The Repertoire of $CD4^+$ $CD28^-$ T Cells in Rheumatoid Arthritis

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ABSTRACT

Background: While oligoclonality of circulating $CD4^-$ $CD8^-$ and of $CD8^+$ T cells is not uncommon, clonal dominance within the CD4 compartment is not frequently found in healthy individuals. In contrast, the majority of patients with rheumatoid arthritis (RA) have clonally expanded $CD4^+$ T cell populations. Previous studies have demonstrated that these clonogenic $CD4^+$ T cells do not express the CD28 molecule. To examine the correlation between CD28 expression and clonal proliferation, we have analyzed the T cell receptor (TCR) diversity of $CD4^+$ $CD28^-$ T cells in normal individuals and in RA patients.

Material and Methods: The size of the peripheral blood $CD4^+$ $CD28^-$ compartment was determined in 30 healthy individuals and 30 RA patients by two-color FACS analysis. In 10 RA patients and five controls with more than 2.5% $CD4^+$ $CD28^-$ T cells, TCR BV gene segment usage was analyzed with 19 BV-specific antibodies. Oligoclonality was assessed in sorted $CD4^+$ $CD28^+$ and $CD28^-$ T cells using TCR BV-BC-specific polymerase chain reaction and size fractionation. Clonal dominance was confirmed by direct sequencing.

Results: The $CD4^+$ $CD28^-$ T cell compartment was expanded to more than 2.5% in 70% of the RA patients and 30% of the normal individuals. Compared with the $CD4^+$ $CD28^+$ T cells, the TCR BV gene segment usage among $CD4^+$ $CD28^-$ cells was grossly skewed with the dominance of single BV elements. Molecular TCR analysis provided evidence for oligoclonality in 17 of 21 expanded BV elements. In two unrelated RA patients who shared both HLA-DRB1 alleles, the TCR β-chain sequences of dominant clonotypes were highly conserved.

Conclusions: Oligoclonality is a characteristic feature of $CD4^+$ $CD28^-$ T cells which are expanded in some healthy individuals and in the majority of RA patients. The lack of CD28 expression is a common denominator of $CD4^+$, $CD8^+$, and $CD4^-$ $CD8^-$ T cells prone to develop clonal dominance. The limited TCR diversity of clonal $CD4^+$ $CD28^-$ populations in RA patients suggests that these T cells recognize a limited spectrum of antigens. The fact that the majority of individuals with marked expansions and oligoclonality of $CD4^+$ $CD28^-$ T cells are RA patients suggests a role for these unusual lymphocytes in the pathogenetic events leading to RA.

INTRODUCTION

Diversity of receptor molecules is considered one of the fundamental principles of T lymphocytes (1–3). The expression of different T cell receptors (TCR) equips the immune system to interact specifically with an enormously wide spectrum of antigen ligands. Receptor diversity is generated through a series of mechanisms including rearrangement of polymorphic TCR gene segments, addition of templated and nontemplated nucleotides, imprecise joining, and α-β-chain pairing.

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Recent data have, however, challenged the view that receptor diversity is a consistent hallmark of the functional T cell repertoire. Studies on specialized lymphocyte subsets have demonstrated that clonal expansion of selected T cell specificities may occur more frequently than previously thought (4–7). Clonal outgrowth of $CD8^+$ T cells is not unusual and seems to be more the rule than the exception (8). CD8 oligoclonality may more commonly occur with increasing age (6,9). Evidence for oligoclonality has also been provided for TCR α/β^+ $CD4^-$ $CD8^-$ T cells (4,5,10). When compared with single positive T cells in the peripheral blood, double negative T cells

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were found to possess unique phenotypic characteristics and to include clonally expanded populations (10). Sharing of sequence motifs in the third complementarity determining region (CDR3) by T cells utilizing different Vβ elements was interpreted as evidence for a common antigen ligand driving the clonal proliferation of double negative T cells.

Mechanisms leading to clonal outgrowth are not understood. Different models have been proposed in an attempt to explain oligoclonality of selected T cells. Possibly, the ligand specifically recognized by clonally expanded T cells is a self-antigen and mechanisms of peripheral tolerance are insufficient in these cells to avoid the expansion of autoreactive cells. Alternatively, expanded clonotypes represent the outcome of unique intrathymic differentiation pathways (11). Differences in selection pressures during thymic selection may indeed lead to the generation of T cell populations with unique functional capabilities. Finally, extrathymic development of T cells with distinguishing phenotypic features and unique repertoire characteristics may represent yet another possibility (12).

