 37°C in the presence of either the above peptide or an irrelevant one (RAKFKQL). (D) Induction of A14.7 clone TNF release by synthetic BZLF1 peptides. TNF production was estimated after a 3-h incubation with peptide SENDRLRL.

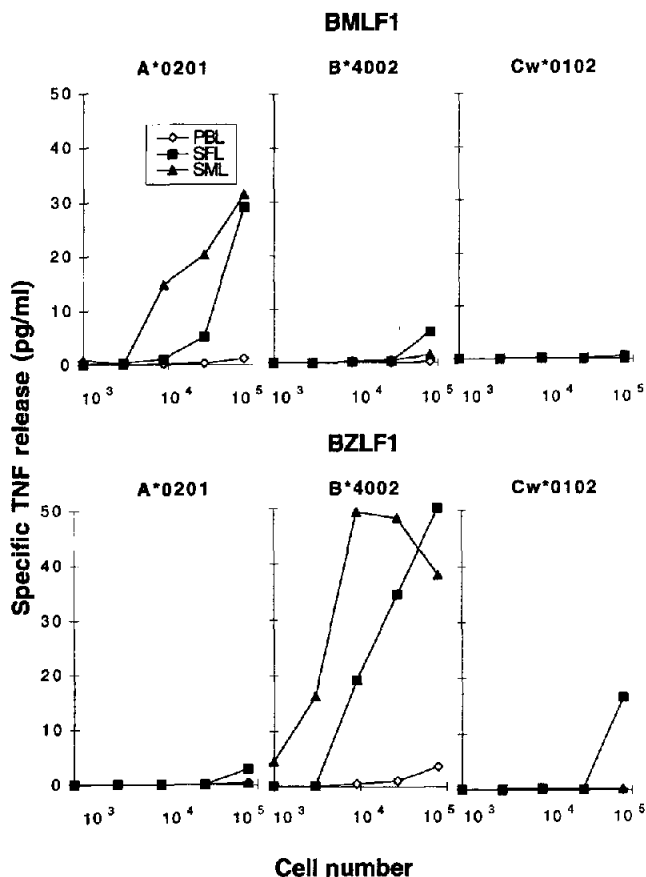


Figure 4. TNF responses of PBL, SFL, and SML from patient 1 to BMLF1- or BZLF1-transfected COS cells. Short-term-cultured PBL, SFL, and SML were incubated with COS cells transfected with either

responses to COS cells transfected with HLA-A*0201 and either BMLF1 or BZLF1 cDNA were also readily detected within short-term-cultured synovial T cells derived from 4 out of 9 HLA-A*02 patients (patients 1, 3, 5, and 6) (Fig. 5, left, and data not shown). Similarly to patient 1, an enrichment for HLA-A*02/BMLF1 reactive cells within the joints of patient 6 was seen when comparing TNF production obtained with PBL and SFL. Taken together, these results suggest that T cells reactive against these EBV transactivators are frequently expanded during chronic RA.

Evaluation of Cytolytic Activity of Synovial T Cell Clones toward BLC. Because BLC are known to be latently infected by EBV, the recognition by BLC-reactive T cells of BMLF1 or BZLF1, which are expressed exclusively during the virus lytic cycle, was rather unexpected and prompted us to analyze further the functional behavior of these T cells toward BLC. Although synovial T cell clones strongly pro-

BMLF1 cDNA together with HLA-A*0201 (upper left), B*4002 (upper middle), or Cw*0102 (upper right), or BZLF1 cDNA together with HLA-A*0201 (lower left), B*4002 (lower middle), or Cw*0102 DNA (lower right). Lymphocytes were added at three concentrations to COS cells 1 d after transfection, and TNF release was measured 1 d later. Specific TNF release was calculated by subtracting values obtained with COS cells transfected with BZLF1 or BMLF1 and an irrelevant HLA DNA (A*0101) from those obtained under the above conditions. Note that dominant responses to BMLF1/A*0201 and BZLF1/B*4002 were observed with both SFL and SML recovered 27 mo later. There was at least a 100-fold difference (on a per cell basis) between the amounts of TNF released by SFL versus PBL after exposure to BMLF1/A*0201- and BZLF1/B*4002-transfected COS cells. Weak but significant responses to BMLF1/B*4002 (upper middle), BZLF1/A*0201 (lower left), and BZLF1/Cw*0102 (lower right) were obtained with SFL and/or SML.

