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# Human γδ T-cell recognition of *Yersinia enterocolitica*

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## SUMMARY

We have studied the human γδ T-cell response to *Yersinia enterocolitica*, a facultative intracellular bacterium which causes gastroenteritis and, particularly in human leucocyte antigen (HLA)-B27$^+$ individuals, reactive arthritis (ReA). A marked proliferation of that cytotoxic γδ T cells is seen when *Yersinia*-infected lymphoblastoid cell lines or fixed intact *Yersinia* are added to cultures of mononuclear cells derived from the synovial fluid of ReA patients or from the peripheral blood of healthy donors. In contrast, heat-inactivated *Yersinia* fail to stimulate the γδ T-cell response. The γδ T-cell lines generated killed both autologous and allogeneic infected cell lines. Interestingly, a T-cell line generated from synovial fluid mononuclear cells (SFMC) killed infected autologous cell lines and a cell line matched for HLA-B27 less well than infected allogeneic target cells. γδ T-cell clones isolated from this line were found to express Vγ9Vδ2 T-cell receptor (TCR) and also killed infected mismatched cells more efficiently than autologous targets. Moreover, from experiments using major histocompatability complex (MHC)-deficient cell lines, it was apparent that target cell recognition was MHC independent. Our results suggest that γδ T cells can be involved in immunity to *Yersinia enterocolitica* and should be taken into account when considering immunopathological mechanisms leading to reactive arthritis.

## INTRODUCTION

A minor T-cell subset can be defined by the expression of the γδ T-cell receptor (TCR). Although this receptor has structural similarities to the αβ TCR, it is becoming clear that antigen recognition by γδ T cells can be quite different from that exhibited by αβ T cells.[1] Thus, while most αβ T cells express either CD4 or CD8 and recognize peptidic antigen presented by an appropriate major histopcompatability complex (MHC) molecule, γδ T cells usually lack CD4 or CD8 and recognize antigens independently of classic MHC molecules. Indeed, experiments with murine γδ T cells have suggested that the γδ TCR may function in an analogous fashion to immunoglobulin, recognizing intact polypeptide rather than small peptide fragments.[2,3] Moreover, work with human γδ T cells has revealed that antigens need not be derived from polypeptides; for example, mycobacteria-reactive γδ T cells can recognize isopentenyl pyrophosphate and related prenyl pyrophosphate derivatives.[4–6] The recognition of these non-peptidic ligands evidently proceeds via a novel MHC-independent extracellular pathway.[7] However, other human γδ T cells require the presence of members of the non-classic MHC-related molecules CD1,[8–10] of which CD1b is known to present non-peptidic antigens such as lipoglycans and mycolic acid.[11–13]

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Despite the delineation of some of the antigens recognized by γδ T cells, the biological role of this T-cell subset is still under investigation. There is a consensus that γδ T cells are involved in the immune response to infections.[14,15] However, the precise function of the γδ T-cell response is not clear. Experiments with gene knockout mice have indicated that γδ T cells may complement αβ T cells resulting in the efficient clearance of infective organisms.[16] It has been suggested that the γδ T cells act as a first line of defence linking the innate and the acquired immune responses.[17] However, experiments in which γδ deficient mice die when challenged with *Mycobacterium tuberculosis* indicate that γδ T cells may be an absolute requirement for resistance to this infection.[18] In addition to their role in resistance to infection, it has also been proposed that γδ T cells may function as regulatory cells to limit immunopathology.[16]

In the present study we have examined the γδ T-cell response to *Yersinia enterocolitica*. Encounter with this bacterium usually results in uncomplicated gastroenteritis. However, in some individuals reactive arthritis (ReA) can ensue (see 19 for review). ReA is an oligoarthritis which develops soon after infection of a mucosal site with a facultative or obligate intracellular pathogen such as *Yersinia*, *Salmonella* or *Chlamydia*. It is widely believed that an inappropriate or defective cellular immune response to the triggering bacteria may be responsible for this immunopathology. As ReA is associated with human leucocyte antigen (HLA)-B27, much interest has been focused on the CD8$^+$ T cells. However, to date, HLA-B27-restricted CD8$^+$ T-cell clones with

specificity for the inducing bacterium have only been isolated by one laboratory.[20] Most reports of cell-mediated immunity in patients have assayed $CD4^+$ T cells and some bacterial antigens recognized by synovial $CD4^+$ T-cell clones have been identified.[21–25] It would therefore appear that $CD8^+$ T cells may not contribute solely to the immunopathology of ReA. The results of the present study suggest that, in addition to $CD4^+$ and $CD8^+$ T-cell activities, T cells expressing the $\gamma\delta$ TCR should not be ignored when considering ReA disease mechanisms. We report that a profound expansion of $\gamma\delta$ T cells is seen after culture of peripheral blood mononuclear cells (PBMC) from normal individuals or synovial fluid mononuclear cells (SFMC) from ReA patients with either *Yersinia*-infected and fixed autologous B lymphoblastoid cell line (BLCL) or with fixed bacteria. In contrast, heat-inactivated *Yersinia* did not support the proliferation of $\gamma\delta$ T cells. One *Yersinia*-reactive T-cell line from an arthritic individual was subjected to further testing and found to kill both infected autologous and allogeneic cell lines. Moreover, $\gamma\delta$ T-cell clones isolated from this line lysed infected targets in an MHC-independent fashion.