While clonal expansion of $CD4^-$ $CD8^-$ and of $CD8^+$ T cells is frequent, oligoclonality within the $CD4^+$ subset is rarely seen in normal individuals (9). We have recently described that oligoclonality of peripheral blood $CD4^+$ T cells occurs with increased frequency in patients with rheumatoid arthritis (RA) (13). Phenotypic studies on in vivo and isolated clonotypes have revealed that they can be distinguished from the majority of mature T cells through the lack of the CD28 molecule (14). While the role of clonal $CD4^+$ T cells in RA is not completely understood, the question arises whether the $CD4^+$ $CD28^-$ T cell compartment is restricted in its diversity and whether certain TCR genes are preferentially utilized by these cells. Here, we report that in RA patients and normals, expansion of the $CD4^+$ $CD28^-$ compartment is associated with clonal outgrowth of selected T cells. Restrictions in the TCR repertoire of clonally expanded T cells suggests that the spectrum of antigens recognized is limited.

MATERIAL AND METHODS

Study Population

Peripheral blood mononuclear cells (PBMC) were obtained from 30 patients who fulfilled the 1988 American College of Rheumatology criteria for the diagnosis of RA (15) and 30 normal individuals of the same age group who did not have a personal or family history of RA.

Fluorescence-Activated Cell Sorting Analysis

PBMC were separated from heparinized venous blood by Ficoll gradient centrifugation. Cells were stained with FITC-conjugated anti-CD4 (Becton Dickinson, San Jose, CA, U.S.A.) and phycoerythrin (PE)-conjugated anti-CD28 (Becton Dickinson) and analyzed on a fluorescence-activated cell sorting (FACS) scan. In 10 RA patients and in 5 normal individuals who had more than 2.5% $CD4^+$ $CD28^-$ cells, the BV gene segment usage was analyzed by three-color FACS analysis with FITC-conjugated BV-specific antibodies (anti-BV2, anti-BV3, anti-BV11, anti-BV13S1, anti-BV13S6, anti-BV14, anti-BV16, anti-BV17, anti-BV18, anti-BV20, anti-BV21S3, and anti-BV22, obtained from Immunotech, Marseilles, France; anti-BV5S1, anti-BV5S2-3, antiBV6S7, anti-BV8, and anti-BV12, obtained from T Cell Diagnostics, Cambridge, MA, U.S.A.; anti-BV7, kindly provided by A. Boylston; anti-BV23S1, kindly provided by O. Kanagawa; PercP-labeled anti-CD4 and PE-conjugated anti-CD28 (Becton Dickinson).

T Cell Receptor β-Chain Sequence Analysis

$CD4^+$ $CD28^-$ and $CD28^+$ cells were purified by cell sorting on a FACSVantage. Total RNA was extracted by guanidinium thiocyanate phenol chloroform extraction using a commercially available kit. cDNA was amplified with BV- and BC-specific primers (9,16). The amplified products were labeled using a primer extension assay and were separated on a 5% denaturing polyacrylamide gel. The distribution of band intensities were analyzed to determine whether the distribution was Gaussian, indicating polyclonality, or whether the diversity was restricted with the emergence of dominant bands, indicating clonal expansion (17,18). In parallel, the amplification products were directly sequenced by reverse transcriptase–mediated dideoxy sequencing as described (19). If the size analysis showed more than one dominant band, bands were eluted and directly sequenced. Previous experiments had shown that this approach yielded an unequivocal sequence if one particular clonotype

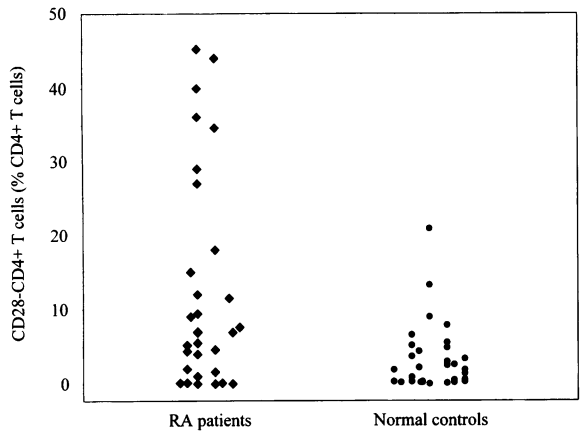

FIG. 1. Expansion of the CD4$^+$ CD28$^-$ compartment in RA patients

PBMC from 30 healthy individuals and 30 RA patients were analyzed by two-color FACS for the presence of CD4$^+$ CD28$^-$ T cells.

constituted more than 25% of T cells sharing the particular BV-BC combination (20). To confirm polyclonality, amplification products of selected samples were reamplified with the appropriate BV primer and a BJ primer. The sequences of the BJ primers have been described (13). Amplified products were again size-fractionated on a polyacrylamide gel.