Table 3. TNF Responses of Synovial T Cell Clones from Patient 19 to BMLF1- or BZLF1-transfected COS Cells

T cell clone	TNF release		
	(-)	B*3501	
		BMLF1	BZLF1
		<i>pg/ml</i>	
B2	-	-	26.5
B3	-	-	32.3
B4	-	-	27.4

TNF production by T cell clones was estimated after incubation with COS cells transfected with HLA-B*3501 cDNA and either BMLF1 or BZLF1 cDNAs. -, TNF secretion <1.0 pg/ml

liferated when exposed to BLC expressing the appropriate HLA allele (Table 4; and reference 20), none of them were able to kill to detectable levels BLC which otherwise triggered their proliferation (Table 4 and data not shown). This could not be explained by a lack of lytic potential because (a) these clones efficiently lysed BLC in the presence of lectin (Table 4), and (b) addition of appropriate synthetic peptides to synovial T cell clones resulted in efficient peptide self presentation and autotoxicity (Figs. 1 and 3). Actually, this peculiar behavior is most probably explained by the T cell clone's fine specificity. Indeed, although BLC are known to express predominantly EBV latent proteins, rare cells supporting a fully productive EBV infection have

Table 4. Cytolytic Activity of Synovial T Cell Clones from Patient 1 toward BLC Triggering their Proliferation

T cell clone	Cytotoxicity				Specific proliferation
	BLC		BLC + lectin		
	20:1	5:1	20:1	5:1	
A2.3	3	1	67	54	26 833
A2.10	2	0	68	55	12 457
A2.19	6	6	70	72	48 129
A14.7	7	6	88	70	70 374
A17.10	4	4	46	35	66 877
A17.11	3	3	74	68	7 795
A22.19	5	4	81	83	61 656
A22.28	10	9	79	61	28 196

Cytotoxic activity of synovial T cell clones toward autologous BLC (no. 1) was assessed at 20:1 and 5:1 E/T ratios. T cell lytic potential was calculated by estimating target killing in the presence of purified PHA (leucoagglutinin) (0.5 µg/ml). Results are expressed as percentage of specific lysis. Tritiated thymidine uptake of synovial T cell clones was estimated after a 2-d coculture with irradiated BLC. Results are expressed as (cpm of T cells + BLC) - (cpm of T cells + cpm of BLC alone). Values correspond to means of duplicate counts (for cytotoxicity assays) or triplicate counts (for proliferation assays).

been detected in most BLC lines studied (30). Such cells could efficiently trigger proliferation of T cell clones directed against lytic proteins, although their lysis by the corresponding CTL might not be detectable in standard cytotoxicity assays.

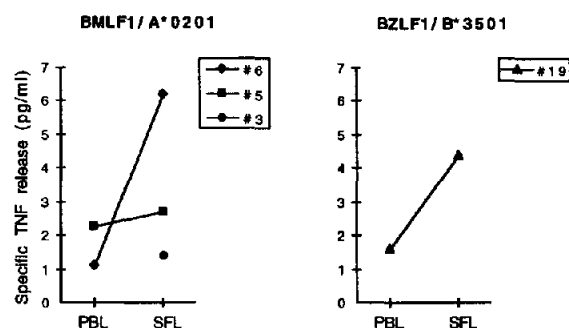


Figure 5. TNF responses of synovial T cells derived from HLA-B*35+ and HLA-A*02+ chronic RA patients to BMLF1- or BZLF1-transfected COS cells. 10⁵ short-term-cultured synovial T cells and PBL derived from nine HLA-A2+ RA patients and one HLA-B*35+ patient were incubated with COS cells transfected with HLA-A*0201 and BMLF1 or with HLA-B*3501 and BZLF1 DNA, respectively. Specific TNF release was calculated by subtracting the amounts of TNF secreted in the presence of COS cells transfected with an irrelevant HLA DNA (HLA-A*0301) and either BZLF1 or BMLF1 from those obtained with the restricting HLA DNA. Significant responses were observed in 5 patients (patients 1, 3, 5, 6, and 19). Shown are the results obtained with three HLA-A*02 patients (patients 3, 5, and 6) (left) and with the HLA-B*35 patient (patient 19) (right). For patient 3, results obtained with PBL were not interpretable because of a high spontaneous TNF release

Discussion

We study here the fine specificity of synovial T cells derived from several chronic RA patients. Our results indicate that in two patients, a large fraction of T cell clones derived from synovial fluid and synovial membranes of inflamed joints reacts against two EBV transactivators, BMLF1 and BZLF1. Moreover, responses against these EBV proteins were evidenced within short-term-cultured synovial T cells derived from several other RA patients. These observations raise questions regarding (a) the mechanisms governing T cell recruitment to the synovium during chronic RA, (b) the implication of EBV in chronic RA pathogenesis and the possible pathogenic role of EBV-reactive T cells, (c) the possible dominance of anti-EBV T cell responses in RA patients toward a specific set of proteins, and (d) the general significance and consequences of EBV transactivator recognition by T cells.

