$CD4^+$ $CD7^-$ $CD28^-$ T Cells Are Expanded in Rheumatoid Arthritis and Are Characterized by Autoreactivity

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Abstract

Clonal expansion of $CD4^+$ T cells is a characteristic finding in patients with RA and is only infrequently found in patients with psoriatic arthritis and healthy controls. Expanded $CD4^+$ clonotypes are present in the blood, infiltrate into the joint, and persist over years. We have now addressed the question of whether the expanded clonotypes have unique functional and phenotypic properties which may explain the preferential in vivo expansion in RA. In contrast to most $CD4^+$ T cells, expanded clonotypes lacked the expression of the CD28 and CD7 cell surface molecules. Accordingly, the subsets of $CD4^+$ $CD28^-$ (9.7 vs. 1.7, $P = 0.00002$) and $CD4^+$ $CD7^-$ T cells (21.5 vs. 12.6, $P = 0.018$) were increased in RA patients compared with age-matched normal individuals. Despite the lack of CD28 expression, clonally expanded $CD4^+$ T cells were not anergic but proliferated in response to immobilized anti-CD3 and could be maintained in tissue culture. In vivo expanded $CD4^+$ T cells were autoreactive to ubiquitously distributed autoantigens. They responded in an autologous mixed lymphocyte reaction, and T cell clones isolated from selected patients proliferated to autologous peripheral blood adherent cells. These data suggest that in RA patients selected $CD4^+$ T cells which share the $CD7^-$ $CD28^-$ phenotype escape from peripheral tolerance. (*J. Clin. Invest.* 1996. 97:2027–2037.)
Key words: rheumatoid arthritis • autoimmunity • T cells • clonal expansion • T cell receptor

Introduction

Rheumatoid arthritis is characterized by dense lymphoid infiltrates in the synovial membrane associated with progressive destruction of the joint architecture (1). Most RA patients have additional disease manifestations not related to the joint inflammation, and ~ 10% develop severe extraarticular organ involvement which often presents as vasculitis or as Felty's syndrome (2). Extraarticular manifestations have a very strong HLA-DRB1 association, and most of these patients are heterozygous or homozygous for two disease-associated HLA-DRB1*04 alleles (3, 4). These findings have led to the hypothesis that T cells influence disease mechanisms in RA beyond the recognition of a defined arthritogenic antigen.

We have recently described that patients with RA express a unique repertoire of T cell receptor (TCR)[1] genes in the naive $CD4^+$ T cell population (5). These repertoire changes are only partially explained by the HLA-DRB1*04 association of the disease, suggesting that additional mechanisms influence the outcome of thymic selection in RA patients (5). Additional evidence for a generalized abnormality in T cell homeostasis in RA comes from the finding that RA patients carry multiple clonally expanded $CD4^+$ T cells (6). Expanded CD4 clonotypes are infrequent in normal HLA-DRB1*04^+ controls, as well as in HLA-DRB1*04^+ patients with a nonrheumatoid polyarthritis such as psoriatic arthritis (6a). Typically these clonotypes are detected in the peripheral blood and, in about similar frequencies, in the inflamed joint, indicating that they do not represent a consequence of ongoing synovitis. While clonotypes vary in their frequency, they persist over several years. A core group of three BV genes, BV3, 14, and 17, is used by the majority of T cells having undergone in vivo clonal expansion in RA patients. This unique TCR repertoire led us to hypothesize that these clonotypes may originate from a specialized subset of T cells with defined immunological functions. To pursue this hypothesis, we have phenotypically and functionally characterized expanded $CD4^+$ clonotypes in RA patients. Their phenotype in vitro and in vivo identifies them as $CD4^+$ T cells lacking the expression of the CD28 and the CD7 molecules. $CD28^-$ $CD4^+$ T cells are rare in normal individuals but expanded in RA patients. Expanded clonotypes exhibited an unusual functional profile in that they proliferated in response to autologous antigen presenting cells (APC). Our findings suggest that RA patients have a unique subset of T cells prone to escape from peripheral tolerance and proliferate in vivo.

Methods

Study population. Peripheral blood was obtained from patients with seropositive erosive RA. Patients were characterized for their HLA-DRB1 alleles by PCR and subsequent oligonucleotide hybridization as described (4). Results are shown in Table I. Normal individuals without personal or family history of RA were age-matched to the disease population. A second control group consisted of 13 HLA-DRB1*04 normal individuals.

Cell purification. PBMC were separated from heparinized venous blood by Ficoll gradient centrifugation. Cells were stained with various combinations of phycoerythrin (PE)- and FITC-conjugated mAb and sorted on a FACSVantage®. The following combinations of antibodies were used: anti-CD3 and anti-CD4, anti-CD4 and anti-CD45RO, anti-CD4 and anti-CD28, anti-CD4 and anti-CD7, and anti-CD4 and anti-CD57 (all from Becton Dickinson, San Jose, CA).

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1. *Abbreviations used in this paper:* AICD, activation-induced cell death; AMLR, autologous mixed lymphocyte reaction; APC, antigen presenting cell; CDR, complementarity determining region; PE, phycoerythrin; RT, reverse transcriptase; TCR, T cell receptor.

Table I. Distribution of HLA-DRB1 Alleles in the Patient Population

Allelic HLA-DRB1 combinations	RA patients
	n
HLA-DRB1*04/04	6
HLA-DRB1*04/01	3
HLA-DRB1*0401/x‡	13
HLA-DRB1*0404/x	5
HLA-DRB1*01/x	2
HLA-DRB1*1402/x	1
HLA-DRB1*x/x	4

‡x, Non–disease-associated HLA-DRB1 allele.

Identification of in vivo expanded clonotypes. Total RNA of 1×10^6 $CD3^+$ $CD4^+$ cells was extracted by guanidinium thiocyanate phenol chloroform extraction using a commercially available kit. cDNA was amplified with six BV specific primers (BV2, 3, 5S1, 8, 14, and 17) and a BC specific primer (7). A 1:100 dilution of the amplified template was reamplified with the appropriate BV primer combined with primers specific for the different BJ elements. The following BJ primers were used: BJ1S1 (TGCCTTGTCCAAAGAAAGC), BJ1S2 (ACCTGGTCCCCGAACCGAAGG), BJ1S3 (TACAACAGTGAGCCAACTT), BJ1S4 (AGACAGAGAGCTGGGTTCCAC), BJ1S5 (GATGGAGAGTCGAGTCCCATCA), BJ1S6 (TCACAGTGAGCCTGGTCCCATT), BJ2S1 (CCTCTAGCACGGTGAGCCGT), BJ2S2 (GGTCAGCCTAGAGCCTT), BJ2S3 (TGCCTGGGCCAAAATACT), BJ2S4 (CAGCACTGAGAGCCGGGTCCCGGCG), BJ2S5 (ACCAGGAGCCGCGTGCCT), and BJ2S7 (ACCGTGAGCCTGGTGCCC). The amplified products were labeled using a primer extension assay with the appropriate end-labeled BJ or BV primer and were separated on a 5% denaturing polyacrylamide gel. For the majority of samples, the analysis showed 8–12 bands for one particular BV-J combination with Gaussian distribution of band intensities. Dominant bands within the Gaussian distribution were taken as evidence for a clonal expansion. A band was defined as dominant if the intensity of the band was larger than the sum of two adjacent bands and at least 30% of the total product. Dominance of a clonotype within one BV-J combination was confirmed by direct sequencing of the total BV-J amplification product by reverse transcriptase (RT)-mediated dideoxy sequencing as described (6, 8). Previous experiments had shown that one particular clonotype had to be $> 25\%$ of all T cells having the particular BV-BJ combinations to give an unequivocal sequence (6). $CD4^+$ T cells were subsequently sorted for the expression of the CD7, CD28, CD45RO, and CD57 markers. BV-J combinations which harbored dominant clonotypes were analyzed in the CD4 subsets for the presence of the clonotype.