## MATERIALS AND METHODS

*Preparation of mononuclear cells from peripheral blood and synovial fluid*

Synovial fluid was collected from three patients with a history of *Yersinia enterocolitica*-associated reactive arthritis. Peripheral blood was also donated by three normal individuals. The synovial fluids were treated with 10 U/ml hyaluronidase (Sigma Chemical Co. Ltd, Poole, UK) for 30–60 min at 37°. SFMC and PBMC were isolated on a Ficoll (Pharmacia, Milton Keynes, UK) gradient. The cells were either used fresh or cryopreserved until required.

*Cell lines*

Most of the B lymphoblastoid lines (BLCL) employed were generated from PBMC. The PBMC were incubated with supernatant from the Epstein–Barr virus (EBV)-producing cell line B95.8 at 37° for at least 1 hr. The cells were washed and cultured at $1–2 \times 10^6$/ml in RPMI (Life Technologies Ltd, Renfrew, UK) supplemented with 5% heat inactivated fetal calf serum (HI FCS; Life Technologies Ltd), 10 mM HEPES (Sigma), 2 mM glutamine (Sigma), 100 μg/ml streptomycin sulphate BP (Evans Medical Ltd, Greenford, UK) and 100 U/ml benzylpenicillin sodium BP (Glaxo, Greenford, UK) in the presence of 1 μg/ml cyclosporin A. The cells were fed at weekly intervals with fresh medium supplemented with cyclosporin A until the line was established when the use of cyclosporin A was discontinued.

RML, HHKB, and PITOUT were given by S. Marsh (ICRF, London, UK). Daudi, C1R and K562 were provided by A. Rickinson (Department of Cancer Studies, University of Birmingham, UK).

Yersinia enterocolitica

*Yersinia enterocolitica*: O9 (Y9) was kindly donated by the Department of Infection, University of Birmingham, UK. The bacteria were grown overnight in 2YT broth in a shaking incubator at 37°. This late time of culture and temperature were chosen to generate stationary phase bacteria similar to those which may be encountered *in vivo*.

*Infection and fixation of stimulatory and target cells*

Cell suspensions were washed at least twice in antibiotic-free RPMI. An aliquot ($\approx 100$ μl/$1 \times 10^6$ cells) of the broth culture of Y9 was added to the cell suspension. The cells were then pelleted and incubated for 1 hr at 37°; this time being determined as sufficient to give infection without leading to premature lysis of cells. The infected cells were then washed three times in RPMI supplemented as described before except that 5% heat-inactivated male AB sera (West Midlands Blood Transfusion Service, Birmingham, UK) was used instead of FCS and 1 mM sodium pyruvate (Sigma), 1 × modified Eagle's medium (MEM) non-essential amino acid solution (Sigma), and 100 μg/ml gentamycin (Sigma) were also added. The cells were used at this stage for cytotoxicity assays. For stimulation of SFMC or PBMC, the infected BLCL was fixed with 0·25% formaldehyde (Sigma) for 10 min, washed twice in 1·5 M glycine (Sigma), incubated for 30 min in RPMI supplemented as described, washed again and re-incubated prior to a last wash. The number of these cells used in stimulation and cloning assays was based on those cells which retained the ability to exclude trypan blue. Control uninfected BLCL were prepared in the same manner. For some experiments intact *Yersinia enterocolitica* were fixed as described above and added directly to cultures.

In vitro *stimulation assays*

The medium employed for all assays was RPMI supplemented as described in the previous section except that gentamycin was not routinely present. SFMC or PBMC were cultured in 2 ml volumes in 24-well plates at $1–2 \times 10^6$/ml in the presence or absence of $3·3–6·7 \times 10^4$/ml fixed infected or non-infected autologous BLCL or with fixed *Yersinia enterocolitica*.

*T-cell cloning*

Cells were cloned after 3–7 days of stimulation at limiting dilution in Terasaki plates (ICN Biomedicals Ltd, Thame, UK). The cells were cloned in the presence of irradiated PBMC obtained from three allogeneic donors ($8 \times 10^3$/well), infected autologous BLCL (1–5/well), 1 μg/ml phytohaemagglutinin (PHA: Murex Diagnostics, Dartford, UK), 100 U/ml recombinant interleukin-2 (IL-2: Eurocetus UK Ltd, Harefield, UK) and 10% supernatant from concanavalin A stimulated PBMC. The isolated clones were maintained with 20–50 U/ml rIL-2 and restimulated, as required, with irradiated allogeneic PBMC from three donors and 1 μg/ml PHA.