Regarding the first issue, it has been widely accepted that synovium infiltration by T cells in chronic RA results from nonspecific trapping of T cells, e.g., in response to

chemotactic factors released during the inflammatory reaction (19, 31). Our results show that a large fraction of synovial T cells from two chronic RA patients recognizes a restricted set of EBV antigens, thus demonstrating that at least in some patients, T cell recruitment to the synovium is an active Ag-driven process. Although conflicting results have been obtained regarding the presence or absence of the EBV genome in synovia (32–34), it is possible that EBV-reactive T cells are expanded locally after recognition of EBV-infected cells within the joint. Alternatively, EBV could trigger cross-reactive T cell responses against cellular proteins expressed in target organs. In this regard, several reports have described shared sequences between MHC alleles linked to increased RA susceptibility and EBV glycoproteins (35–38).

With respect to the second issue, implication of EBV in RA was first proposed several years ago, based on several clinical studies demonstrating increased EBV loads and anti-EBV Ab titers in the sera of RA patients (39, 40). However, since no evidence for increased anti-EBV responses in RA lesions was provided, the physiopathological significance of these findings has remained controversial. In particular, the possibility that these clinical observations reflected a general alteration of immune responses due to chronic inflammation rather than an active participation of EBV infection in RA pathogenesis could not be ruled out. In this regard, our demonstration that EBV-reactive T cells are enriched within the joints of chronic RA patients provides, for the first time, a direct link between EBV and RA. Furthermore, the fact that these patients showed concomitantly high anti-EBV Ab titers suggests that increased anti-EBV serological responses previously found in a large fraction of chronic RA patients (39, 40) is associated with specific activation of EBV-reactive T cells within the lesions. Although our results suggest that EBV-reactive T cells in RA lesions are very likely to be pathogenic, it remains to be established definitively whether these cells exert harmful effects, for example, through direct cytotoxicity or release of inflammatory cytokines activating T cell-independent joint erosion (e.g., mediated by synoviocytes) (19). Analysis of the *in vivo* behavior of these cells (e.g., in SCID/human models) will certainly help resolve these issues.

Whether or not anti-EBV responses in RA are directed against a restricted set of antigens is another interesting question. At present, a comparison of EBV responses between RA and non-RA individuals remains difficult, because none of the studies performed to date have really addressed T cell responses against EBV lytic proteins in non-RA EBV-infected

individuals (30). Moreover, the fact that some BLC-reactive synovial T cell clones do not respond to BMLF1 or BZLF1 in COS transfection assays strongly suggests recognition of a larger set of EBV proteins by RA patient-derived T cells. Despite this, biased anti-EBV responses toward proteins of the virus replicative cycle in RA patients is strongly suggested by the following observations. As mentioned before, EBV is known to enter the lytic cycle only in a minority of cells in BLC cultures. Therefore a dominant response against proteins expressed at that stage could explain both the decreased cytotoxic responses against autologous BLC frequently observed in RA patients (41, 42) and our inability to detect significant killing of BLC by synovial CTL in standard cytotoxicity assays (Table 4). It is noteworthy that this peculiar behavior could also explain why synovial CD8⁺ T cell responses to EBV have not been detected before, because specificity of these cells is classically established through cytotoxicity assays.

Beyond the field of autoimmunity, these observations provide new insights into the fine specificity of the anti-EBV T cell response and the immune control of EBV reactivation, because they provide the first clear-cut evidence that the BZLF1 and BMLF1 EBV-transactivating proteins can be targets for CD8⁺ T cells. T cell recognition of such proteins has been suspected in several EBV-linked pathological situations (e.g., acute infectious mononucleosis, nasopharyngeal carcinoma, EBV reactivation in immunodepressed patients) during which serological Ab titers against BZLF1 product and other early antigens are significantly increased (for review see reference 30). However, technical limitations have hampered direct analysis of T cell responses directed against EBV lytic antigens. The physiological significance of BZLF1/BMLF1 recognition by T cells can be inferred from the function of these proteins during virus replication. BZLF1 is the first gene expressed during the immediate early stage of the EBV lytic cycle, and it is thought to turn on expression of many other immediate early genes (43). BMLF1 is a delayed immediate early gene with promiscuous transactivating properties that acts synergistically with BMLF1 and another EBV transactivator, BRLF1, in *in vitro* assays (43). It is therefore likely that T cell responses against these two key transactivators of EBV lytic infection play a central role in controlling virus spreading under physiological and pathological situations. With the recent development of murine models allowing *in vivo* analysis of anti-EBV human T cell responses (44), this hypothesis can now be tested.

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