RESULTS

Expansion of the CD4$^+$ CD28$^-$ T Cell Compartment in RA Patients

Most mature CD4$^+$ α/β T cells express the CD28 molecule which is critically involved in transmitting costimulatory signals (21,22). Optimal activation of T cells requires triggering of the specific TCR accompanied by costimulatory signals (23,24). In the absence of costimulation, T cells acquire a functional state of nonresponsiveness or anergy. Unless the function of CD28 can be substituted by an alternate molecule, CD4$^+$ CD28$^-$ T cells should be functionally impaired. In general, the frequency of CD4$^+$ CD28$^-$ T cells in human peripheral blood is low. Results for 30 normal donors are shown in Fig. 1. The majority of the normal individuals had few, if any, CD28$^-$ T cells within the CD4$^+$ population of the peripheral blood as determined by two-color FACS analysis. However, in a few individuals, CD28$^-$ T cells accounted for a significant proportion of the CD4$^+$ compartment. In contrast to healthy individuals, the CD4$^+$ CD28$^-$ compartment is largely expanded in patients with RA. Figure 1 shows a cohort of RA patients which was age- and sex-matched with the control group. While the group of RA patients included individuals with CD28$^-$ frequencies below 5% in the peripheral blood, more than half of the patients carried high numbers of CD4$^+$ CD28$^-$ T cells. In a subset of patients CD4$^+$ T cells lacking the CD28 surface marker accounted for one-third to almost one-half of the circulating CD4 compartment.

TCR Vβ Gene Usage in CD4$^+$ CD28$^-$ T Cells

To analyze the T cell repertoire of CD4$^+$ CD28$^-$ T cells in normal individuals and RA patients, the expression of 19 TCR BV gene elements was examined by three-color FACS analysis in 10 RA patients and five normal controls with an expanded subset of CD4$^+$ CD28$^-$ T cells. The 10 RA patients had between 4.6 and 34.6% CD4$^+$ CD28$^-$ T cells; in the five normal individuals, CD4$^+$ CD28$^-$ T cells ranged from 2.9 to 9.4% (Table 1). The BV gene segment distributions of three representative individuals are shown in Fig. 2. In Patient RA-9, BV18 and BV22 were majorly expanded. BV2 and BV20 did not show the expected concomitant decrease, while all the other BV elements were infrequent in the CD4$^+$ CD28$^-$ population. Similar results were obtained for Individual C-2. While BV6S7 and BV22 were majorly expanded, all BV elements except BV12 showed the expected concomitant decline. In the third individual, Patient RA-7, all BV elements within the window tested were infrequent in the CD4$^+$ CD28$^-$ population, suggesting that at least one of the BV elements which was not included in the test panel was markedly increased in frequency. Results from all RA patients and normal individuals are summarized in Table 1. All 10 RA patients and all 5 normal controls with expanded CD4$^+$ CD28$^-$ populations had significantly distorted BV repertoires compared with the CD4$^+$ CD28$^+$ population. In 7 of the 10 RA patients and in 4 of the 5 normal controls a dominance of one to three BV elements was observed. Comparison of the different patients did not show a strong bias as to which BV element was found to be expanded. Ten different BV elements were found in 13 expansions. Only the BV17 gene element was represented three times. Two of the patients with markedly increased BV17$^+$ CD28$^-$ cells typed HLA-DRB1*0101/0404 heterozygous. The BV18 element was found in two patients;

TABLE 1. TCR BV expansions in $CD4^+$ $CD28^-$ T cells

	HLA-DRB1 Allele	$CD4^+$ $CD28^-$ Compartment Size (%)	BV Families with Expansion in the $CD28^-$ Compartment
RA Patients			
RA-1	0401/08	4.6	BV17
RA-2	0404/0101	4.9	BV14, BV17
RA-3	0404/0101	7.1	BV6S7, BV17
RA-4	0401/11	9.9	None detected
RA-5	0401/07	13	BV7
RA-6	03/03	17	BV5S1, BV21S3, BV22
RA-7	0404/0404	19.3	None detected
RA-8	15/15	22.4	None detected
RA-9	0401/03	29	BV18, BV22
RA-10	0401/03	34.6	BV13, BV18
Controls			
C-1	0101/0101	2.9	BV6S7, BV20
C-2	0401/0101	5.1	BV6S7, BV22
C-3	0101/07	6.6	BV2, BV13S1
C-4	0404/15	7.9	None detected
C-5	04/07	9.4	BV2, BV21S3

both of whom also shared both DR haplotypes (DRB1*03/0401). BV22 was encountered in two patients, however, these two patients only shared one HLA-DRB1 allele. A similar diversity was seen in the five normal individuals. Increased usage of BV2 and BV6S7 was documented in two donors each.