*Analysis of TCR gene usage*

TCR $\gamma$ and $\delta$ chain sequences were determined for a number of the T-cell clones using a procedure similar to that previously described for TCR $\alpha$ and $\beta$ chain analysis.[26] Briefly, cDNA was prepared from RNA extracted from the T-cell clones, modified with a poly diguanosine triphosphate (dGTP) tail and subjected to anchor polymerase chain reaction (PCR). PCR for both $\gamma$ and $\delta$ TCR used the anchor primer (5′CTATCTAGAGAGAGCTCGCGGCCGC$_{13}$3′) and either the 3′ C$\delta$ primer (5′CGCAGATCTCAGACAAGCGACATTTG3′) or the 3′ C$\gamma$ primer (5′CGCAGATCTGACAAAGGTATGTTCCAGCCTTCTGG3′). The amplification was performed in a Hybaid omnigene thermocycler (Hybaid Ltd. Teddington, UK) using 32 cycles of 93° for 45 s, 57° for 30 s and 72° for 1 min followed by extension at 72° for 4 min. The

PCR products were digested with the restriction enzymes *Not*I and *Bgl*II and cloned into a M13mp18 cloning vector (kindly supplied by P. Moss and J. Bell, Oxford, UK). DNA sequencing was by the dideoxy chain termination method using a Sequenase version 2.0 reagent kit (Amersham International plc, Little Chalfont, UK).

*Flow cytometry*

Cells to be stained were washed and resuspended in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA). The cells were stained with fluoroscein isothiocyanate (FITC)-conjugated monoclonal antibodies or control FITC–immunoglobulin G1 (IgG1) (Dako A/S, Glostrup, Denmark) in 96U-well floppy plates (Falcon Cell Culture Products, Cowley, UK) in 100 $\mu$l volumes. The monoclonal antibodies employed were against CD3 (Dako), CD4 (Dako), CD8 (Dako), CD16 (Dako), TCR$\alpha\beta$ (Becton-Dickinson UK Ltd, Cowley, UK) and TCR$\gamma\delta$ (Becton-Dickinson). The cells were analysed with a FACSort® flow cytometer (Becton-Dickinson) and the data analysed using WinMDI version 1.3.1 (contact trotter@flosun.salk.edu).

*Cytotoxicity assay*

For most experiments the non-radioactive cytotoxicity kit, CytoTox-96™, was used according to the manufacturer's directions (Promega Corporation, Southampton, UK). This is a colorimetric assay which measures the lactate dehydrogenase released from lysed cells. Target cells were added at 5000/well and effector cells were added at the indicated E:T ratio in a total of 100 $\mu$l. The cells were incubated at 37° for 4 hr prior to harvesting 50 $\mu$l supernatant. We found that the non-radioactive cytotoxicity kit gave comparable results to the traditional $^{51}$chromium release assay which was employed for the experiment shown in Fig. 6. In this case, aliquots of $1 \times 10^6$ target cells were labelled with 0·1 mCi/ml of $^{51}$Cr (Amersham International) in 100 $\mu$l for 45 min, washed and then half the cells were infected with *Yersinia*, as described above. The assay was set up in triplicate in 200 $\mu$l volumes with 5000 target cells and 50 000 effector cells. Maximum release was determined by adding Triton-X (Sigma) to cultures of target cells and minimum release from cultures of target cells in the absence of effector cells. After 4 hr, 50 $\mu$l of supernatant was spotted onto a filter mat (Pharmacia) and radioactivity present assayed using a 1205 $\beta$-plate counter (LKB-Wallac, Turku, Finland). Percentage specific cytotoxicity was calculated in the standard manner.

## RESULTS

A substantial proliferative reaction was noted when autologous BLCL infected with *Yersinia* were cultured with SFMC or PBMC. Flow cytometry analysis after 7–10 days of stimulation, revealed an expansion of TCR$\gamma\delta$-bearing cells in the cultures (Fig. 1). Mononuclear cells from each of the individuals tested displayed less than 10% TCR$\gamma\delta^+$ cells prior to culture or after culture in the absence of infected BLCL (results not shown). The nature of the stimulus required for proliferation was further studied and the results from culture of SFMC from one individual, CM, are shown in Fig. 2. It is apparent that incubation of BLCL with *Yersinia* was required to promote expansion of $\gamma\delta$ T cells. Unfixed infected BLCL cultured with SFMC in the presence of gentamycin also stimulated $\gamma\delta$ T cell proliferation (results not shown). Interestingly, $\gamma\delta$ T-cell proliferation was also provoked by the addition of fixed intact *Yersinia* to SFMC. This stimulatory

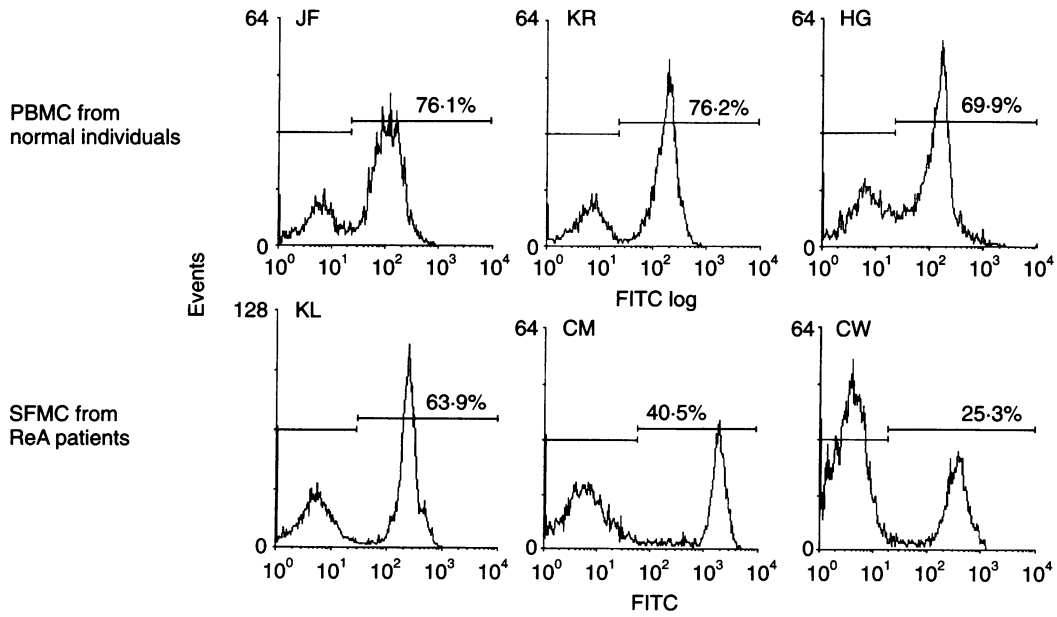


**Figure 1.** Analysis of $\gamma\delta$ TCR expression after stimulation with fixed infected autologous BLCL. PBMC from normal individuals (top row) or SFMC from ReA patients (bottom row) were cultured for 7–10 days with fixed autologous BLCL infected with *Yersinia enterocolitica*. Expression of the $\gamma\delta$ TCR analysed by flow cytometry is shown. Each panel represents an independent experiment. In each case, TCR$\gamma\delta$ expression in unstimulated cultures was less than 6% and is not shown. No staining was apparent with an IgG1–FITC isotype control antibody.

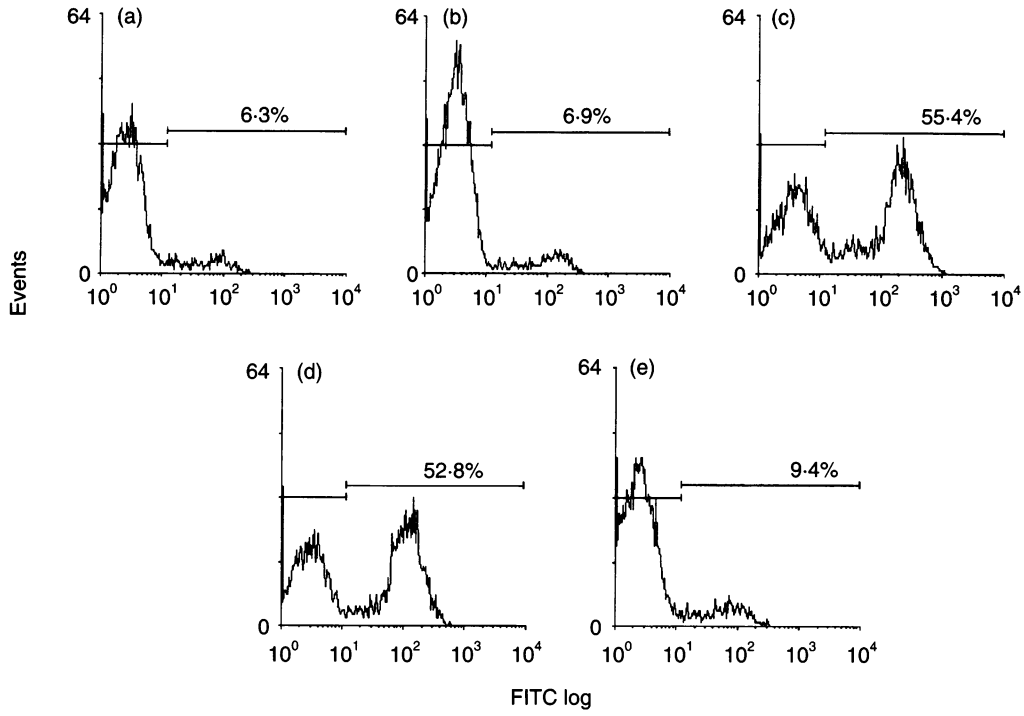


**Figure 2.** TCR$\gamma\delta$ expression after culture of SFMC with various stimuli. TCR$\gamma\delta$ expression was determined by flow cytometry following culture of SFMC without added stimulators (a) or with fixed autologous BLCL (b), fixed infected BLCL (c), fixed *Yersinia* (d) or heat-inactivated *Yersinia* (e). No staining was detected with an isotype matched IgG1–FITC control (not shown).

capacity was almost completely ablated by heat inactivation of the *Yersinia*.

Cell lines generated from stimulation of CM SFMC were tested for their cytotoxic capacity. As shown in Table 1, when the line was tested against a panel of BLCL, although uninfected cell lines were killed to various degrees, enhanced killing was exhibited against the infected target cells irrespective of MHC allele expression. Cell lines derived from the PBMC of the three normal donors also killed infected target cells in an MHC-unrestricted fashion (results not shown).

T-cell clones were generated to study further the apparent MHC-unrestricted killing. Thirty-six of the 67 T-cell clones obtained were found to be TCR$\gamma\delta^+$. The remaining 31 clones expressed the $\alpha\beta$ TCR and CD4; no CD8$^+$ T-cell clones were isolated. These CD4$^+$ T cell clones were not analysed further. Twelve of the 36 TCR $\gamma\delta^+$ clones killed infected autologous targets to various degrees. Figure 3 shows cytotoxicity of eight representative clones. Flow cytometry was used to determine the expression of various surface markers and the results from a representative clone are depicted in Fig. 4. All of the clones had the T-cell markers CD2$^+$ and CD3$^+$ in conjunction with the $\gamma\delta$ TCR. The clones failed to express either of the co-stimulatory molecules CD4 or CD8. Staining for the natural killer (NK) marker, CD16 and the $\alpha\beta$ TCR was also negative. The $\gamma\delta$ TCR expressed by eight of the 12 *Yersinia*-reactive T-cell clones was examined by sequencing. As shown in Table 2, all of the clones were found to express V$\gamma$9 in conjunction with V$\delta$2. Moreover, with the exception of clone 5.32, all clones used the same J genes; JD1 and JGP. Two pairs of identical sequences (both DNA and deduced protein sequences) were found: 5.55 and 0.5.8, 5.23 and 5.4. The $\delta$ TCR CDR3 all exhibited a small hydrophobic amino acid (L, I or V) at position 97 and were of similar sequence length, being larger than those used for the $\gamma$ chain. Analysis of 5.27 revelaed two in-frame sequences for $\delta$ gene usage, V$\delta$2 and V$\delta$1. However, only one in-frame sequence for $\gamma$ gene products was found.

The T-cell clones were further tested for their ability to kill allogeneic infected BLCL. As shown in Fig. 5, BLCL were susceptible targets following their infection with *Yersinia*, irrespective of their MHC expression. Indeed, the allogeneic infected targets were killed more efficiently than the autologous infected cell line. An examination of the kinetics of the cytotoxic response revealed that killing could be detected as

**Table 1.** MHC-unrestricted killing of target cells by a *Yersinia*-stimulated cell line

| Target cell | MHC matches | Specific cytotoxicity | |
|---|---|---|---|
| | | Uninfected | Infected |
| CM | Autologous | 19·2 | 45·3 |
| GS | B27 | 11·8 | 47·2 |
| MR | B40 | 6·2 | 73·2 |
| HHKB | A3 | 26·6 | 60·5 |
| RML | Allogeneic | 15·1 | 58·5 |
| PITOUT | Allogeneic | 30·8 | 65·2 |

A cell line generated by culturing SFMC from a patient, CM, with *Yersinia*-infected autologous BLCL was tested for its ability to kill various infected and uninfected target cell lines at E:T ratio of 10:1. The percentage specific cytotoxicity noted after 4 hr and MHC identities between autologous and other target cells are recorded.

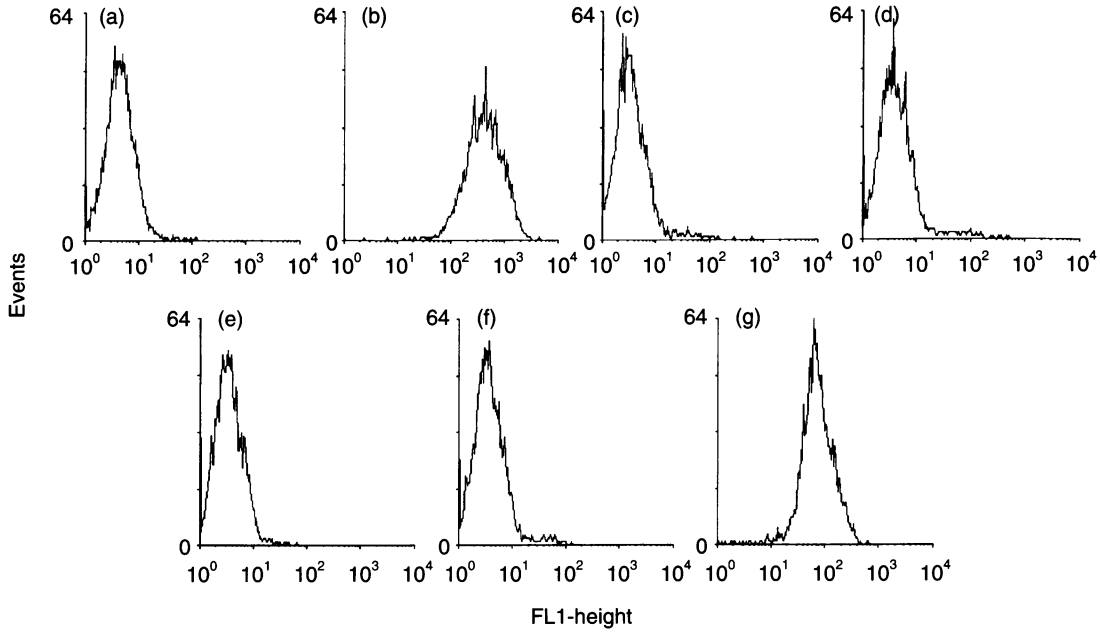


**Figure 4.** Phenoype of a *Yersinia*-reactive T cell clone. Flow cytometry analysis of a T-cell clone stained with a control IgG1–FITC (a) or FITC-conjugated antibodies specific for CD3 (b), CD4 (c), CD8 (d), CD16 (e), TCR$\alpha\beta$ (f), or TCR$\gamma\delta$ (g) is shown.

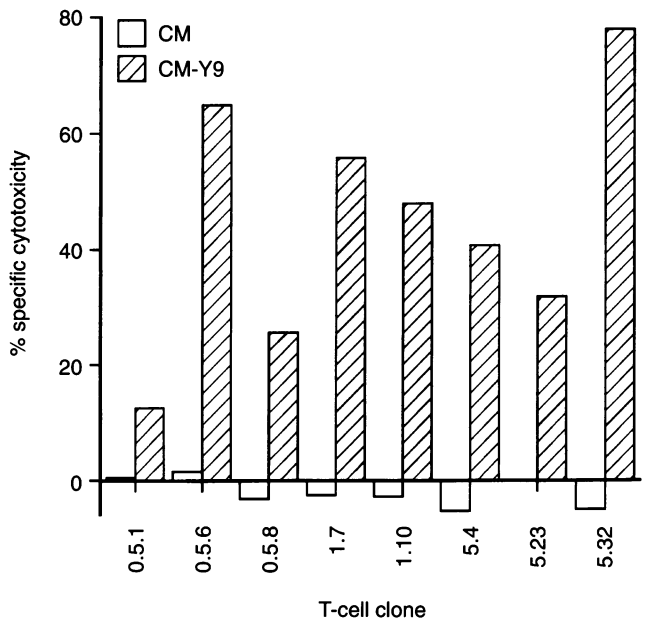


**Figure 3.** TCR$\gamma\delta^+$ T cell clones kill *Yersinia*-infected but not uninfected autologous BLCL. Eight of the $\gamma\delta^+$ T-cell clones were tested for their ability to lyse uninfected and *Yersinia*-infected autologous BLCL at E:T=5:1. The percentage specific cytotoxic responses measured after 4 hr of incubation are shown.

early as 1 hr and was already maximal by 2·5 hr (results not shown). Cell lines deficient in MHC expression were also tested for their sensitivity to cytolysis by the T-cell clones (Fig. 6). Infection of Daudi cells (class $I^-$), C1R cells (class $I^{lo}$) and K562 cells (class I and $II^{lo}$) enhanced their susceptibility to lysis by the T-cell clone. As for the allogeneic targets, cytolysis of these infected MHC-deficient cell lines was consistently greater than that observed for the autologous infected BLCL. Five of the 12 $\gamma\delta$ T-cell clones were tested against MHC-deficient cell lines and all exhibited this MHC-independent killing of infected targets.

## DISCUSSION

Our results indicate that cytotoxic T lymphocytes (CTL) with reactivity for *Yersinia*-infected targets can be readily generated from both PBMC of normal individuals as well as from SFMC of ReA patients. The CTL lines obtained were found to express predominantly the $\gamma\delta$ TCR. This profound expansion of TCR$\gamma\delta$ T cells in response to *Yersinia* is reminiscent of the reported stimulation of V$\gamma$9V$\delta$2 T cells in response to other bacteria such as mycobacteria, listeria and salmonella.[27–29] Indeed, an analysis of the TCR$\gamma\delta$ gene usage from clones isolated from the *Yersinia*-stimulated T-cell lines revealed V$\gamma$9V$\delta$2 TCR products. Thus, *Yersinia enterocolitica* also has the potential to stimulate this T-cell subset. The extreme nature of the *in vitro* expansion (5–15-fold increase in percentage expressing TCR$\gamma\delta$) of the $\gamma\delta$ T cells was further reflected in the isolation of two sets of clones bearing identical TCR sequence; as the DNA sequences were invariant, these clones probably represent *in vitro*-derived sister clones. Although two $\delta$ gene products were found when 5.27 was analysed, only one in-frame $\gamma$ gene product was found, despite analysis of multiple cloned PCR products. This suggests that either 5.27 is not clonal or allelic exclusion had failed, resulting in the generation of two $\delta$ gene products; such $\gamma\delta$ T-cell clones have been described.[30]

The *Yersinia*-reactive T-cell lines were found to kill infected target cells in an MHC-unrestricted fashion. Curiously, the T-cell line from the ReA patient, CM, exhibited poorest cytolysis of autologous or HLA-B27 matched infected BLCL. Results of recent experiments suggest that human $\gamma\delta$ T-cell clones, like NK cells, express receptors for self-MHC molecules which down-regulate killing of target cells expressing the

**Table 2.** T-cell receptor sequences of *Yersinia*-reactive T-cell clones

| Clone | Vδ | n-D-n | Jδ | | Vγ | n | Jγ | |
|---|---|---|---|---|---|---|---|---|
| 5.55 | CACD | ALGLNGQE | KLIFGKG | VD2/JD1 | CALWEV | | QELGKKIKVFGPG | VG9/JGP |
| 0.5.8 | CACD | ALGLNGQE | KLIFGKG | VD2/JD1 | CALWEV | | QELGKKIKVFGPG | VG9/JGP |
| 5.23 | CACD | TLLGDTY | KLIFGKGTRVT | VD2/JD1 | CALW | DQK | ELGKKIKVFGPG | VG9/JGP |
| 5.4 | CACD | TLLGDTY | KLIFGKG | VD2/JD1 | CALW | DQK | ELGKKIKVFGPG | VG9/JGP |
| 5.30 | CAC | ESLGDTKS | TDKLIFGKG | VD2/JD1 | CALWEV | QGGS | GKKIKVFGPG | VG9/JGP |
| 0.5.6 | CACD | PLGSGD | TDKLIFGKG | VD2/JD1 | CALWE | EHR | ELGKKIKVFGPG | VG9/JGP |
| 5.27 | CACD | SILA | TDKLIFGKG | VD2/JD1 | CALWEV | LG | LGKKIKVFGPG | VG9/JGP |
| | YFC | GSVVVGVLG | DKL | VD1/JD1 | | | | |
| 5.32 | CACD | PVEKRDTRQ | LIFGKGTRVT | VD2/JD1 | CALWEV | QLARW | KLFGSGTTLVVT | VG9/JG1/2 |

The in-frame deduced amino acid sequences of TCR δ and γ chains are shown.

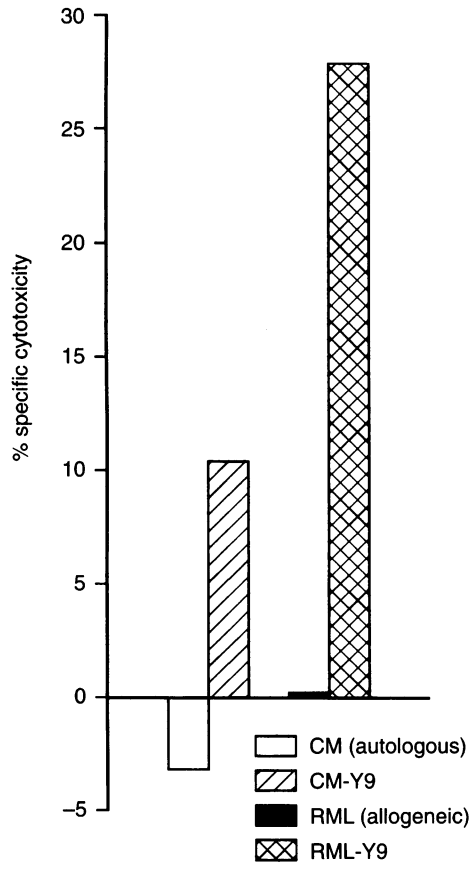


**Figure 5.** MHC-unrestricted cytotoxicity of infected target cells. Uninfected and infected autologous or allogeneic BLCL were incubated in a $^{52}$Cr-release cytotoxicity assay with one of the γδ T-cell clones (0.5.1) at an E:T ratio of 10:1 for 4 hr. The percentage specific cytotoxicity is shown.

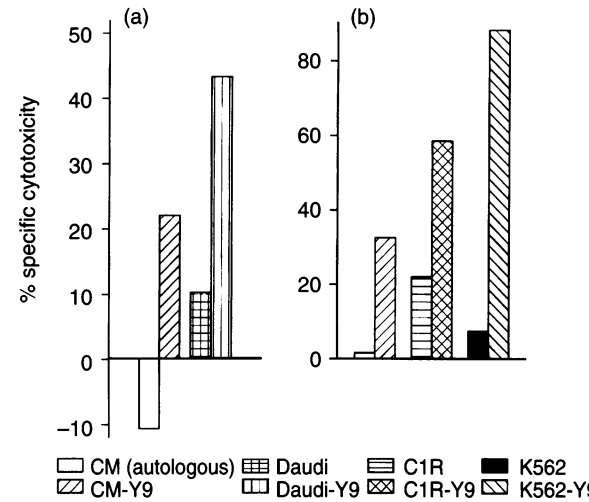


**Figure 6.** MHC-independent cytotoxicity of infected target cells. The γδ T-cell clone was incubated at an E:T ratio of 10:1 with the indicated infected or uninfected target cells for 4 hr. The percentage specific cytotoxic response for each target is shown. (a) and (b) represent independent experiments.

relevant MHC molecules.[31] Thus, the poor killing of HLA-B27 target cells may be due to a negative signal mediated via binding to a B27-reactive receptor on the TCRγδ CTL. A further explanation for the poor killing of B27 expressing targets is suggested by the work of Kapasi and Inman[32,33] who reported that cell lines expressing this MHC allele exhibited reduced rates of infectivity with gram-negative bacteria associated with reactive arthritis. Thus, infected cells expressing HLA-B27 may display a reduced number of T-cell ligands compared to infected target cells lacking this MHC molecule.

To confirm that the MHC-unrestricted killing exhibited by the T-cell lines was not due to mixed populations we generated T-cell clones. The γδ T-cell clones, like the cell lines, exhibited MHC-unrestricted killing of infected cell lines. Moreover, from the results with MHC-deficient cell lines, it is also apparent that the γδ T cells recognized infected target cells in an MHC-independent manner. As we did not analyse the cytotoxic capabilities of the CD4$^+$ TCRαβ T-cell clones, we cannot affirm that the pattern of killing documented is unique to the γδ T-cell clones. Nevertheless, TCRγδ$^+$ T cells exhibiting such MHC-independent activities have been described.[34] It has been suggested that some γδ T cells may, as has been demonstrated for a CD4$^-$ CD8$^-$ αβ T cell,[12,13] rely on antigen presented by CD1, a family of non-polymorphic β2 microglobulin-associated glycoproteins. However, as the γδ T cells reported here recognize infected Daudi cells (a cell line lacking β2-microglobulin), they cannot require the expression of CD1. Non-peptidic, phosphorylated ligands for TCRγδ T cells, particularly those expressing a Vγ9Vδ2 TCR, have been identified from mycobacteria.[4–6] Such compounds can stimulate the proliferation of Vγ9Vδ2 T cells in the absence of classic MHC molecules or classic MHC-presentation pathways.[7] Given the

preponderance of Vγ9Vδ2 TCR gene usage in the isolated T-cell clones, it is possible that *Yersinia* contain such non-peptidic ligands or give rise to the expression of equivalent cellular derived compounds upon infection of cells. Precisely how the phosphorylated ligands are recognized by the γδ T cells is, as yet, unknown. However, evidence is accumulating suggesting that at least some γδ T cells may be stimulated by native unprocessed antigens, in an analogous fashion to the engagement of the immunoglobulin receptor on B cells.[34] The CDR3 regions of immunoglobulin light and heavy chains and TCR γ and δ chains are more similar in length distribution than the CDR3 regions of the α and β TCR molecules.[35] Thus the γδ complex may be able to fold in an immunoglobulin-like manner to permit the recognition of antigenic shapes outside the constraints of the MHC–peptide complex used by the αβ TCR. In agreement with this study, the CDR3 loop lengths reported here were found to be larger for the δ chains compared to the γ chains. However, the δ CDR3 regions showed remarkable consistency in length and were shorter than most of the sequences reported by Rock *et al.*[34] Moreover, our results indicate the occurrence of a small hydrophobic amino acid (L, I or V) at position 97 in the δ sequences. This sequence is poorly represented in fetal γδ T cells, and it has been suggested that peripheral antigenic stimulation leads to the expansion of γδ T cells with such a motif.[36,37] We have not determined the nature of the stimulatory ligand for the *Yersinia*-reactive γδ T cells reported here. However, formaldehyde-fixed, but not heat-inactivated, bacteria were able to stimulate the proliferation of γδ T cells when added to mononuclear cells. Thus, the stimulation of γδ T cells was not dependent upon the addition of B lymphoblastoid cell lines to the synovial fluid mononuclear cell cultures. Our results are compatible with a native, heat-labile ligand for γδ T-cell stimulation. Moreover, since active infection was not required to provoke the expansion of the γδ T cells, the stimulatory ligand is likely to be a *Yersinia*-derived antigen rather than a self antigen provoked by infection (e.g. heat-shock protein).

Regardless of the nature of the stimulatory ligand for the *Yersinia*-reactive γδ T cells, the question arises as to the involvement of this T-cell subset in ReA. Previous studies of SFMC from ReA patients have also described a proliferation of γδ T cells in response to live but not dead *Yersinia enterocolitica*.[38] Moreover, cytotoxicity of bacteria-infected target cells was shown for five of eight Vγ9Vδ2 T-cell clones isolated by random cloning from SFMC, with four of those clones thought to recognize heat shock protein 65.[39] These clones, like the ones described here, exhibited MHC-unrestricted cytotoxicity. From the present results, it is clear that γδ T-cell proliferation can be induced by *Yersinia* not only from SFMC but also from PBMC donated by normal individuals. Whilst it is generally believed that γδ T cells are important in immune responses to infectious agents, studies have also implicated a pathological role for this subset of T cells.[14] It is clear that the γδ T-cell response to *Yersinia* is not absent in ReA patients. However, we did not determine whether there was a quantitative or qualitative difference in the γδ T-cell responses between normal and arthritic individuals. