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Evidence for Clonal Selection of γ/δ T Cells in Response to a Human Pathogen

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Summary

T cells bearing γ/δ antigen receptors comprise a resident population of intraepithelial lymphocytes in organs such as skin, gut, and lungs, where they are strategically located to contribute to the initial defense against infection. An important unsolved question about antigen-driven γ/δ T cell responses regards the breadth of their T cell receptor (TCR) repertoire, since many specific epithelial compartments in mice display limited diversity. We have examined the diversity of TCR δ gene expression among human γ/δ T cells from skin lesions induced by intradermal challenge with *Mycobacterium leprae*. We show that the vast majority of γ/δ cells from *M. leprae* lesions use either Vδ1-Jδ1 or Vδ2-Jδ1 gene rearrangements and, within a given region of the lesion, display limited junctional diversity. This contrasts markedly with the extensive diversity of γ/δ T cells from peripheral blood of these same individuals, as well as skin from normal donors. These results indicate that the γ/δ response to *M. leprae* involves the selection of a limited number of clones from among a diverse repertoire, probably in response to specific mycobacterial and/or host antigens.

One of the fundamental aspects of cellular immunology is the selection and clonal expression of T cells bearing specific receptors by antigens. While the selection of α/β T cells has been studied in detail, little is known about the selection of γ/δ T cells by antigen. The genetic diversity of the TCR provides a measure of the scope of the T cell repertoire. In contrast to the α/β TCR, the germline gene segment diversity for both the TCR γ and δ chains is small. The further limitation of this diversity by the preferential usage of only a few variable (V) genes or V gene pairs at specific anatomical locations (1–3) suggests a particularly narrow TCR repertoire, likely reflecting recognition of a limited number of ligands. On the other hand, there is unprecedented junctional diversity, particular in the δ chains of this receptor (4–6). We reasoned that analysis of the TCR γ/δ repertoire of a specific immune response would provide clues about the set of antigens recognized: whether diverse or limited, conventional, or superantigen-like. The presence of antigen-reactive TCR γ/δ-bearing cells in leprosy skin lesions (7) provides a unique in vivo model to examine the γ/δ receptor repertoire at the site of immunopathologic reaction.

Much experimental evidence indicates that γ/δ T cells contribute to the granuloma formation in response to mycobacterial infection (7). First, TCR γ/δ cells comprise a strikingly high percentage of the T cell population in infectious disease lesions that contain recently formed granulomas. These include lepromin skin tests (Mitsuda reactions), which are experimental DTH reactions induced by intradermal injection of *Mycobacterium leprae*, reversal reactions in leprosy, which represent a naturally occurring DTH response to *M. leprae*, and localized American cutaneous leishmaniasis. Second, the γ/δ T cells from these infectious lesions proliferate to mycobacterial antigens in vitro. Third, the γ/δ T cells from lesions appear to release a lymphokine(s) that synergizes with granulocyte/macrophage (GM)-CSF to induce macrophage adhesion, aggregation, and proliferation, cellular events that would be necessary for the granulomatous response.

The present study was undertaken to determine the distribution and TCR diversity of γ/δ T cell subpopulations in lepromin skin tests. This was accomplished by immunopathologic analysis according to Vδ chain expression and molecular analysis of Vδ, Jδ, and junctional elements. The repertoire in these infectious lesions was compared with the

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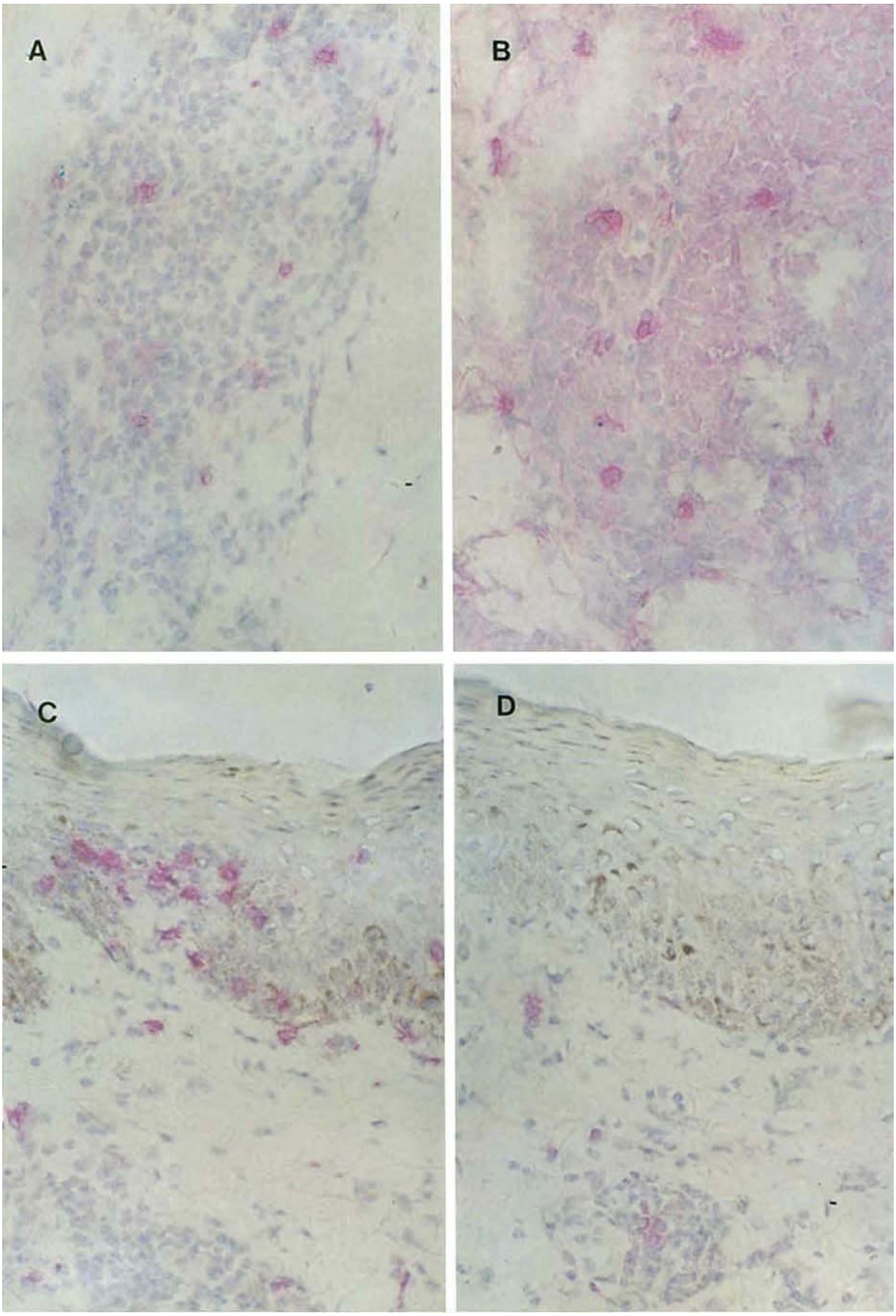
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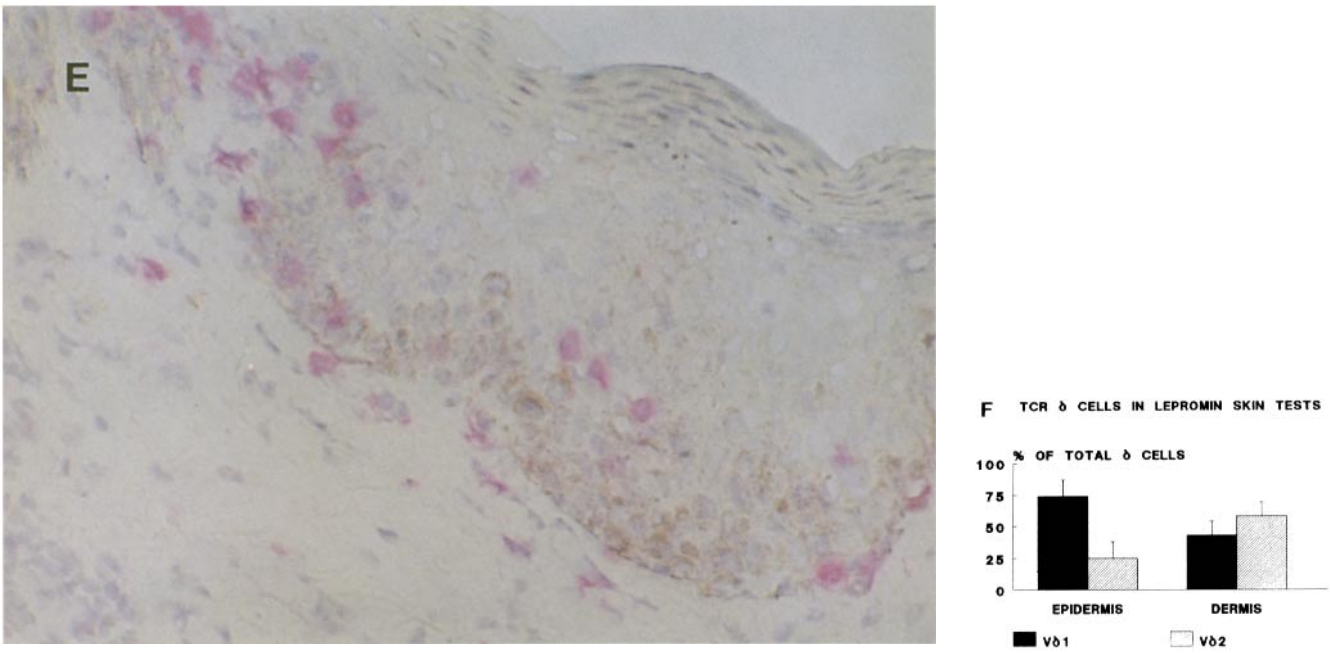


Figure 1 A–F. Immunohistochemical analysis of Vδ phenotypes in lesions and peripheral blood. Both Vδ1 (*A*) and Vδ2 (*B*) -bearing T cells were present in the dermal granulomas of lepromin skin tests. In contrast, Vδ1 cells (*C*) were present in the epidermis in greater numbers than Vδ2 cells (*D*), which were virtually absent. A more extensive view of the epidermis (*E*) shows Vδ1 cells throughout. The percentages of Vδ1- and Vδ2-bearing T cells (± SD) are shown in *F*.

peripheral blood of the same individual and the repertoire in normal human skin.

Materials and Methods

Immunoperoxidase Staining of Biopsies. Lepromin skin tests (3-wk Mitsuda reactions) were studied from 14 tuberculoid leprosy patients (8). All specimens were obtained with informed consent. The patients were distributed among the different diagnostic groups showing no segregation according to sex, race, or age. Skin biopsy specimens were obtained by punch or scalpel technique at the time of diagnosis, embedded in OCT medium (Ames Co., Elkhart, IN), and snap frozen in liquid nitrogen. The tissues were stored at −70°C until sectioning. Sections (3–5 μm) were acetone fixed before undergoing incubations with the mAbs for 45 min. mAbs included the Cδ-specific antibody anti-TCRδ1 (9) (1:100, T-cell Sciences, Boston, MA), the Vδ1-Jδ1-specific δ antibody, δTCS1 (1:10, T-cell Sciences), and the Vδ2-specific antibody, BB3 (1:3,000; a generous gift of Dr. L. Moretta, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). The results obtained with δTCS1 were corroborated with the Vδ1-reactive antibody A13 (1:1,000; kindly provided by Dr. S. Ferrini, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). The primary antibody was visualized using the alkaline phosphatase anti-alkaline phosphatase technique (Dako Corp., Carpinteria, CA). Slides were sequentially incubated with rabbit anti-mouse antibody for 30 min, alkaline phosphatase mouse anti-alkaline phosphatase immune complex for 30 min, and then developed with Vector Red substrate for 30 min (Vector Laboratories, Burlingame, CA). Slides were washed in Tris buffer between incubations. Endogenous alkaline phosphatase was blocked using 125 mM levamisole (Vector Laboratories). Slides were counterstained with hematoxylin and then mounted in glycerine-gelatin. Some slides were stained using the ABC Elite system (Vector Laboratories). The numbers of Vδ-positive cells in the epidermis and dermis of an entire tissue section (~5 × 5 mm) were enumerated and expressed as the percentage of total TCRδ1$^+$ cells in each microanatomic location. Peripheral blood was obtained from the same individuals and analyzed by flow cytometry.