T cell cloning. $CD4^+$ T cells from selected patients were stimulated with immobilized anti-CD3 for 6 h and cloned by limiting dilution on irradiated EBV-transformed cell lines in the presence of 20 U/ml recombinant IL-2. Established T cell clones were screened for the expression of the TCR β chain sequence corresponding to the sequence which had been found to be clonally expanded in vivo. To determine antigen specificity, 6×10^4 T cell clones were cocultured with 1×10^5 irradiated (3,000 rad) autologous mononuclear cells for 72 h. Proliferative responses were determined by [^{3}H]thymidine incorporation. In parallel cultures, cloned T cells were cultured with autologous cells and 5 μg/ml L243 (anti-HLA-DR) and L227 (anti-HLA-DR and DQ), respectively.

Immunophenotyping. T cell clones were stained with the following mAbs: FITC anti-CD3, PE and FITC anti-CD4, FITC anti-CD5, FITC anti-CD7, PE anti-CD8, PE anti-CD28, PE anti-CD45RO, PE anti-CD56, and FITC anti-CD57 (all from Becton Dickinson). PBMC from RA patients and from normal controls and mononuclear cells from synovial fluid were stained with anti-CD4 PERCP, anti-CD28 PE, and either anti-CD57 FITC or anti-CD7 FITC. Samples were analyzed with a FACScan® using the PC Lysis software.

T cell assays. PBMC were depleted of $CD8^+$ cells by incubation with 40 μg anti-CD8/10^6 cells (OKT8, CRL 8014, American Type Culture Collection, Rockville, MD) at 4°C for 30 min and subsequent incubation with magnetic beads coupled with anti–mouse Ig (Perceptive Diagnostic, Cambridge, MA). 1×10^5 cells were cocultured with 1×10^5 irradiated autologous adherent cells (obtained by plastic adherence of PBMC on serum-coated Petri dishes for 2 h). Cells were harvested on days 7 and 10 and total RNA was obtained. Parallel cultures were assayed for proliferative responses by [^{3}H]thymidine incorporation. Control cultures included cells cultured on immobilized anti-CD3 for 5 d and cells cultured with 1 μg/ml SE B and SE C2 (Toxin Technology, Sarasota, FL) for 5 d. Total RNA was amplified by RT-PCR with BV and BJ specific primer sets, and the amplified products were size fractionated as described above. The intensity of dominant bands corresponding to in vivo expanded clonotypes was semiquantified using phosphorimage analysis (Bio-Rad Laboratories, Richmond, CA) and the phosphoranalysis™/Macintosh software. The intensities before and after culture were compared.

Results

Phenotype of clonally expanded $CD4^+$ T cells in RA patients. To test the hypothesis that the $CD4^+$ T cells, which clonally expand in the peripheral repertoire of RA patients, have unique functional properties, expanded clonotypes were isolated in in vitro culture. RA patients were selected who carried several $CD4^+$ clonotypes in the peripheral blood as determined by PCR with BV and BJ specific primers, subsequent complementarity determining region 3 (CDR3) length analysis, and direct sequencing. These clonotypes were stable over time and found in at least two different blood samples several months apart. PBMC were obtained and randomly cloned by limiting dilution after stimulation with immobilized anti-CD3. Established T cell clones were screened for the expression of the TCR β chain sequence which had been found in the original blood sample. Eight of such T cell clones from five different RA patients were analyzed for the expression of nine cell surface markers by FACS® analysis. The T cell clones derived from the in vivo expanded T cells shared the $CD3^+$ $CD4^+$ $CD8^-$ $CD2^+$ phenotype. The profile of the cell surface molecules shown in Table II was unusual for $CD4^+$ T cells. All eight clones lacked the expression of CD7 and seven lacked CD28 expression. The clone KD1.1 which expressed CD28 could also be isolated as a $CD28^-$ variant (data not shown). Four of the clones expressed CD57 and one was negative for the CD5 molecule. In two of the clones, a small number of $CD56^+$ cells was detected with the majority of the cells negative for this marker. To exclude that this profile of cell surface markers may relate to the in vitro culture, we tested seven $CD3^+$ $CD4^+$ T cell clones which had been isolated from healthy control individuals and which had been kept under the same tissue culture conditions. These T cell clones showed a more uniform pattern, in particular, all of them expressed CD28. However, all but one were also negative for CD7, raising the possibility that CD7 was lost during the tissue culture.

The lack of CD28 expression is an infrequent finding in human peripheral blood $CD4^+$ T cells, suggesting that expanded T cell clones were derived from a small subset of peripheral blood $CD4^+$ T cells (9). Most human peripheral T cells are also positive for the CD7 marker (10); however, the significance of

Table II. Phenotypic Characterization of T Cell Clones

T cell clone	CD5	CD7	CD56	CD57	CD28
In vivo expanded RA clones					
HD1.1	+	−	−	(+)*	−
P5	+	−	−	(+)	−
V1	+	−	−	+	−
KD1.1	+	−	−	−	+
7A4.1	(−)	−	(−)‡	−	(−)
12A5.2	+	(−)	(−)	(+)	−
H1-67	+	−	−	−	−
HOP2-3	+	−	−	−	(−)
Control clones					
PMP9	+	(+)	−	−	+
JJGP23	+	−	−	−	+
TG3.3	+	−	−	−	+
SJ1.13	+	−	−	−	+
SJ1.58	+	−	−	−	+
JF1.26	(+)	−	−	−	(+)
JF1.68	(+)	−	−	−	+

PBMC from five RA patients were randomly cloned after activation with immobilized anti-CD3. T cell clones expressing a TCR β chain sequence which had been found to be dominant in the peripheral blood $CD4^+$ T cell compartment were identified and characterized for their cell surface markers. Control clones were derived from normal individuals and randomly chosen. *50–80% of all T cells expressed the respective marker; ‡20–50% positive for the marker.

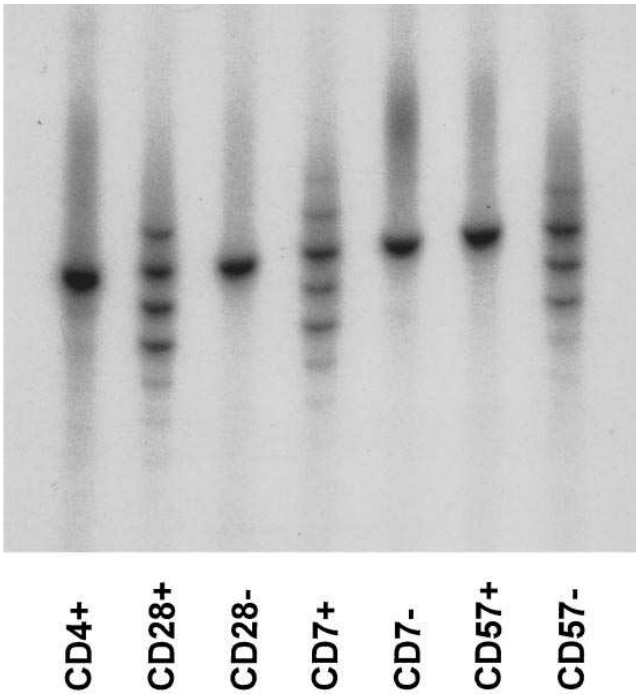

Figure 1. Identification of expanded clonotypes in $CD4^+$ T cell subsets. To confirm the cell surface phenotype of expanded clonotypes in vivo, $CD4^+$ T cells were sorted from PBMC of RA patients and analyzed for the presence of expanded clonotypes by PCR with primer sets for 72 BV-BJ combinations (6 BV and 12 BJ gene segments), subsequent spectrotyping and direct sequencing. A representative example of a BV17-BJ1S1$^+$ clonotype is shown. $CD4^+$ T cells were subsequently separated according to the expression of the CD28, the CD7, and the CD57 marker. The $CD4^+$ $CD28^+$, $CD4^+$ $CD7^+$, and the $CD4^+$ $CD57^-$ populations showed Gaussian distribution of BV17-BJ1S1 transcripts indicating T cell polyclonality. In contrast, the T cell clone could be detected within the $CD28^-$, $CD7^-$, and the $CD57^+$ population of CD4 T cells. Clonal identity was confirmed by sequencing the CDR3 region. All dominant bands expressed the same sequence (BV17-CASSARRGARAF-BJ1S1).