Oligoclonality within the $CD4^+$ $CD28^-$ T Cell Population

To determine whether the preferential usage of certain BV gene segments within the $CD4^+$ $CD28^-$ compartment was caused by a polyclonal BV-specific stimulation or by the clonal proliferation of single T cell clonotypes, the diversity of the repertoire of T cells sharing a BV element was analyzed. $CD4^+$ $CD28^-$ T cells were purified from PBMC by cell sorting. TCR β-chain sequences were amplified by polymerase chain reaction (PCR) using primers specific for the expanded BV gene segment and a BC primer. The amplified product was subsequently size-fractionated. Representative experiments on three BV elements from four patients are shown in Fig. 3. In all patients, the population of $CD4^+$ $CD28^+$ T cells showed a polyclonal pattern; that is, the bands representing the different TCR size classes were spaced by three nucleotides and the intensities of the bands followed a Gaussian distribution. In contrast, the repertoire of $CD4^+$ $CD28^-$ T cells sharing an expanded BV element showed a distorted Gaussian distribution with the dominance of single bands in 17 out of 21 BV-BC combinations analyzed (Fig. 3). All amplified products that were suspicious for clonal proliferation of a single T cell specificity were analyzed by direct sequencing of the total PCR product to confirm clonality. Oligoclonality was found in RA patients as well as in the normal controls. In 14 of the 21 expanded BV elements, a single clonotype was identified. In three BV elements, several clonotypes were found. TCR length analysis demonstrated a polyclonal pattern in four amplified products (data not shown). To address the question how oligoclonal the $CD4^+$ $CD28^-$ compartment is in general, selected BV-BC amplification products were reanalyzed at the BV-BJ level. This approach allows to detect clonotypes which are less frequent than 1%. Size fractionation of different BV18-BJ products of patient RA-10 are shown in Fig. 4. The Gaussian distribution of band intensities were maintained, demonstrating polyclonality of

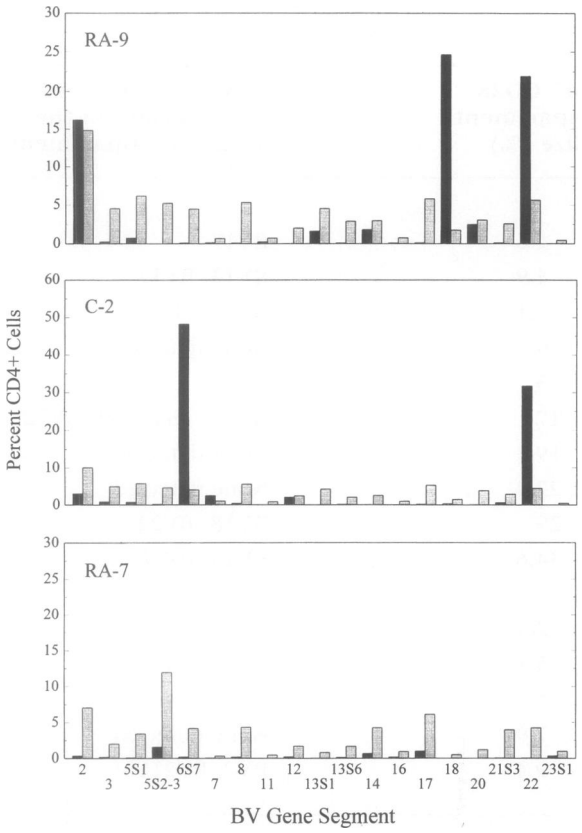

FIG. 2. TCR BV gene segment usage in $CD4^+$ $CD28^-$ T cells

Five normal individuals and 10 RA patients were identified who had more than 2.5% of $CD4^+$ $CD28^-$ T cells (Fig. 1). PBMC were analyzed by three-color FACS analysis with PercP–anti-CD4, PE–anti-CD28, and FITC–anti-BV element. Compared with the $CD4^+$ $CD28^+$ compartment (▨), the TCR β-chain repertoire of $CD4^+$ $CD28^-$ T cells (■) was strongly skewed toward the preferential usage of few BV elements. Three representative individuals are shown; results for all normal individuals and RA patients are summarized in Table 1.

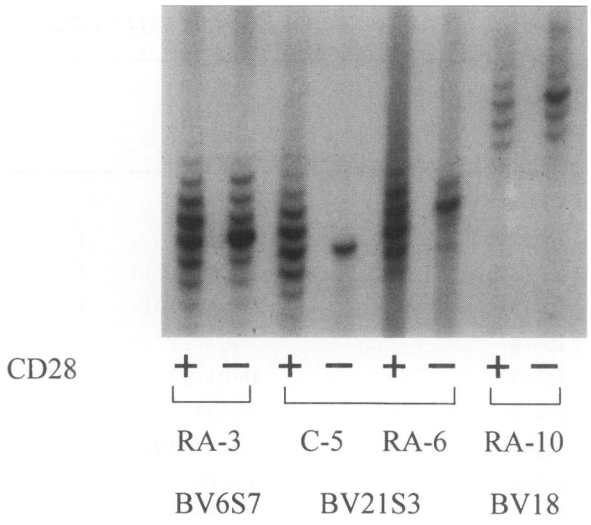

FIG. 3. Clonal T cell expansions within the $CD4^+$ $CD28^-$ compartment

To examine the nature of the skewed BV gene segment usage, the TCR repertoire of $CD4^+$ $CD28^+$ and $CD4^-$ $CD28^-$ T cells were analyzed by BV-BC-specific PCR of TCR sequences and subsequent size fractionation. Results for four BV-BC combinations from three individuals are shown. The $CD4^+$ $CD28^+$ population always showed a Gaussian distribution of band intensities, suggesting polyclonality. In contrast, dominant bands emerged in the $CD4^+$ $CD28^-$ T cells. Clonality was confirmed by sequencing (Table 2). Of the 21 expanded BV elements (Table 1), 17 harbored one or more T cell clones.

the population. These data suggest that the $CD4^+$ $CD28^-$ compartment is primarily polyclonal. Oligoclonality is frequently found for expanded BV elements, however, clonal expansion is not always responsible for the increased BV gene segment usage.

To estimate the contribution of clonal expansion to the increased size of the $CD4^+$ $CD28^-$ compartment, the total number of clonotypes within one patient, the size of individual clonotypes and the total size of the $CD4^+$ $CD28^-$ compartment which ranged from 2.9 to 34.6% were compared. The size of the $CD4^+$ $CD28^-$ compartment did not correlate to the total number of clonally expanded T cell specificities or with the size of the individual clonotypes (data not shown). In contrast, patients who had less of a skewing in the BV repertoire tended to have a larger $CD4^+$ $CD28^-$ compartment, suggesting that the initial expansion of the $CD4^+$ $CD28^-$ compartment is polyclonal.

Conserved Junctional TCR Sequence Motifs

All BV-specific TCR products that contained dominant bands suggestive for expanded clonotypes were directly sequenced to confirm clonality (Table 2). If the PCR products contained more than one dominant band, individual bands were eluted and sequenced. In two instances, distinct donors shared amino acid sequence motifs in the V-D-J junction. In Patient RA-9, an expanded BV18-BJ2S3 clonotype was found. Patient RA-10 had a BV18-BJ2S5 clonotype. Those clonotypes had the identical junctional motif (PELAGP) and differed in the CDR3 only

TABLE 2. TCR β-chain sequences of dominant CD4+ CD28− T cell clones

	BV Segment	CDR3 Sequence	BJ Segment
RA patients			
RA-1	BV17	CASS TRVK NTEAFF	BJ1S1
RA-2	BV14	CASS LREG EQFF	BJ2S1
	BV17	CASS TSGQV TDTQYF	BJ2S3
RA-3	BV6S7	Polyclonal	
	BV17	CASS QDR NSPLHF	BJ1S6
RA-5	BV7	CASS PEDSPR ETQYF	BJ2S5
RA-6	BV5S1	Polyclonal	
	BV21S3	CASS DNGRRMA TF	BJ1S2
	BV22	CASS EEN TQYF	BJ2S5
RA-9	BV18	CASS PELAGP DTQYF	BJ2S3
	BV18	CASS PQRS NYGYTF	BJ1S2
	BV18	CASS PGYSGSDD EQYF	BJ2S7
	BV22	CASS ELWQ SGANVLTF	BJ2S6
	BV22	CASS SYLHREG QFF	BJ2S1
RA-10	BV13	CASS YPETG SYEQYF	BJ2S7
	BV18	CASS PELAGP ETQYF	BJ2S5
Controls			
C-1	BV6.7	CASS VRDER NEKLFF	BJ1S4
	BV20	Polyclonal	
C-2	BV6.7	CASS PHYREG DTQYF	BJ2S3
	BV22	CASS EASPQGAG GNTIYF	BJ1S3
C-3	BV2	Polyclonal	
	BV13S1	CASS QDR NTEAFF	BJ1S1
C-5	BV2	CASS RSRLARK DTQYF	BJ2S3
	BV2	CASS AASRGWLTMA LFF	BJ1S4
	BV21S3	CASS QLF QETQYF	BJ2S5

in an aspartic acid versus glutamic acid substitution in the J gene segment. In contrast to the amino acid sequence, the nucleotide sequence differed at three positions (Table 3). It is of particular interest to note that these two patients shared both HLA-DRB1 alleles. Both patients typed HLA-DRB1*03/0401. Within the group of 10 patients, Patients RA-2 and RA-3

TABLE 3. Nucleotide sequences of TCR β-chains with shared amino acid motifs

Patient	HLA-DRB1 Allele	BV Gene Segment	N-D-N Region Sequence						BJ Gene Segment
RA-9	DRB1*0401/03	BV18	**P**	**E**	**L**	**A**	**G**	**P**	BJ2S3
			CC**A**	GAA	CTA	GC**G**	GG**A**	CC**A**	
RA-10	DRB1*0401/03	BV18	**P**	**E**	**L**	**A**	**G**	**P**	BJ2S5
			CC**C**	GAA	CTA	GCG	GG**G**	CC**G**	

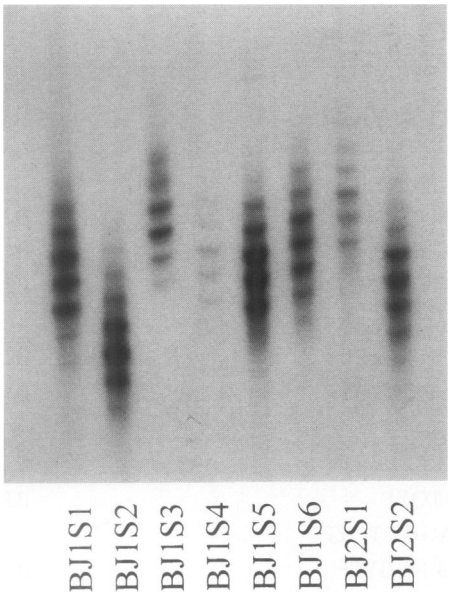

FIG. 4. Diversity of $CD4^+$ $CD28^-$ T cells

To examine the question of whether the repertoire of $CD4^+$ $CD28^-$ T cells is generally restricted or whether few T cell clones have emerged from a polyclonal repertoire, TCR sequences sharing selected BV elements were reamplified with BV- and BJ-specific primers and size-fractionated. Results are shown for BV18-BJ combinations from the same patient, all of which had a Gaussian distribution of band intensities, suggesting polyclonality. No additional dominant clonotype was identified at the more sensitive resolution level, indicating that the $CD4^+$ $CD28^-$ T cell compartment is primarily polyclonal with the emergence of single clonotypes.

also shared both HLA-DRB1 alleles (HLA-DRB1*01/0404). These patients had expanded $BV17^+$ T cell clones, however, with different junctional sequences. A second shared junctional sequence motif was identified in Patient RA-3 and in C-3. Both individuals shared a QDRN motif. However, the amino acid stretch was present in different frameworks (BV17-BJ1S6 in Patient RA-3 and BV13S1-BJ1S1 in C-3). Patient RA-3 and the control individual both expressed an HLA-DRB1*01 allele but were discordant for the second HLA-DR haplotype.

DISCUSSION

Based on the observation that RA patients carry multiple clonal $CD4^+$ T cell populations with a $CD28^-$ phenotype (14), the current study was designed to examine the repertoire of $CD4^+$ $CD28^-$ T cells in normal individuals and RA patients. Lack of the CD28 molecule on the T cell surface should have consequences for T cell function (23,24). Triggering of the TCR by specific antigen is considered insufficient to induce expansion and lymphokine secretion unless supplemented by costimulatory signals. Interaction of the CD28 molecule with its ligands on the surface of antigen presenting cells provides the necessary costimulatory signals to drive T cell activation. However, accumulating evidence indicates that $CD28^-$ cells are not always functionally impaired but may contribute significantly to immune responses. Data presented here indicate that repertoire formation follows different rules in $CD28^-$ than $CD28^+$ cells with the frequent emergence of oligoclonality. This observation may not be restricted to the CD4 compartment; it may also apply to the $CD28^-$ subsets of $CD8^+$ and $CD4^-$ $CD8^-$ T cells. Clonally expanded $CD4^+$ T cells appear to be nonrandomly selected with very similar TCR β-chain sequences isolated from different individuals. These findings are most compatible with the model that a very limited spectrum of antigens drives the response of $CD4^+$ $CD28^-$ T cells.

In contrast to the murine system, $CD28^-$ T cells in humans constitute a significant part of the immune system. Approximately half of the circulating $CD8^+$ as well as the $CD4^-$ $CD8^-$ T cells do not express the CD28 molecule. Among $CD4^+$ T cells, CD28 negativity is generally infrequent (22). However, we and others have found that a subset of normal donors can carry more than 5% of $CD4^+$ $CD28^-$ T cells (21). Expansion of the $CD4^+$ $CD28^-$ T cell subset is a characteristic finding for patients with RA. About two-thirds of all RA patients express a high frequency of $CD4^+$ $CD28^-$ T cells with up to 50% of all $CD4^+$ T cells missing the CD28 molecule. Expansion of the $CD4^+$ $CD28^-$ compartment is a feature of patients with more severe disease. Particularly, the presence of highly frequent $CD4^+$ $CD28^-$ T cells is tightly associated with the development of extraarticular manifestations, such as rheumatoid organ involvement (own unpublished observations). Longitudinal studies suggest that the emergence of $CD28^-$ T cells is not a consequence of the disease but rather can precede disease onset (Ref. 20 and our unpublished observations). The molecular and functional characteristics of $CD4^+$ $CD28^-$ T cells are of particular interest since they can be suspected to play a role in the disease process. The two characteristics of the $CD4^+$ $CD28^-$ compartment studied here, expansion and oligoclonality, were

not specific for RA patients but were more frequent and more pronounced in patients than in normal controls.

Oligoclonality of T cells is not as uncommon as previously believed. Among $CD8^+$ T cells, it appears to be a regular phenomenon. In a study of 56 normal individuals, 72% exhibited evidence for oligoclonal T cell growth of $CD8^+$ T cells (9). The repertoire of $CD8^+$ clonotypes was found to be highly diverse, and no examples of TCR sequence homologies were detected in unrelated controls and siblings. Interestingly, in three of six sets of monozygotic twins clones with extensive sequence homology were described, suggesting that genetic factors are critical in selecting clonal specificities prone to expand. Other predisposing factors include age and possibly the presence of a chronic inflammatory disease (6,8,25,26).

Readily detectable oligoclonality has also been described for the $CD4^-$ $CD8^-$ TCR α/β T cell compartment. The diversity of expanded clonotypes among double negative T cells appears to be more restricted than among CD8 cells. Porcelli et al. (4) have demonstrated that $CD4^-$ $CD8^-$ T cells preferentially utilize a restricted spectrum of BV genes and an invariant TCR α-chain. In the study by Brooks et al. (10), oligoclonality of $CD4^-$ $CD8^-$ T cells involved all different BV families. However, these authors were able to identify several junctional region amino acid sequence motifs combined with different BV elements which were conserved in TCRs isolated from distinct donors. Finally, Delabonna et al. (5) were able to show the utilization of an invariant AV20-BJQ/BV11 TCR by clonogenic $CD4^-$ $CD8^-$ T cells. This particular TCR could be identified in every individual tested. The $AV24^+$ $CD4^-$ $CD8^-$ T cells derived from a single or a few expanded T cell clones, as demonstrated by γ-chain rearrangement and N-D-N region diversity (5,27).

In contrast to $CD8^+$ and $CD4^-$ $CD8^-$ T cells, oligoclonality within the CD4 compartment in normal individuals is much less pronounced. Montaro et al. (9) did not find any instance of CD4 T cell clonal dominance in 12 normal individuals. In contrast, we have frequently observed clonal expansion of $CD4^+$ T cells in the peripheral blood compartment of RA patients (13). In general, the size of the $CD4^+$ T cell clones is smaller than that of the CD8 T cell clones. Subsequent studies showed that the clonally expanded CD4 T cells in RA patients had a unique cell surface phenotype and that they lack the expression of the CD28 and the CD7 molecules (14). It is possible that genetic factors determine the propensity to develop clonogenic T cell populations. Sibpair studies in RA multicase families demonstrated that oligoclonality in the CD4 compartment is equally frequent in affected and unaffected siblings (13). This observation raises the possibility that genetic factors regulate the size and the diversity of the $CD28^-$ $CD4^+$ compartment.

By focussing on $CD4^+$ $CD28^-$ T cells in this study, we could clearly demonstrate that oligoclonality is a general phenomenon in this compartment. In 7 of the 10 RA patients and 4 of the 5 normal individuals with expanded $CD4^+$ $CD28^-$ populations, clonal T cell expansions were identified. The three patients and the one normal control in whom expanded clonotypes were not found had a grossly distorted BV gene segment repertoire, suggesting that these donors had clonal expansion involving BV gene elements for which no specific antibody was available.

The finding of sequence homologies in the TCR β-chains in unrelated individuals suggests that the repertoire of clonally expanded T cells is highly restricted. From two RA patients, clonotypes were isolated that shared the identical BV gene segment, identical amino acid motifs in the N-D-N region, and identical CDR3 length but differed in the BJ gene segment usage (BJ2S3 versus BJ2S5). Interestingly, these two RA patients expressed identical HLA-DRB1 genes on both haplotypes. Sequence homology was also found for a clone derived from an RA patient and a normal donor. In this case, the same N-D-N region amino acid motif was combined with different BV and BJ gene elements. Conserved structural features of receptors used by clonally expanded populations are highly suggestive for shared antigen driving the expansion (28–30). Our data suggest that the spectrum of antigens recognized by $CD4^+$ $CD28^-$ T cells is limited and closely related TCRs are selected in the setting of shared HLA-DR molecules.

The nature of the antigens driving the clonal proliferation remains elusive. Among CD8 T cells, sequence homologies expressed by dominant clonotypes were only found for monozygotic twins and not for siblings or adoptees, emphasizing the impact of shared genetic determinants rather than similar environmental factors (9). In contrast, within $CD4^+$ $CD28^-$ (Table 2) and $CD4^-$ $CD8^-$ $CD28^-$ T cells (4,5,10) TCR β-chains with similar sequence motifs have

been derived from unrelated individuals. Provided this difference reflects distinct rules in the formation of the TCR repertoire of these T cell subsets, it could be proposed that the antigen spectrum recognized is subset specific. $CD4^-$ $CD8^-$ T cells have been demonstrated to be reactive to microbial antigens which they recognize in restriction to CD1 molecules (31). We have evidence that $CD4^+$ T cell clones in RA patients exhibit autoreactivity and proliferate in response to autologous antigen presenting cells (14). It is intriguing that the individuals from whom TCR β-chains with almost identical sequences were isolated shared both HLA-DR haplotypes. This suggests that these T cells were HLA-DR restricted and that HLA-DR–imposed repertoire selection is important in shaping the selection of clonotypes undergoing clonal proliferation. Also, both donors had active RA, raising the possibility that a disease-related antigen is recognized.

Data presented here add to the evidence that CD28 negativity is associated with oligoclonality. Lack of CD28 expression is the common denominator among $CD4^+$, $CD8^+$, and $CD4^-$ $CD8^-$ T cell subpopulations harboring dominant clonotypes. It cannot be excluded that the absence of $CD28^-$-transmitted signals is directly involved in the clonal expansion. However, the CD28 pathway has been shown to be important in the prevention of apoptosis by inducing Bcl-x_L expression and not in clonal downsizing (32). Experiments in the CD28-deficient mouse have shown that the absence of CD28 does not favor peripheral clonal proliferation (33). In contrast, $CD28^+$ T cells generally outcompete their $CD28^-$ counterpart in bone marrow chimeras of $CD28^-$ and $CD28^+$ cells. In contrast to CD28, CTLA-4, which also binds to the CD80/CD86 molecules, has been implicated in clonal downsizing (34–36). However, CTLA-4 can be induced in $CD4^+$ $CD28^-$ T cell clones (our unpublished observations), suggesting that the lack of CD28 expression is not associated with an inability to upregulate CTLA-4.

It is also possible that human $CD28^-$ T cells are independent from costimulatory signals or use different signals and therefore can escape peripheral tolerance mechanisms. Since expansion of the $CD4^+$ $CD28^-$ compartment and clonal dominance are not necessarily linked (Fig. 4), an increase in the number of $CD28^-$ cells may be the primary event rendering the individual susceptible to oligoclonal proliferation. Shifts in the size of the $CD28^-$ compartment may only occur in predisposed individuals resulting in the heterogeneity of $CD4^+$ $CD28^-$ frequencies encountered in the normal population. Accumulation of individuals with high concentrations of $CD28^-$ T cells among RA patients suggests a role of these cells in chronic inflammatory processes. Understanding the nature and function of costimulatory molecules in $CD28^-$ cells may therefore be crucial in exploring peripheral tolerization mechanisms of these particular cells as well as their contribution to RA. We have preliminary evidence that $CD4^+$ $CD28^-$ T cells require costimulatory signals for the expression of the IL-2 receptor α-chain and the prevention of anergy induction. These costimulatory signals do not involve the CD28/CTLA-4-CD80/CD86 interaction. Alternate costimulatory molecules have also been suggested by Behar et al. (37), studying $CD4^-$ $CD8^-$ $CD28^-$ T cells. These cells utilize costimulatory molecules expressed on activated monocytes but not on lymphoblastoid B cell lines. Differential expression of costimulatory molecules for $CD28^-$ cells on accessory cells may be relevant to determine the functional profile of $CD4^+$ $CD28^-$ T cells and may contribute to their role in RA.

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