PCR Analysis. 20 5-μm sections from each of five lepromin skin tests were placed in 0.5 ml of 4 M guanidinium isothiocyanate buffer. Additionally, 10^6 PBMC were obtained by Ficoll-Hypaque density centrifugation. Genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation with yields of 1–3 μg of DNA. For each PCR reaction, ~100 ng of DNA was used in a 25-μl reaction. By calculating the numbers of γ/δ cells/mm^2 of granuloma as detailed previously (10), and knowing the volume of tissue utilized, we determined that each PCR reaction of lesions contained DNA from ~500 γ/δ T cells. Similar calculations for peripheral blood indicated identical numbers of γ/δ T cells per each reaction. Titration of Vδ-Jδ containing plasmid confirmed that the sensitivity of our PCR amplification was on the order of 10^2 or 10^3 copies. PCR amplification mixtures contained 10 pmol of each oligonucleotide primer, 2.5 U Taq polymerase (Promega Corp., Madison, WI), and 2.5 mM $MgCl_2$ in PCR buffer (Promega Corp.). Oligonucleotide primers were tested on a panel of γ/δ T cell clones with known rearrangements. Sequences of oligonucleotide primers were selected to yield ~300 bp products (11). 30 cycles of PCR amplification were performed using the following conditions: 1 min of denaturation at 94°C and 2 min of annealing/extension at 65°C. Amplified products were subjected to electrophoresis on 1.5% agarose gels and visualized as single bands by ethidium bromide. Verification of product was accomplished by nucleotide sequencing. Two skin biopsy specimens were obtained from normal donors after informed consent. DNA was extracted from the entire 4-mm punch biopsy and 100 ng used for PCR amplification followed by sequencing of the V-J junctions.

Nucleotide Sequence Analysis of TCR δ V-J Junctions. To facilitate cloning into sequencing vector, oligonucleotide primers for

PCR amplification were designed to contain a SalI or EcoR1 restriction site in Vδ and Jδ primers, respectively. Amplified product was digested with both restriction enzymes and gel purified. Purified products were ligated into pUC18 vector and used to transform DH5α (BRL Laboratories, Gaithersburg, MD) and selected for ampicillin resistance. Plasmid DNA from insert containing colonies was sequenced (Sequenase 2.0 Kit; U.S. Biochemicals, Cleveland, OH) using the method of Sanger et al. (12). Precautions taken to avoid PCR contamination artifact included the use of positive displacement pipettes, assembling of PCR reactions in a laminar flow hood in a separate room from plasmid preparations, and inclusion of negative and well-characterized controls in the PCR reactions.

Results

Distinct Microanatomic Localization of Vδ T Cell Populations. The microanatomic distribution of γ/δ T cell subpopulations within lepromin skin tests was investigated by immunohistologic staining of frozen sections with mAbs directed against Vδ-encoded determinants (Fig. 1). Within the dermal granulomas, Vδ1- and Vδ2-bearing cells accounted for the majority of infiltrating γ/δ cells, with a Vδ2/Vδ1 ratio ~2:1 compared to 9:1 in the peripheral blood of these same individuals. Unexpectedly, in contrast to the predominance of Vδ2-bearing cells in the peripheral blood and in the dermis, TCRδ$^+$ cells infiltrating the epidermis primarily expressed the Vδ1-encoded TCRs. Vδ2$^+$ cells were rarely detected in the epidermal layer.

Preferential Usage of Jδ1 by γ/δ T Cells in Lesional Skin. To further characterize the repertoire of the γ/δ T cells in response to mycobacterial stimulation in vivo, we performed PCR amplification of tissue-derived vs. peripheral blood–derived DNA using paired V and joining (J) oligonucleotide primers. PCR conditions were established such that each PCR reaction included DNA derived from ~500 γ/δ T cells. PCR analysis confirmed that Vδ1 and Vδ2 were used by γ/δ T cells in these lesions, as amplified product was obtained from all five lesions for Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements (Fig. 2). In contrast, Vδ2-Jδ3 rearrangements were detected in 1/5 lesions, but 5/5 blood samples (Figure 2). These data suggest a selective localization of the Vδ2-Jδ1 subpopulation of Vδ2$^+$ cells to lesions. PCR using other Vδ and Jδ combinations did not reveal additional gene rearrangements (data not shown).

Limited Junctional Diversity of γ/δ TCRs in Leprosy Skin Lesions. The junctional diversity of γ/δ T cells in lepromin skin tests was determined by cloning and sequencing PCR amplified products. We chose to focus on TCR δ chain diversity for two reasons: it encodes the greatest amount of diversity in the γ/δ receptor; and it is specific for γ/δ T cells since the δ locus is deleted during rearrangement of the TCR α chain. By using DNA rather than mRNA, bias towards activated lymphoblasts containing abundant mRNA is eliminated as each γ/δ T cell contains only one copy of its functionally rearranged δ gene. Strikingly, in each of the three lepromin skin tests subjected to nucleotide sequencing analysis, the majority of Vδ1-Jδ1 and Vδ2-Jδ1 junctional sequences were found to be identical, but distinct for each patient (Fig. 3). This was clearly different from peripheral blood of these