the lack of CD7 expression by the in vivo expanded T cell clones was indeterminate. To address the question of whether the phenotypic profile of the isolated $CD4^+$ T cell clones reflected the cell surface expression of clonal CD4 population in vivo, $CD4^+$ T cells from RA patients were screened for clonally expanded populations by PCR and spectrotyping. $CD4^+$ cells were subsequently sorted for the expression of the CD7, CD28, CD45RO, and CD57 markers. $CD4^+$ T cell subpopulations positive and negative for the respective marker were compared by PCR with the appropriate BV-BJ primer set and spectrotyping for the presence of the dominant TCR transcript which had been identified in the unseparated CD4 populations. Fig. 1 shows a Gaussian distribution of TCR transcripts with different CDR3 lengths after BV17-BJ1S1 specific amplification in the $CD4^+$, $CD57^-$, $CD28^+$, and $CD7^+$ populations. A dominant band of equal size corresponding to the clonal specificity was identified in the $CD7^-$, $CD28^-$, and $CD57^+$ subsets. Results for 21 different clonotypes from six RA patients are summarized in Table III. All expanded clonotypes expressed the CD45RO molecule, characterizing them as memory T cells. 4 of 11 clonotypes analyzed were detected in the $CD45RO^+$, as well as the $CD45RO^-$ population, raising the possibility that either some of the clonally proliferating cells revert back to the naive phenotype or clonal expansion is a feature of naive cells which eventually acquire the CD45RO phenotype. Without an exception, all clonal T cell populations were identified among $CD28^-$ $CD4^+$ T cells. In 3 of the 16 clonotypes, a fraction of the cells was positive for the CD28 molecule. Also, all of the analyzed clonally proliferating T cells lacked the expression of CD7. Two clonotypes could also be detected in the $CD7^+$ population. 10 of 11 tested clonotypes were $CD57^+$ in vivo. Four clones were found among $CD57^-$ $CD4^+$ T cells.

Expansion of $CD4^+$ $CD7^-$ $CD28^-$ T cells in RA patients. The finding that the in vivo expanded clonotypes predominantly expressed the $CD4^+$ $CD28^-$ $CD7^-$ phenotype raised the question of whether the $CD4^+$ $CD28^-$ and the $CD4^+$ $CD7^-$ compartments are expanded in RA. To address this question, the frequencies of $CD4^+$ $CD7^-$, $CD4^+$ $CD28^-$, and $CD4^+$ $CD57^+$ T cells were determined by two-color FACS® analysis in 22 patients with seropositive RA and 22 age-matched controls (Fig. 2). In control individuals, CD28 was absent on a small population of $CD4^+$ T cells. A median fraction of 12.6% of $CD4^+$ T cells was negative for CD7. CD57 was expressed on a small subset of $CD4^+$ T cells accounting for a median of 1.5%. All three $CD4^+$ T cell subsets were expanded in RA patients. Most significantly, the percentages of $CD4^+$ $CD28^-$ T cells were increased in patients (9.7 vs. 1.7%; $P = 0.00002$). The size of the $CD4^+$ $CD7^-$ subset was almost doubled in the patient cohort with a median frequency of 21.5% ($P = 0.018$). The expansion was least marked for $CD4^+$ $CD57^+$ T cells (3.2 vs. 1.5%; $P = 0.13$). In addition to age, HLA-DR

Table III. Profile of Cell Surface Markers of In Vivo Expanded CD4 Clones

	Cell surface marker							
T cell clone	CL45RO$^+$	CD45RO$^-$	CD28$^+$	CD28$^-$	CD7$^+$	CD7$^-$	CD57$^+$	CD57$^-$
BV2 BJ2S3			−	+	−	+	+	−
BV3 BJ1S5	+	−	+	++				
BV3 BJ1S6	+	−	−	+	−	+	+	−
BV3 BJ2S2	+	−			−	+		
BV5 BJ1S5							+	−
BV5 BJ2S4			−	+	+	ND	ND	+
BV8 BJ1S3	+	−						
BV8 BJ2S3			−	+			+	−
BV14 BJ1S3 A	+	−	−	+			+	−
BV14 BJ1S3 B	+	+	+	+				
BV14 BJ1S3 C					+	+	ND	+
BV14 BJ1S4	+	−	−	+				
BV14 BJ2S2			−	+	−	+	ND	+
BV14 BJ2S7			+	+	−	+	+	−
BV17 BJ1S1 A	+	+	−	+	−	+	+	−
BV17 BJ1S1 B			−	+	−	+	+	−
BV17 BJ1S4					−	+	+	−
BV17 BJ1S5	+	+	−	+	−	+	+	−
BV17 BJ2S2 A	+	−	−	+				
BV17 BJ2S2 B			−	+	−	+	−	+
BV17 BJ2S4	+	+	−	+				

CD4$^+$ T cells were purified from PBMC of six RA patients by cell sorting. Expanded CD4$^+$ clonotypes were identified by TCR BV-BJ specific RT-PCR and subsequent CDR3 length analysis as described in Fig. 1. Fresh PBMC were separated into CD4$^+$ CD45RO$^+$ and CD4$^+$ CD45RO$^-$, CD4$^+$ CD28$^+$ and CD4$^+$ CD28$^-$, CD4$^+$ CD7$^+$ and CD4$^+$ CD7$^-$, and CD4$^+$ CD57$^+$ and CD4$^+$ CD57$^-$ cells by FACS® sorting. The T cell subsets were then analyzed for the presence of the clonal specificity which was originally identified in the unseparated CD4 population by RT-PCR and CDR3 length analysis. Blank areas indicate experiments not done; ND, not detectable; FACS® sorted cell population was too small for RNA extraction.

polymorphism might influence the frequency of the CD4$^+$ CD28$^-$ population. 19 of the 22 RA patients expressed one of the disease-associated HLA-DRB1*04 alleles. Therefore, Fig. 2 contains a second control group of 13 healthy HLA-DR4 individuals. There was no evidence that the HLA-DRB1*04 polymorphism alone was sufficient to explain the expansion of the CD4$^+$ CD28$^-$ compartment in the RA patients.

The phenotype expressed by the expanded clonotypes sug-

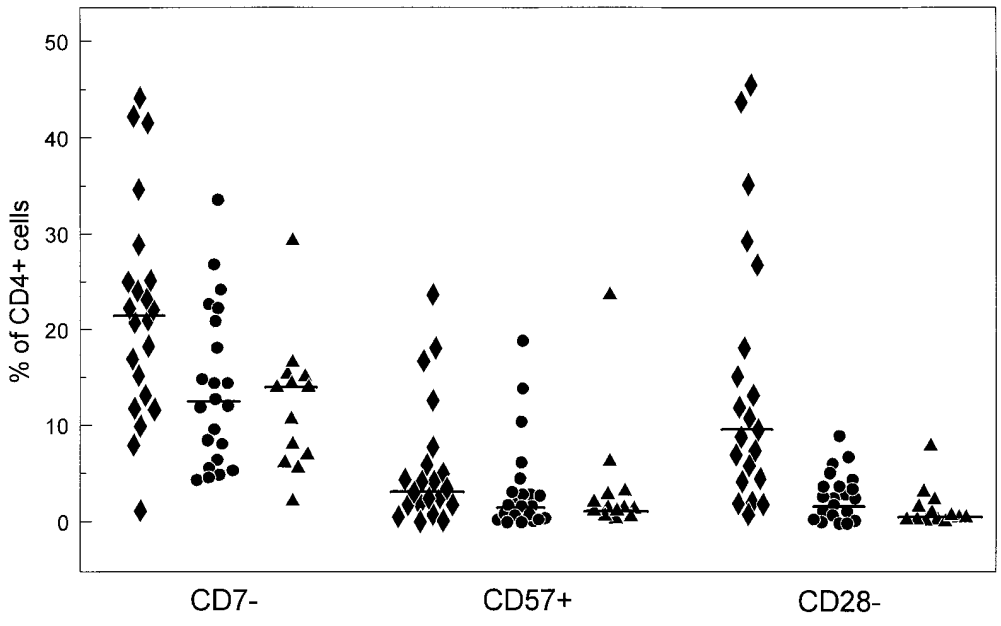

Figure 2. Expansion of CD4$^+$ CD28$^-$ CD7$^-$ T cells in patients with RA. PBMC from 22 patients with seropositive RA (*diamonds*), 22 age-matched normal individuals (*circles*), and 13 HLA-DRB1*04 normal individuals (*triangles*) were analyzed by two-color FACS® analysis. Medians are indicated by bars. RA patients had a higher fraction of CD4$^+$ CD28$^-$ and CD4$^+$ CD7$^-$ T cells in the CD4 compartment compared with age-matched controls ($P = 0.00002$ and $P = 0.018$, respectively, by Mann-Whitney test), and compared with HLA-DR4–matched controls ($P = 0.00001$ and $P = 0.006$, respectively) while the fraction of CD4$^+$ CD57$^+$ T cells was only slightly increased.

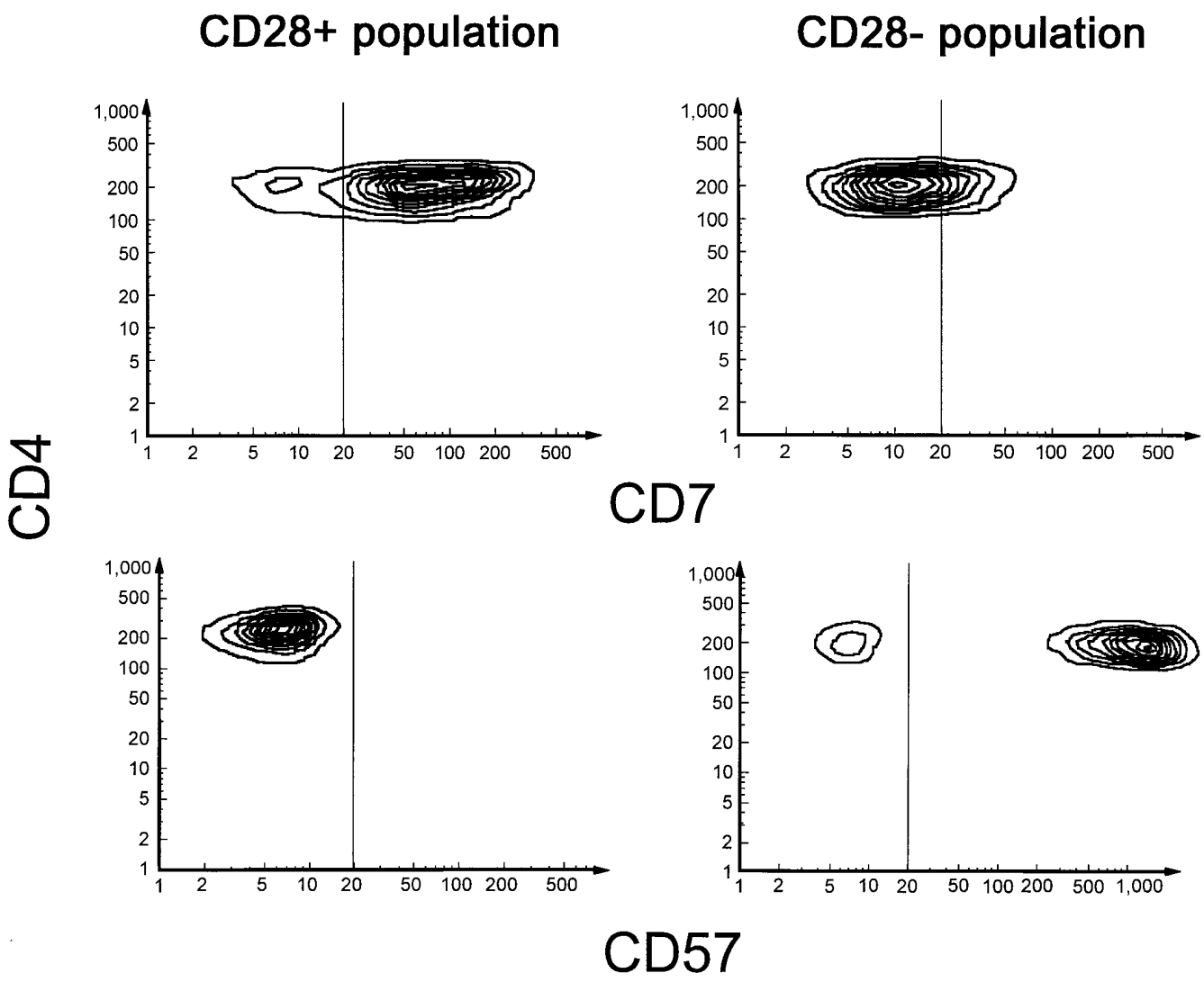

Figure 3. Profile of cell surface antigens expressed by $CD4^+$ $CD28^-$ T cells in patients with RA. PBMC of patients with RA were analyzed by three-color FACS® analysis. Results are shown for $CD4^+$ $CD28^+$ and $CD4^+$ $CD28^-$ T cells. The vast majority of $CD28^+$ T cells expressed the CD7 marker while $CD28^-$ $CD4^+$ T cells expressed low levels or no CD7. Expression of the CD57 marker correlated with lack of CD28 expression.

gested a correlation between the expansion of the $CD28^-$ and $CD7^-$ compartments. To address that question, $CD4^+$ T cells from patients with RA were analyzed by three-color FACS® analysis. These studies demonstrated that the majority of $CD28^-$ $CD4^+$ T cells were also lacking the CD7 marker and virtually all $CD57^+$ $CD4^+$ T cells were included in the $CD28^-$ subset (Fig. 3). We concluded from these experiments that RA patients have an abnormality in their $CD4^+$ T cell compartment which can be assigned to a subpopulation expressing the $CD7^-$ $CD28^-$ $CD57^+$ phenotype. This T cell compartment is expanded in RA and the expansion is at least partially due to the emergence of oligoclonal T cell populations.