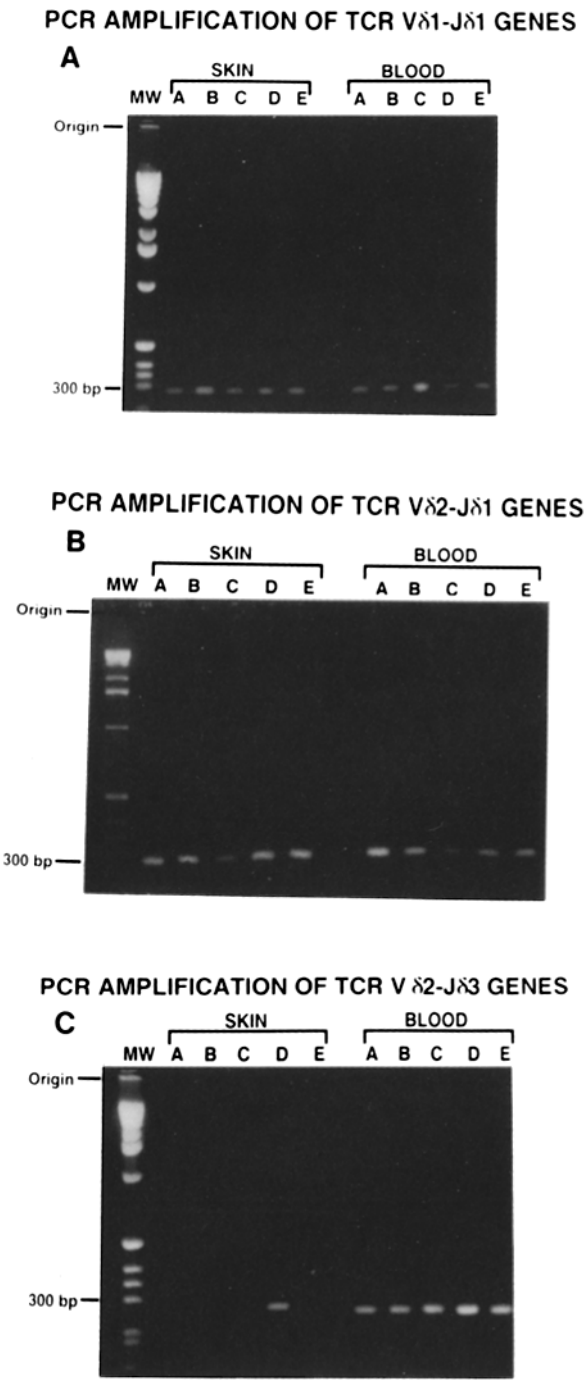


Figure 2. PCR analysis of δ chain V-J gene segment usage in lesions and peripheral blood. V-J junctions were PCR amplified from DNA extracted from five lepromin skin tests (lanes *A–E*) and the peripheral blood of the same individuals. PCR-amplified product was detected in all five skin lesions and blood for Vδ1-Jδ1 (*A*) and Vδ2-Jδ1 (*B*). In all five blood samples, but only one of five skin lesions, Vδ2-Jδ3 rearrangements could be amplified (*C*). No rearrangements were detected for any other Vδ-Jδ pairs (data not shown).

A Vδ1-Jδ1 JUNCTIONAL NUCLEOTIDE SEQUENCES FROM BIOPSY OF LEPROMIN SKIN TESTS

	Vδ1	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	CTCTTGGGGAC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
PT A	CTCTTGGGGAC	AT			CTA	ACCCCGACA	GGGAT	TA	ACCGATAA	(5/8)*
	CTCT	C			TCCTA	AGGGTAG	CTGGGG	TTCCC	ACACCGATAA	(3/8)
PT B	CTCTTGGGGA	TGCG			CTA	TTTGA	GGGGGATACG	CGAAATA	ACACCGATAA	(9/9)
PT D	CTCTTGG	TGACCCC	GAA	GGGAC	TCC	AG			ACACCGATAA	(8/8)

B Vδ2-Jδ1 JUNCTIONAL NUCLEOTIDE SEQUENCES FROM BIOPSY OF LEPROMIN SKIN TESTS

	Vδ2	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	GTGACACC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
PT A	GTGACACC	TTGC					ACTGGGGGA	GACGGG	ACACCGATAA	(6/6)
PT B	GTGA	TCG					ACTGGGGG	GCCCGT	ACACCGATAA	(8/8)
PT D	GTGACACC	G					TGGGGG	GAACCTCGT	ACACCGATAA	(11/13)
	GTGACA	GACC			CCTT	GAC	CTGGGGGA	GACGGGGAGG	AA	(1/13)
	GTGACAC	CC					CTGGGG	CCCACAT	ACACCGATAA	(1/13)

C Vδ1-Jδ1 JUNCTIONAL NUCLEOTIDE SEQUENCES FROM PATIENT PBMC

	Vδ1	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	CTCTTGGGGAC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
PT A	CTCTTGGGG	CC					GGGATAC	GAG	ACACCGATAA	(1/5)
	CTCTTGGGGA	TCA					TGGGG	ACT	ACACCGATAA	(1/5)
	CTCTTGGGGA	T	AAT				CTGGGGG	CCA	ACACCGATAA	(1/5)
	CTCTTGGGGAC				CTT	ATGA	GGGGAT	T	ACACCGATAA	(1/5)*
	CTCTTGGGGAC	TGG			CCTAC		TGGGGG	T	ACACCGATAA	(1/5)*
PT B	CTCTTGGGGAC	A			TCCT	T	ACTGGGGG	GT	ACACCGATAA	(1/7)
	CTCTTGGGGAC	GA			TCCT	TAGCC			ACACCGATAA	(1/7)*
	CTCTTGGGGAC	TCG					ACTGGGGG	CCGT	ACACCGATAA	(1/7)*
	CTCTTGGGGAC	CACG					TGGGGG	G	ACACCGATAA	(1/7)
	CTCTTGGGGAC	ACGCGC					GGGGA	GA	ACACCGATAA	(1/7)
	CTCTTGGGGAC	TGCG			TCCT	TGTG	TGGGG	CC	ACACCGATAA	(1/7)
	CTCTTGGGGA	TTGCGT					TGGGGG	TG	ACACCGATAA	(1/7)*
PT D	CTCTTGGGGAC	CCCGGTG					TGGGGG	CATCC	ACCGATAA	(1/6)
	CTCTTGGGGAC	TCGAGGG			TTCC	A	CTGGG	AT	ACACCGATAA	(1/6)
	CTCTTGGGGAC	GGGACGAGG					ACTGGG		ACACCGATAA	(1/6)*
	CTCTTGGGGAC	AACCTGTCG			TCCT	GCT			ACACCGATAA	(1/6)
	CTCTTGGGGAC	G					TGGGG	C	ACACCGATAA	(1/6)
	CTC		AAT	CT			ACTG	CCAGCTGC	ACACCGATAA	(1/6)

D Vδ2-Jδ1 JUNCTIONAL NUCLEOTIDE SEQUENCES FROM PATIENT PBMC

	Vδ2	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	GTGACACC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
PT A	GTGACACC	G	ATAG	G	CCTT	GCAAT			ACCGATAA	(1/13)
	GTGAC	CCGGCC					CTGGGG	CCCACAT	ACACCGATAA	(1/13)
	GTGACAC	CCGGCCCG					ACTGGGG	CCCACATCACT	CGATAA	(1/13)*
	GTGACACC	TGAC	AAT	GC	TCCT	CGT			ACACCGATAA	(1/13)*
	GTGACACC	C	TAGT	GAAGG	CTT	ACG			ACACCGATAA	(1/13)
	GTGAC	CT					ACTGGGGGAT	T	ACACCGATAA	(1/13)
	GTGA	ATCGCTT					TGGGGG	T	ACACCGATAA	(1/13)
	GTGACACC	GTG					CTGGGG	CC	ACACCGATAA	(1/13)*
	GTGAC	CCCTTA	GAAATA	CGCT					ACACCGATAA	(1/13)
	GTGAC	TCAA					TGGGGGATACG	CAGG	CCGATAA	(1/13)
	GTGAC	CC					ACTGGGGGAC	CCAG	ACACCGATAA	(1/13)
	GTGAC	CAG					CTGGGGG	TTACTGAGG	ACACCGATAA	(1/13)
	GTGACACC	CTTTCGAGGG			TCC	CAG			ACACCGATAA	(1/13)
PT B	GTGAC	CCCTGT					ACTGGGGG	TACGTGT	ACACCGATAA	(2/12)
	GTGACACC	GCAACCACC					GGGGG	T	ACACCGATAA	(1/12)
	GTGAC	C			CCT	CTCGGTTG	TGGGG	TC	ACACCGATAA	(1/12)
	GTGAC	CTAG					TGGGGGA	CC	ACACCGATAA	(1/12)
	GTGAC	GCGT					ACTGGGGG	GGAAAG	ACACCGATAA	(1/12)*
	GTGACACC	G			TCCTAC	G	TGGGGG	CCCTC	ACACCGATAA	(1/12)
	GTGACAC	GGG	ATAG	AAGCACAA			GGGGGAT		ACACCGATAA	(1/12)
	GTGACACC	TT					ACTGGGGG	CACCGA T	ACACCGATAA	(1/12)*
	GTGAC	GGC					CTGGGGG	GTATAAAGCGTG	ACACCGATAA	(1/12)
	GTGACA	A					ACTGGGGG	GTGCCTTT	ACACCGATAA	(1/12)*
	GTG	TGAC					GGGGG	TTCAGGAGTTTTT	AA	(1/12)*
PT D	GTGAC	CCCCG			TCC	CT	ACTGGGGG	CTACCGGGCG	CACCGATAA	(1/9)*
	GT	CTGGTGCATGCCCTG	TAGT	CCCAG	CTAC	TC			ACACCGATAA	(1/9)
	GT	CATCG					ACTGGGGG	GCCCGT	ACACCGATAA	(1/9)
	GTGAC	GGCGC					ACTGGGGG	CGAAGA	ACACCGATAA	(1/9)
	GTGAC	CCTTGCTCCGGGGC	AGT				ACTGGGGGAT	GCCGT	ACACCGATAA	(1/9)*
	GTGAC	CCAC					ACTGG	AGGCGAGCCCCAG	ACACCGATAA	(1/9)
	GTGACACC	GTC					GGGGA	GA	ACACCGATAA	(1/9)
	GTGAC	CAGGTC					CTGGGGGATACG	CGGGGAC	CCGATAA	(1/9)
	GTGACACC	AGT					ACTGGGGGAT	TCCTCGT	ACACCGATAA	(1/9)*

Figure 3. Nucleotide sequence analysis of TCRδ V-J junctions of skin test lesions and blood. Sequencing of PCR products was performed to analyze the diversity at the V-J junction. Limited diversity was found in both the Vδ1-Jδ1 (*A*) and Vδ2-Jδ1 (*B*) junctions of γ/δ T cells from lesions in patients A, B, and D. In contrast, there was marked genetic diversity in both the Vδ1-Jδ1 (*C*) and Vδ2-Jδ1 (*D*) junctions from the blood of the same individuals. Nonfunctional sequences are marked with an asterisk.

same individuals, which exhibited extensive diversity (Fig. 3). The data indicate clonal selection by antigen with oligoclonal expansion within the lesion. The occurrence of identical sequences that are nonproductive in a given sample is likely to represent the nonfunctional allele in the cell undergoing clonal expansion.

To determine the extent of oligoclonal expansion throughout the lesion, spatially separated regions of one biopsy specimen were studied. Within each region of the biopsy specimen, there was limited diversity of the V-J junctions, although the predominant sequence differed from site to site (Fig. 4). Given the limited diversity observed in these lesions, we addressed the possibility of a PCR artifact producing the surprising results. Extensive precautions were undertaken to avoid PCR contaminations as outlined in the Materials and Methods. Simultaneous PCR and cloning was performed on lesions and blood, so that finding of limited diversity in lesions was accompanied by finding of expected diversity in blood. The finding of different predominant sequences in separate areas of the biopsy argues against sample to sample contamination. The finding of limited diversity was obtained by complete workup of tissue samples by three separate investigators in three separate laboratories. We are confident that the PCR reaction was performed on DNA from ~500 cells and not on a single cell based upon: (*a*) calculation of the number of γ/δ cells in the biopsy specimen; (*b*) comparison of PCR product to PCR titration of known amounts of plasmid DNA containing δ gene segments; (*c*) appearance of several unique sequences along with the predominant sequence in many of the clonings of lesional skin; and (*d*) derivation of the same sequence by separate PCR reactions and cloning of the tissue sample.

Extensive Diversity of γ/δ TCRs in Normal Human Skin. The resident γ/δ population in normal human skin is small, with few cells identified by immunohistologic techniques (13). We wanted to determine whether the limited TCR diversity among γ/δ T cells from leprosy lesions reflected a limited diversity within the resident γ/δ population in skin or rather clonal expansion of specific TCRs in response to antigenic selection. The γ/δ TCR repertoire analysis of normal skin indicated the presence of Vδ2-Jδ1 rearrangements, with minor Vδ1-Jδ1 products and undetectable Vδ2-Jδ3 gene rearrangements (Fig. 5). This indicates that in diseased or normal skin, there is selective localization of γ/δ T cells expressing the Jδ1 gene product. Lack of δ chain–bearing Jδ3 rearrangements in DNA derived from normal skin further supports our conclusion that the PCR products are derived from a resident γ/δ population, not from T cells within blood vessel lumina. In contrast to diseased skin, the analysis of TCR junctional sequences derived from normal skin indicates extensive diversity equivalent to peripheral blood (Fig. 5).

Conserved Motif in Predicted Amino Acid Sequences of Vδ2-Jδ1 Junctions. The predicted amino acid sequences of predominant lesional Vδ2-Jδ1 gene junctions indicated conservation of amino acids from site to site within a biopsy and from patient to patient (Fig. 6). The conserved motif comprised the 5′ end of the V-J junction: T·L/V·G·G/D, beginning in the third amino acid position after the second cysteine of Vδ2. This motif results primarily from the contribution of Vδ2, P, and Dδ3 gene segments. A similar motif is seen in the peripheral blood of these individuals, but only in those clones that solely use the Dδ3 gene segment (Fig. 6). This motif was rarely observed in normal skin (Fig. 6). In addition, the length of the V-J junction was relatively conserved in the lesional sequences, as compared to the peripheral blood.

To ascertain whether the conserved motif we observed in the Vδ2-Jδ1 junctions of leprosy lesions was present in other granulomatous diseases, we translated the Vδ2-Jδ1 nucleotide sequences of γ/δ T cells from the lungs of patients with

Vδ-Jδ JUNCTIONAL NUCLEOTIDE SEQUENCES FROM PATIENT B BIOPSY OF LEPROMIN SKIN TEST

Vδ1-Jδ1

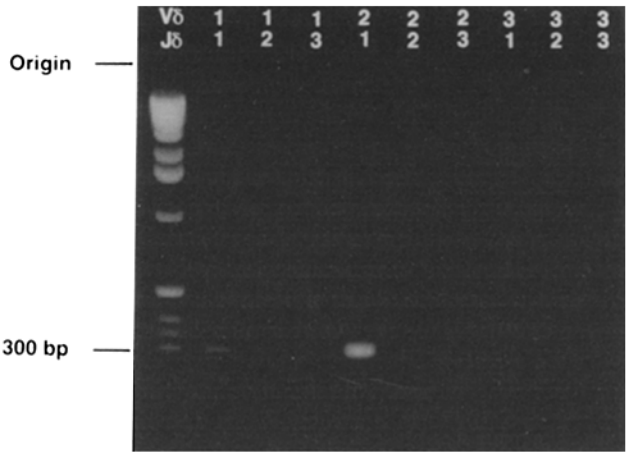
	Vδ1	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	CTCTTGGGGAC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
AREA 1	CTCTTGGGGA	TGCG			CTA	TTTGA	GGGGGATACG	CGAAATA	ACACCGATAA	(9/9)
AREA 3	CTCTTGGG	ACGGGGGTGA					GGGGA	CTCCAGG	ACACCGATAA	(12/12)

Vδ2-Jδ1

	Vδ2	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	GTGACACC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
AREA 1	GTGA	TCG					ACTGGGGG	GCCCGT	ACACCGATAA	(8/8)
AREA 2	GTGAC	GGG					CTGGGGGATAC	TA	ACACCGATAA	(10/17)
	GTGACACC	GTA					GGGGG	TCGGT	ACACCGATAA	(6/17)
	GTGAC	CCCC					CTGGGG	CCCACAT	ACACCGATAA	(1/17)*
	GTGAC				TCC	ATT	ACTGGGGGA	GAACAGT	ACACCGATAA	(1/17)
AREA 4	GTGACACC	GT					CTGG	A	ACACCGATAA	(15/30)#
	GTGACACC	A					TGGGGGA	CCCACGG	GATAA	(8/30)
	GTGACACC	GTGAG					ACTGGGGG	GTACGCTGAG	CGATAA	(1/30)
	GTGACACC	GTC			CCTA		CTGGGGG	GGCCCT	CACCGATAA	(1/30)
	GTGA				TCC	C	CTGGGGG	GGAGGG	ACCGATAA	(1/30)*
	GTGAC	GG					ACTGGG		CACCGATAA	(1/30)
	GTGACACC	TGTA					GGGGG	G	ACACCGATAA	(1/30)
	GTGACA	TG			CCTT	G	GGGGGATACG	G	ACACCGATAA	(1/30)
	GTGAC	CG			CCT		GGGGG	GCG	ACACCGATAA	(1/30)

Figure 4. Microheterogeneity of γ/δ T cell receptors in lesions. Within different regions of a biopsy specimen, distinct clonal junctional nucleotide sequences were identified. Nonfunctional sequences are marked with an asterisk. Sequences marked by a pound sign were obtained by three separate PCR reactions and cloning.

A TCR δ Gene Usage in Normal Skin



B Vδ2-Jδ1 JUNCTIONAL NUCLEOTIDE SEQUENCES FROM BIOPSY OF NORMAL SKIN

	Vδ2	P/N	Dδ1	P/N	Dδ2	P/N	Dδ3	P/N	Jδ1	FREQUENCY
germline	GTGACACC		GAAATAGT		CCTTCCTAC		ACTGGGGGATACG		ACACCGATAA	
NL F	GTGACACC	GA			TCCT	G	ACTGGG	AT	ACCGATAA	(1/9)
	GTGAC	TCCC			TCCT	TT			ACACCGATAA	(1/9)
	GTGACAC	T			CTT	T	ACTGGGGGATACG	CGAG	CGATAA	(1/9)*
	GTGACACC				TCCT	T	ACTGGG		ACACCGATAA	(1/9)*
	GTGACACC	TCCC			TCCT	TT			ACACCGATAA	(1/9)
	GTGACA		AGT	GT			ACTGGGGGGA	CTATTT	ACACCGATAA	(1/9)
	GTGA	GATGGGAG					ACTGGGGGATA	T	ACACCGATAA	(1/9)
	GTGACACC	CCAAG			CTT	GCATG			ACACCGATAA	(1/9)
	GTGAC	CAT	ATAGT	AGTT					ACACCGATAA	(1/9)
NL G	GTGACACC	AGGGTT					ACTGGGG	CTGT	ACACCGATAA	(1/4)
	GTGA	TC			CCTT	AC	TGGGGG	CTGT	ACACCGATAA	(1/4)*
	GTGA	TC			CCTT	A	CTGGGGGAT		CACCGATAA	(1/4)*
	GTGA	AGT					ACTGGGGG	GCCGGT	ACACCGATAA	(1/4)

Figure 5. PCR amplification and TCR V-J junctional sequences in normal skin. Analysis of Vδ and Jδ gene usage by PCR amplification revealed product for both Vδ1-Jδ1 and Vδ2-Jδ1 but not other Vδ-Jδ combinations (*A* shows the results for normal donor F; donor G gave identical results). Nucleotide sequences of Vδ2-Jδ1 junctions revealed extensive nucleotide diversity for specimens from normal donors F and G (*B*). Nonfunctional sequences are marked with an asterisk.

sarcoidosis (14). This comparison revealed a relatively high frequency of the T·L/V·G·G/D motif in Vδ2-Jδ1 junctional sequences (data not shown). This motif occurred in the same amino acid positions as in sequences from leprosy lesions, but only when the Dδ3 segment was the only D segment used. The length of the V-J junction in the repeated sequences of the sarcoidosis patients was conserved, at a length similar to that observed in the leprosy lesion sequences. This was in contrast to the variable length of the Vδ2-Jδ1 junction sequences of normal blood γ/δ T cells reported (14).

Discussion

Distinct Microanatomic Patterns of Vδ Populations in Leprosy Lesions. Immunohistologic identification of Vδ T cell populations in leprosy lesions reveals distinct microanatomic patterns for Vδ1- and Vδ2-bearing cells: the dermal infiltrate containing both populations, but the epidermal γ/δ T cells preferentially expressing the Vδ1 chain. The localization of Vδ1$^+$ cells to epidermis in these mycobacterial lesions suggests either homing, retention, and/or in situ expansion of Vδ1$^+$ cells during the immunopathogenic reaction. This localization of Vδ1$^+$ cells to an epithelial surface may be characteristic of the inflammatory process. The gut epithelia of patients with Celiac disease, a gluten sensitive enteropathy, was found to contain Vδ1$^+$ cells (15).

The experimental evidence indicates that Vδ1 and Vδ2 populations may recognize distinct antigens and/or presentation molecules. Some human Vδ1$^+$ cells appear to have cytotoxic activity against allogeneic targets; this cytotoxicity is blocked by a mAb that recognizes a 43-kD protein termed TCT.1 (16). Also, Vδ1$^+$ cells have been reported to recognize CD1 molecules (17, 18). Langerhans cells express CD1 molecules, can present antigen, and are increased in the epidermis and dermis of these lesions (8). Perhaps, the expression of the TCT.1 molecule and/or CD1 molecules by diseased epithelia results in the stimulation of the Vδ1 population. In contrast, human Vδ2$^+$ cells expand in response to *M. tuberculosis* antigens in vitro. The ability of these same Vδ2$^+$ cells to proliferate to Daudi cell lines is blocked by a polyclonal serum to a human HSP 58-kD protein (19).

Limited Diversity of γ/δ TCRs in Lesional Microenvironments Suggests Clonal Selection by Antigen. The most striking data presented here are the limited diversity of the V-J junctional elements of γ/δ T cells in leprosy lesions. The junctional sequences of γ/δ T cells from lesions were not found in the respective individual's peripheral blood, indicating that the lesional sequences do not represent an already existing clonal

LEPROSY SKIN BIOPSY SPECIMENS

PT A			C	D	T	L	H	W	G	R	R	D	K
PT B			C	D	R	L	G	G	P	Y	T	D	K
			C	D	G	L	G	D	T	N	T	D	K
			C	D	T	V	G	G	R	Y	T	D	K
			C	D	T	W	N	T	D	K			
			C	D	T	M	G	D	P	R	T	D	K
PT D			C	D	T	V	G	G	T	S	Y	T	D	K

PBMC FROM LEPROSY PATIENTS

PT A			C	D	T	D	T	P	C	N	T	D	K	
			C	D	S	M	G	D	T	Q	A	D	K	
			C	D	P	L	G	D	P	D	T	D	K	
			C	D	Q	L	G	V	T	E	D	T	D	K
			C	D	T	Q	C	S	S	Y	T	D	K	
			C	E	S	L	W	G	Y	T	D	K		
			C	D	P	L	E	I	R	Y	T	D	K	
			C	D	T	H	W	G	I	T	P	T	D	K
PT B			C	D	P	C	T	G	G	T	C	T	D	K
			C	D	P	S	R	L	W	H	G	T	D	K
			C	D	L	V	G	D	H	T	D	K		
			C	D	T	V	L	R	G	G	P	H	T	D	K
			C	D	G	L	G	G	I	K	R	D	T	D	K
PT D			C	D	P	R	P	Y	W	G	L	P	G	A	P	K	
			C	A	G	A	C	P	V	V	P	A	T	H	T	D	K
			C	H	R	L	G	G	P	Y	T	D	K				
			C	D	G	A	L	G	A	K	N	T	D	K			
			C	D	P	C	S	G	A	V	L	G	D	A	V	D	K
			C	D	P	H	W	R	R	A	P	D	T	D	K		
			C	D	T	V	G	E	N	T	D	K					
			C	D	Q	V	L	G	D	T	R	G	P	D	K		

NORMAL SKIN

NL F			C	D	T	D	P	W	K	T	D	K	
			C	D	H	I	V	V	T	D	K		
			C	D	S	L	L	Y	T	D	K		
			C	D	T	S	L	L	Y	T	D	K	
			C	E	M	G	D	W	G	I	Y	T	D	K
			C	D	T	P	S	L	H	D	T	D	K	
NL G			C	D	T	R	V	T	G	G	C	T	D	K
			C	D	R	L	G	G	P	Y	T	D	K	

Figure 6. Predicted amino acid sequences based on Vδ2-Jδ1 nucleotide sequences. The predicted amino acid sequences for the predominant lesional Vδ2-Jδ1 junctions are shown. The conservation in amino acid sequences, the T·L/V·G·G/D motif beginning in the third amino acid position after the second cysteine of Vδ2, is primarily encoded by the Vδ2, P, and the Dδ3 gene segments. Although Dδ3 gene segment usage is frequent in the peripheral blood, the conserved motif does not appear in the same location within the V-J junction with the high frequency of leprosy lesional sequences. In addition, the junctional amino acid lengths of peripheral blood Vδ2$^+$ cells are more variable, occasionally showing a greater length than the lesion-derived sequences. The amino acid sequences of normal skin show even greater heterogeneity.

expansion in the peripheral repertoire. It is noteworthy that in each spatially separated region of the biopsy specimen, a limited number of junctional nucleotide sequences were represented multiple times, although the predominant sequence differed from site to site. Since there was conservation in the predicted amino acid sequence in various areas of the biopsy and among individuals, we hypothesize that the γ/δ T cell expansion in lesions is selected by a limited set of antigens within the tissue microenvironment. The limited microheterogeneity implies that a very small number of γ/δ clones initiate the oligoclonal expansion and that their progeny do not disperse homogeneously throughout the lesion. Studies of murine γ/δ T cells indicate the positive selection of γ/δ T cells in the periphery (20). The study of leprosy lesions indicates that the clonal selection by foreign antigen occurs at the site of infection. Furthermore, γ/δ T cells in the lungs and blood of patients with sarcoidosis (14) may similarly result from the oligoclonal expansion to antigen.

The nucleotide sequence analysis of lesional γ/δ T cells revealed that while Vδ1-Jδ1 T cells utilize multiple Dδ segments at the V-J junction, the Vδ2-Jδ1 T cells use only the Dδ3 gene segment in 98% of the isolates. This is in contrast to the 67% usage of Dδ3 alone in sequences derived from the blood of the same individuals, or the 33% usage in normal skin. The differences in Dδ usage suggest a fundamental distinction in the Vδ1 and Vδ2 populations in skin lesions. The exclusive use of the Dδ3 gene segment with minimal use of N segments is a feature of γ/δ T cells representing an early fetal thymic wave (11). Since Vδ2-Dδ3-Jδ1 cells in leprosy skin lesions were found to contain N segments, they are likely derived from a later developmental stage when the levels of terminal deoxynucleotide transferase are higher. The finding of conserved amino acid residues and length of the predominant lesion-derived Vδ2-Jδ1 junctions suggests that the junctional region, and specifically the Dδ3-encoded sequence, participates in antigen recognition (Fig. 4). This is supported by murine studies indicating that the junctional sequences influence the specificity of the γ/δ TCR (21). When compared to the limited TCR diversity displayed by γ/δ T cells from leprosy lesions, the normal skin data suggests that the γ/δ T cells in leprosy lesions represent clonal selection from among a genetically diverse peripheral blood or resident skin population. The reported diversity of V-J junctions in mycobacterial-reactive murine γ/δ T cells derived from antigen-unselected neonatal thymus indicates that the breadth of the antigen-reactive repertoire is large (22).

Human vs. Murine Skin γ/δ T Cells. The normal murine epidermis contains a dendritic cell population that is Thy-1$^+$ and bears γ/δ TCRs. This resident population is of extremely limited genetic diversity, with virtually exclusive usage of a single Vγ gene and single Vδ gene and no junctional diversity (1). Similar to the murine intraepidermal T cells, we find the normal human TCR γ/δ skin population to be encoded by a restricted set of V and J genes (Vδ2-Jδ1). In response to infection, the epidermis is found to contain a specific TCR δ population (Vδ1-Jδ1), and all TCRs are of limited genetic diversity.

Our findings indicate clear differences between human versus murine skin γ/δ T cells. The resident human skin population, known to be significantly smaller than the murine resident population (13), displays extensive, rather than limited, genetic diversity of junctional gene elements. Even in human skin lesions of infectious etiology, the number of epidermal γ/δ T cells was relatively few as compared to the resident murine population. However, limited genetic diversity occurs in γ/δ T cells responding to infection and appears to be due

to the oligoclonal expansion of specific TCR-bearing cells from a diverse pool.

Implications. γ/δ T cells appear to function as a first line of defense against infectious pathogens (23). This hypothesis is based on the finding of large numbers of γ/δ T cells at peripheral interfaces, including normal murine skin (24), gut (25, 26), and lungs (3). γ/δ T cells derived from antigen-unselected murine neonatal thymocytes (27) respond to mycobacterial antigens in vitro. Furthermore, the initial immune response to *M. tuberculosis* (28), *Listeria monocytogenes* (29), and *Trypanosoma cruzi* (30) infection is characterized by expansion of γ/δ T cells. In humans, γ/δ T cells accumulate in early lesions of leprosy and leishmaniasis (7). Studies of human γ/δ have indicated reactivity to a number of infectious agents, including mycobacteria (7, 31–33), listeria (34), streptococcus (34, 35), staphylococcus (34–36), plasmodium (37, 38), and herpes viruses (39). The present data indicate that the γ/δ T cell response to infection involves clonal selection from a diverse TCR repertoire and further expansion by antigen in situ. The limited junctional diversity in leprosy lesions is not consistent with current knowledge of superantigen responses (40). Our data suggest that V-J junctions are critical for antigen recognition.

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