Clonally expanded T cells express CD28 in the synovial compartment. $CD4^+$ $CD28^-$ $CD7^-$ T cells are selectively expanded in RA patients; however, it is not clear how they relate to the synovial inflammation. To compare the representation of these cells in the peripheral blood and in the joint, paired samples collected from the blood and from synovial fluid were studied. $CD4^+$ T cells, which were clonally expanded in the peripheral blood, were also encountered in the synovial fluid and in the synovial tissue. Analysis of CD4 subsets of the paired samples demonstrated that T cell clonotypes which lacked the CD28 marker in the peripheral blood compartment could be identified in the $CD4^+$ $CD28^+$ and the $CD4^+$ $CD28^-$ compartments in the synovial fluid (Fig. 4). Also, expansion of the $CD4^+$ $CD28^-$ T cell compartment was a characteristic finding for the blood but not for the synovial environment. Percentages of the $CD28^-$ T cells were reduced in the synovial fluid in four of the six patients studied. In contrast, $CD4^+$ $CD57^+$ and $CD4^+$ $CD7^-$ T cells were even more enriched in the synovial fluid (Fig. 5). These results suggest that either $CD4^+$ $CD7^-$ $CD28^-$ T cells home to the joint and regain CD28 expression in the synovial compartment or that these clonotypes are primarily $CD28^+$ in the joint and subsequently lose the expression of this cell surface marker.

$CD4^+$ $CD28^-$ clonotypes are not anergic and respond to autologous cells. Signaling of the TCR in the absence of costimulatory signals provided by the CD28 pathway results in anergy

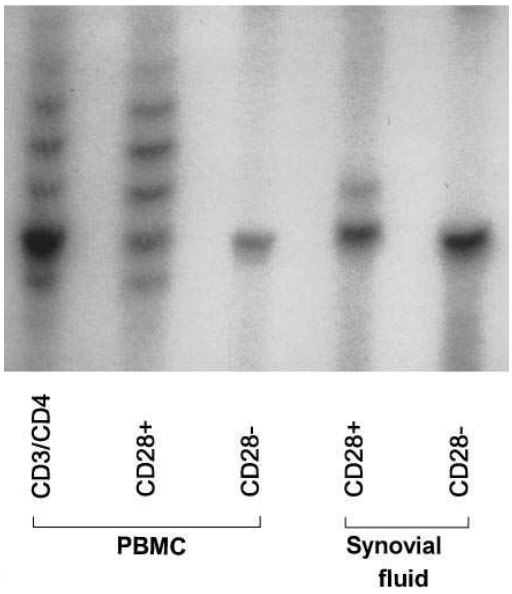

Figure 4. Expanded clonotypes in the synovial compartment express CD28. $CD4^+$ $CD28^+$ and $CD4^+$ $CD28^-$ T cells were purified from peripheral blood and from synovial fluid of an RA patient. cDNA was analyzed for the presence of dominant clonotypes by BV-BJ amplification and subsequent CDR3 length analysis. In the peripheral blood, the BV17-BJ1S1 CD4 clonotype was identified only in the $CD28^-$, but not in the $CD28^+$ compartment. In contrast, the clonotype was present in the $CD4^+$ $CD28^-$ and the $CD4^+$ $CD28^+$ T cell populations derived from the synovial fluid. Identity of the dominant band was confirmed by direct sequencing (BV17-CASSTRVCNTEAF-BJ1S1).

of the responding T cell (11, 12). To examine the responsiveness of expanded clonotypes, purified $CD4^+$ T cells from RA patients were activated by either immobilized anti-CD3 or by the bacterial superantigens SE B and SE C2 and autologous adherent cells. In vivo expanded clonotypes were identified and semiquantified in the unstimulated CD4 population by PCR with BV-J primer sets, including BV3, 14, and 17 specific primers. This spectrum of BV primers was chosen because these BV elements are frequently used by expanded clonotypes in RA. The two enterotoxins were selected because they are stimulatory for T cells expressing these BV elements. Cells were harvested 5 d after anti-CD3 and superantigen stimulation. The T cell repertoire before and after stimulation was compared by BV-BJ specific PCR, size fractionation according to the CDR3 length, and subsequent quantification by phosphorimage analysis. Clonal expansion generally results in the expansion of the fraction of TCR sharing a particular CDR3 length. Determining the fraction of TCR sharing one CDR3 size by phosphorimage analysis, therefore, allowed an approximate estimate of the clonal size. An increase or persistence of the peak corresponding to a particular CDR3 length indicated proliferation of the T cell clone. Normalization of the distribution and disappearance of the peak indicated that the clone did not proliferate under the conditions used. Results of 37 in vivo expanded clonotypes are summarized in Fig. 6. More than 70% of all clonotypes persisted after anti-CD3 stimulation. A minority of CD4 clonotypes decreased or disappeared after anti-CD3 stimulation; however, all were retrieved in the superantigen-driven cultures. These data clearly demonstrated that the expanded clonotypes are not anergic in vivo although they lack the CD28 costimulatory molecule.

To test the question of whether the antigen recognized by these clonotypes represents an endogenous self-antigen, an autologous mixed lymphocyte reaction (AMLR) was used. All 37 in vivo expanded clonotypes which had been tested for the proliferative response to immobilized anti-CD3 and the superantigens SE B and SE C2 were also analyzed in AMLR. Expanded clonotypes were semiquantified after 7 and 10 d of culture with autologous adherent cells. Results at the two time points of the culture period showed a high correlation. A representative example of a phosphorimage analysis of a BV14-BJ1S3 amplification product which included an expanded clonotype is shown in Fig. 7. TCR sequences showing the CDR3 length representing the clonotype constituted 35% of the total BV14-BJ1S3 amplification in unstimulated PBMC compared with 44% after 10 d of AMLR. Results of the AMLR experiments on all clones are summarized in Fig. 6. 71% of all clonotypes responded to autologous cells. Most clonotypes (56% of all clones tested) were reactive to immobilized anti-CD3 as well as in the AMLR. The interpretation that $CD4^+$ $CD28^-$ $CD7^-$ T cell clones proliferated in the AMLR was further supported by phenotypic studies. Cultures obtained after AMLR showed an enrichment of $CD4^+$ $CD7^-$ $CD28^-$ cells. A representative experiment is shown in Fig. 8. In this RA patient, the CD4 population before stimulation included 39% of $CD28^-$ T cells. Upon coculture with autologous adherent cells, this percentage increased to 75%. To further address the hypothesis that the expanded clonotypes recognize endogenous antigen on autologous APC, six T cell clones, which had been isolated from three RA patients and which were derived from in vivo expanded clonotypes, were cultured in the presence and absence of autologous irradiated PBMC. The autologous cells were able to induce proliferation of all of these T cell clones (Fig. 9). Addition of the HLA-DR specific antibody L243 and the HLA-DR/DQ specific antibody L227 did not inhibit the proliferative response, suggesting that the antigen recognized is not presented by the disease-associated HLA-DR molecules (data not shown).

Discussion

Experiments presented here were designed to address the question of whether clonally expanded $CD4^+$ T cell populations in patients with RA are derived from a phenotypically and functionally characterized subset of $CD4^+$ T cells which may be prone to clonal proliferation. Several lines of evidence indicate that clonal $CD4^+$ T cell populations which dominate the peripheral repertoire have disease relevance. They are consistently found in RA patients but are infrequent in patients with psoriatic polyarthritis and thus are not simply a consequence of a chronic inflammatory response. This interpretation is emphasized by the finding that the clonally expanded populations are present very early in the disease process and do not further accumulate within the first decade of disease progression. Also, we have identified expanded $CD4^+$ clonotypes in unaffected siblings of RA patients, raising the possibility that they represent the consequence of a gene inherited in RA families and thus could be a risk factor for the disease. Further evidence for their role in the disease process comes from their infiltration into the joint where they are consistently found (6).

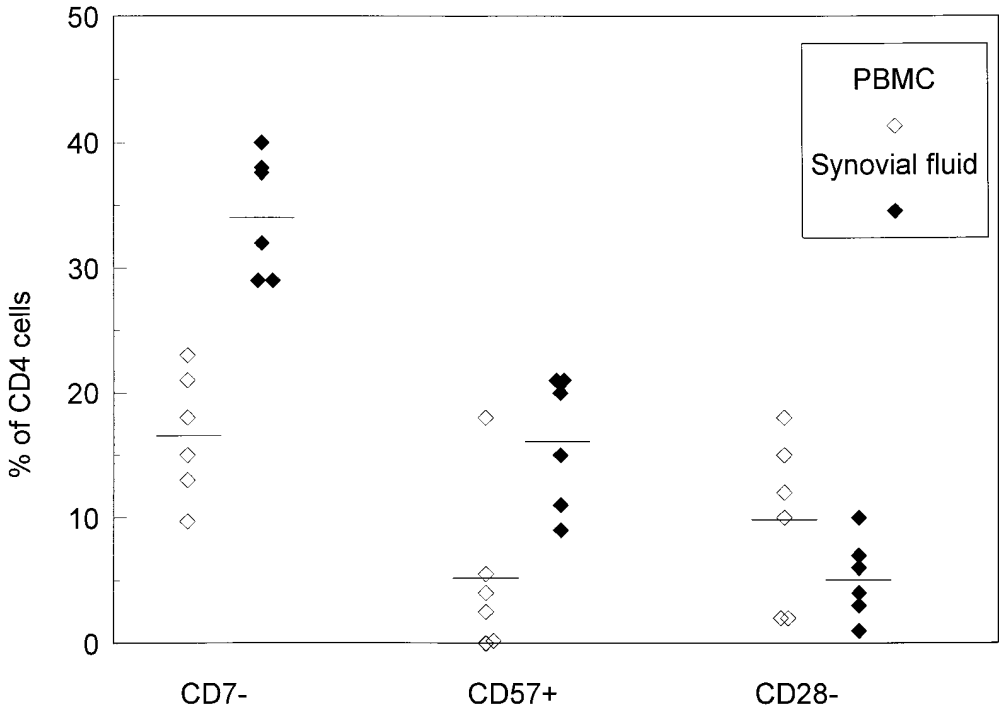

Figure 5. Expansion of $CD7^-$ $CD57^+$ but not $CD28^-$ $CD4^+$ T cells in synovial fluid. PBMC and synovial fluid mononuclear cells from six patients with RA were analyzed by two-color FACS® analysis for the expression of the CD7, CD28, and the CD57 marker in $CD4^+$ T cells. While there was significant enrichment of $CD7^-$ ($P = 0.03$) and $CD57^+$ ($P = 0.03$) $CD4^+$ T cells in the synovial fluid, the $CD28^-$ subset was smaller in the synovial fluid than in the peripheral blood.

Phenotypic analysis of expanded clonotypes isolated from RA patients indicated that they exhibited a unique phenotype, in particular, they lacked the expression of the CD28 molecule. In vivo studies on a large panel of expanded clonotypes confirmed the phenotypic profile as $CD4^+$ $CD7^-$ $CD28^-$ $CD57^+$. Two models could explain the compartmentalization of the expanded clonotypes. Possibly expanding clonotypes could be derived from a separate lineage of $CD4^+$ T cells. Alternatively, $CD4^+$ $CD7^-$ $CD28^-$ T cells originate from $CD4^+$ $CD7^+$ $CD28^+$ cells but undergo a functional and phenotypic change in response to a defined environment. Chronic polyarthritis could represent such an environment. The low frequency of expanded clonotypes in psoriatic arthritis, their presence in very early disease, and their expression in unaffected siblings all argue against chronic inflammation providing the setting for $CD4^+$ $CD7^+$ $CD28^+$ cells to acquire the $CD7^-$ $CD28^-$ phenotype. Some of the expanded CD4 clonotypes were found among $CD45RO^-$ and $CD45RO^+$ subsets. We cannot distinguish between the two models that oligoclonal populations exist within the subset of $CD4^+$ $CD45RO^-$ cells before they acquire the CD45RO phenotype or whether $CD4^+$ $CD45RO^+$ T cells revert to the naive phenotype (13, 14). Data have been presented that the thymic selection of murine $CD4^-$ $CD8^-$ T cells is biased by dominant HLA–peptide complexes and that therefore clonotypes with identical amino acid sequences are generated during thymic selection (15, 16). We have sequenced multiple independently derived clonotypes from three patients and have not found evidence for sequence heterogeneity at the nucleotide level (reference 6 and data not shown). Thus, our evidence suggests that these clonotypes derived from the same progenitor cell and were not independently selected in the thymus.

$CD4^+$ $CD28^-$ T cells represent a very minor cell population in normal individuals and usually account for 0.1–2.5% of $CD4^+$ T cells (9). The biological function of these cells is unclear; however, the absence of the costimulatory molecule CD28 sets them apart from other $CD4^+$ T cells. CD7 is present on the majority of mature T cells, but expression is lost on a subset of $CD4^+$ memory T cells (10). Increased frequencies of $CD7^-$ T cells have been described in the blood and synovial compartment of RA patients (17). Expansion of the $CD7^-$ T cell subset is also a frequent finding in patients with HIV infection (18, 19). $CD7^-$ $CD4^+$ T cells may not represent an end stage of T cell differentiation, but may be associated with special function as suggested by the homing pattern of $CD7^-$ $CD4^+$ T cells. Sezary cells which are pathognomic for the leukemic variant of the cutaneous T cell lymphoma mycosis fungoides are typically $CD7^-$ (20). $CD7^-$ $CD4^+$ T cells also represent a major subpopulation of the infiltrates in inflammatory skin diseases suggesting that these cells tend to accumulate at inflammatory sites (21).

The sizes of the clonal T cell populations presented here are rather small, in the order of 0.1–1% of the total CD4 T cell repertoire. Thus, if all T cell clones are $CD7^-$ $CD28^-$ and the majority are $CD57^+$, one would expect a modest expansion of these compartments in RA patients. Results shown in Fig. 2 demonstrate that this is the case for the $CD7^-$ and the $CD28^-$ $CD4^+$ compartments. In some of the RA patients, the expansion of the $CD28^-$ $CD7^-$ $CD4^+$ T cell compartment was larger than one would expect from the presence of several clonotypes. 3 out of the 22 RA patients studied had a frequency of $CD28^-$ cells of $> 35\%$ of $CD4^+$ T cells. Also, the expansion of the $CD28^-$ subset in RA patients was much more pronounced than the expansion of the $CD4^+$ $CD57^+$ subset. These data suggest that the expansion of $CD28^-$ $CD4^+$ cells may not only be a consequence of clonal expansion but may be a genuine phenomenon in RA patients preceding clonal expansion. In support of this hypothesis, we have analyzed expanded $CD4^+$ $CD28^-$ populations sharing a BV element and have found that these populations also include polyclonally expanded T cells (data not shown). Interestingly, we were also able to identify a few normal donors who had elevated levels of $CD4^+$ $CD28^-$

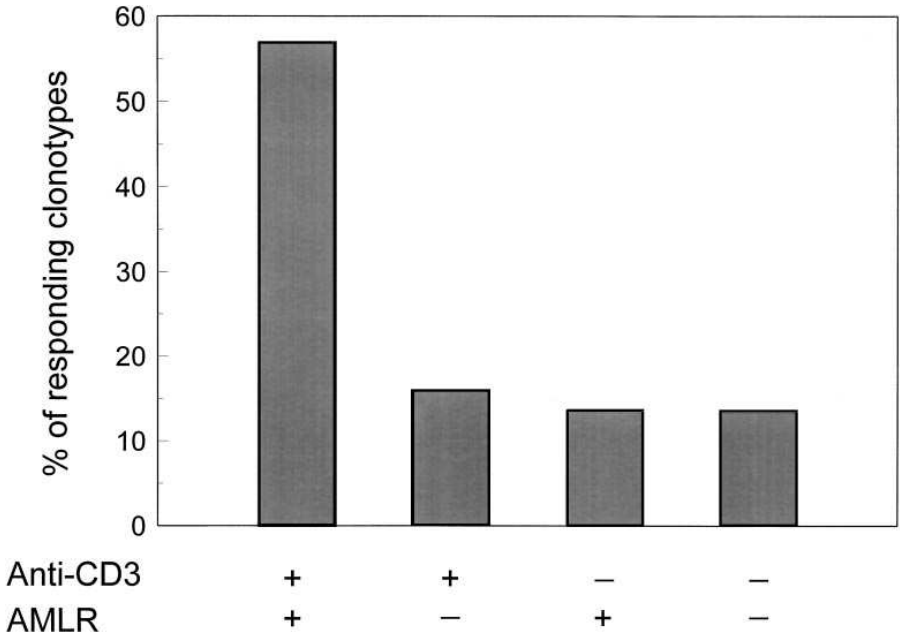

Figure 6. Responsiveness of expanded $CD4^+$ clonotypes. Purified $CD4^+$ T cells were stimulated with immobilized anti-CD3 for 5 d and with autologous irradiated mononuclear cells for 7 and 10 d. Total RNA was harvested and the T cell receptor repertoire before and after stimulation was compared by RT-PCR with BV-BJ specific primer sets and CDR3 length analysis. Expanded T cell clonotypes were identified and the percentage of clonotypes which persisted and expanded under the stimulation was determined. Results for 37 clones derived from 10 patients are summarized.

T cells when compared with most normals. Therefore, it is possible that a subset of normal individuals expresses a phenotype reminiscent of that found in RA patients, an interpretation which would be consistent with a genetic control of the $CD4^+$ $CD28^-$ T cell subset. The HLA-DR4 molecule appears not to be a risk factor to develop an expression of the $CD4^+$ $CD28^-$ subset. $HLA\text{-}DR4^+$ normal individuals did not have a higher number of $CD4^+$ $CD28^-$ cells (Fig. 2) and the expansion in the RA patients did not correlate with the expression of disease-associated HLA-DRB1 alleles.

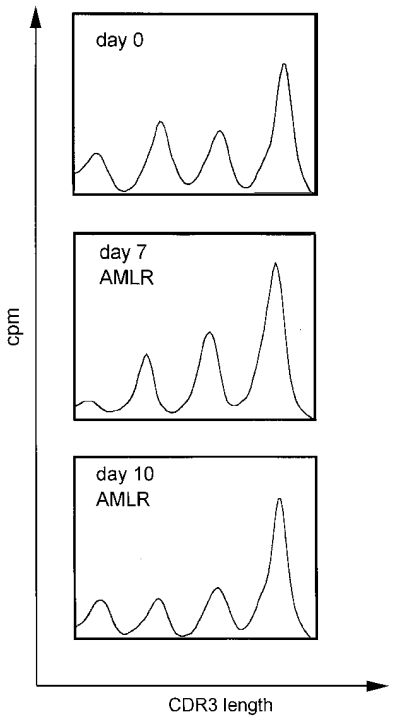

Figure 7. Semiquantification of clonally expanded $CD4^+$ T cells in the AMLR. PBMC depleted of $CD8^+$ T cells were stimulated with autologous peripheral blood adherent cells. Total RNA was obtained before and on days 7 and 10 of the AMLR. TCR sequences were amplified with BV-BJ primer sets and size fractionated as described in Fig. 1. Gels were scanned with phosphorimage analysis. An experiment representative of the T cell clones summarized in Fig. 6 is shown. Size fractionation of the PCR product of the TCR BV14-BJ1S3 combination did not show a Gaussian distribution of band intensities but yielded a dominant peak containing an expanded T cell clone. This clone persisted in the AMLR cultures.

It cannot be decided whether the high frequency of $CD28^-$ $CD4^+$ T cells predisposes for clonal expansion or whether the expansion of the $CD28^-$ compartment is the consequence of numerous $CD4^+$ T cell clones, many of which may be below the detection threshold. An intriguing observation is that clonally expanded $CD8^+$ T cells are also characterized by the lack of CD28 expression. $CD8^+$ $CD28^-$ T cells are not as uncommon as $CD4^+$ $CD28^-$ T cells. In fact, they represent a major fraction of the CD8 compartment (22). Clonality within the CD8 subset is a frequent finding in normal individuals, particularly in the elderly (23–25). However, clonal proliferation of peripheral $CD8^+$ T cells is more pronounced in RA patients than in normal individuals (26, 27). Interestingly, clonal CD8 populations share a phenotypic similarity with the expanded CD4 clonotypes identified in the RA patients. $CD4^+$ and $CD8^+$ cells which have undergone clonal amplification in vivo are $CD28^-$ and frequently $CD57^+$ (23, 25). The expression of the CD7 molecule has not been analyzed on $CD8^+$ T cells. In contrast to $CD4^+$ T cells, $CD8^+$ T cells generally maintain the expression of CD7. $CD7^-$ T cells in the peripheral blood are almost exclusively CD4. The common feature of lack of CD28 expression suggested that there might exist shared mechanisms controlling clonal downsizing of proliferating $CD4^+$ and $CD8^+$ T cells.

Programmed cell death represents a crucial mechanism controlling clonal outgrowth of peripheral T cells and thus appears to be essential in maintaining diversity in the TCR repertoire (28–31). The tight association of antigen-induced proliferation and activation-induced cell death (AICD) does not only serve as a mechanism preventing chronic amplification of selected T cell specificities but also leads to clonal exhaustion and subsequently to the inability of the immune system to recognize a chronically persisting antigen (32, 33). Boise et al. (34) have shown that stimulation of the CD28 pathway induces bcl-x_L expression. Thus, one would expect that $CD28^-$ T cells are extremely sensitive to apoptosis-inducing signals; however,

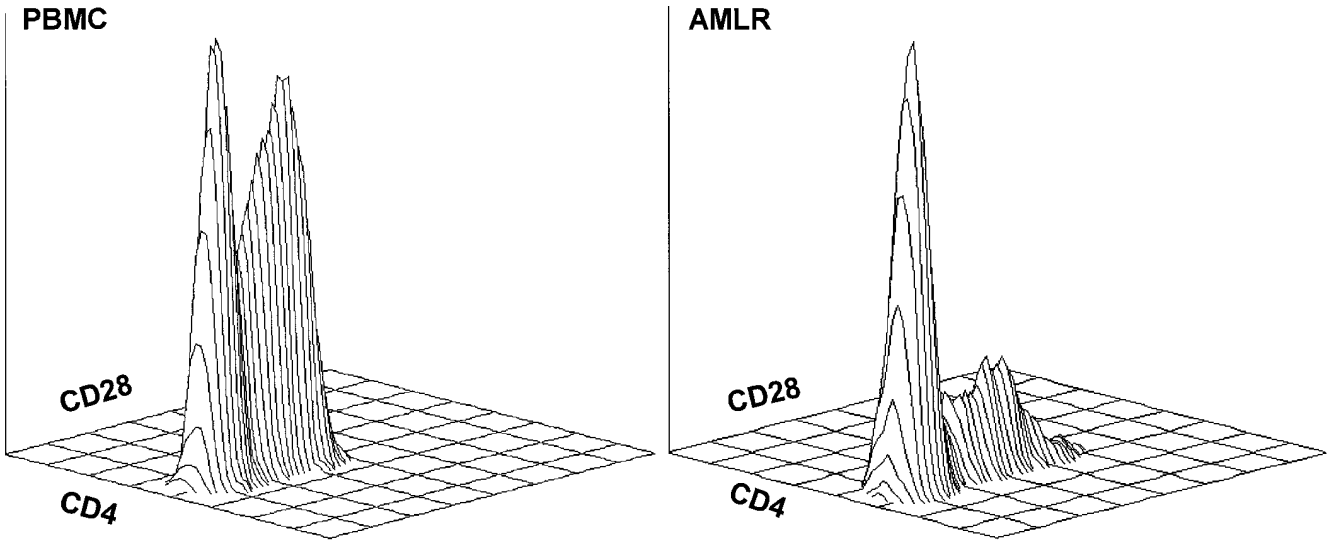

Figure 8. Selection of $CD4^+$ $C28^-$ T cells in the AMLR. $CD4^+$ T cells were analyzed before the AMLR and after restimulation with autologous adherent cells for the expression of the CD28 marker.

the in vivo expansion suggests that the opposite may be the case. Therefore, we tested the susceptibility of $CD4^+$ $CD28^-$ in vivo expanded T cell clones isolated from RA patients toward AICD and apoptosis induced by IL-2 withdrawal. After both apoptosis-inducing signals, expanded T cells were less susceptible to undergo programmed cell death (our unpublished observations).

Several reports have indicated that heterogeneity exists within the CD4 compartment regarding the responsiveness to apoptosis-inducing signals. CD45RO memory T cells express less bcl-2 than naive cells and are therefore more prone to undergo apoptosis when cultured in vitro in the absence of growth factors (35). Ramsdell et al. (36) have described differences between TH1 and TH2 cells in their ability for AICD. These authors have reported that TH1 cells synthesize Fas ligand upon activation and can therefore provide the signal for AICD. TH2 cells do not produce Fas ligand and therefore do not induce AICD. In contrast, Singer and Abbas (37) did not find a difference in AICD analyzing in vitro generated TH1 and TH2 T cell clones from a TCR transgenic mouse. The mechanism underlying the differential susceptibility to apoptosis-inducing signals in the $CD4^+$ $CD28^-$ T cell population is unclear. Preliminary experiments have shown that $CD28^-$ and $CD28^+$ T cell clones do not differ in their expression of bcl-2 (our unpublished observations). Also, we have analyzed the lymphokine pattern of the T cell clones isolated in vitro and have not found predominant production of TH2 specific cytokines.

The memory phenotype and the oligoclonality of $CD4^+$ $CD7^-$ $CD28^-$ T cells in RA suggest previous antigen contact of these cells. Several lines of evidence indicate that the expanded clonotypes are not only the result of defective downsizing but that they are also driven by antigen stimulation. Antigen selection is suggested by the restricted BV repertoire of

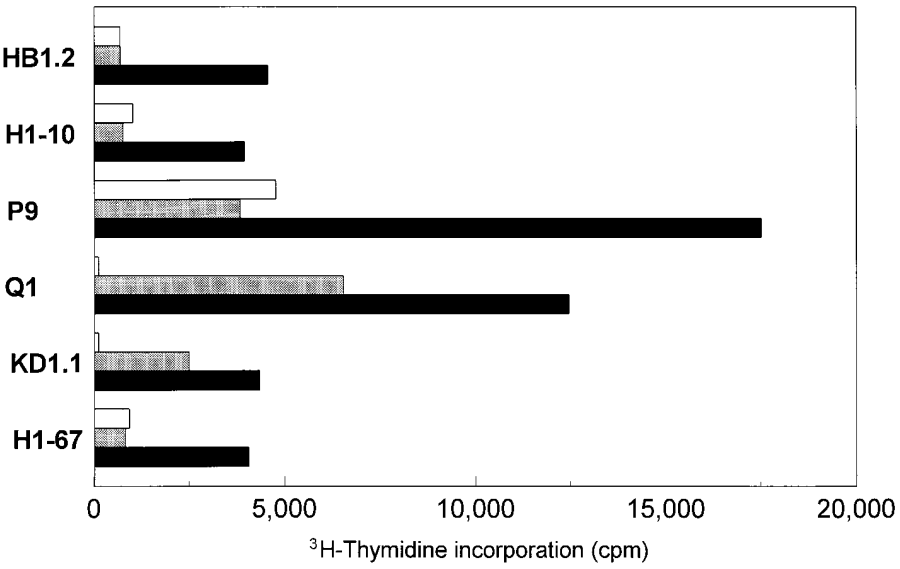

Figure 9. Proliferative response of in vivo expanded T cell clones to autologous APC. T cell clones which had been derived from in vivo expanded T cells of RA patients as described in Table II were cocultured with irradiated autologous PBMC (*solid bars*). Control cultures included T cell clones (*shaded bars*) or irradiated PBMC (*open bars*) only. Proliferative response was determined by [^{3}H]thymidine incorporation after a total culture period of 72 h. Results are shown as mean of triplicates.

the proliferating T cells. Most expanded cells use a BV3, 14, or 17 gene segment (6). We have not seen sequence similarities in the CDR3 region, which presumably contacts the antigen but their heterogeneity might be due to plasticity in the TCR repertoire of antigen-specific T cells. Expanded clonotypes within unseparated T cells as well as isolated T cell clones representing the clonally expanded population reacted to autologous APC, suggesting that they recognize ubiquitous self-antigens (Figs. 6 and 9).

It is not clear how the recognition of a ubiquitous autoantigen may relate to the synovial inflammation. The finding that the same clonotype is also $CD28^+$ in the synovial fluid but, almost exclusively, $CD28^-$ in the peripheral blood suggests that the synovial compartment provides a unique environment for these clonotypes. It is possible that $CD28^-$ T cells are activated in the synovia and express CD28; however, we have failed to induce CD28 expression in vitro in such cells. Alternatively, the $CD28^+$ variant of the clonotype may either preferentially home to the synovial fluid or these cells may have a selective advantage to proliferate in the synovia.

Several characteristics of $CD4^+$ $CD28^-$ cells identify them as a particularly interesting subset of cells in patients with RA. They are a consistent finding in RA and are infrequent in psoriatic polyarthritis, their repertoire is unique due to clonal expansion, they undergo apoptosis less efficiently, and they proliferate in response to autologous APC. Lymphoproliferation of $CD4^-$ $CD8^-$ T cells has recently received attention as a potentially important mechanism in the MRL/lpr and gld models of autoimmune disease (38, 39). Autoimmune phenomena in the MRL/lpr mouse are dependent on $CD4^+$ T cells (40). Whether a clonal outgrowth of selected $CD4^+$ T cells contributes to autoimmune disease in the MRL/lpr mouse is not understood. The disease in MRL/lpr mice shares many features with RA which are distinctly absent in other animal models of chronic inflammatory joint disease. MRL/lpr mice develop rheumatoid factor–positive disease with erosive destruction of joint tissues. Understanding lymphoproliferation and its role in disease manifestations might provide valuable information for both diseases.

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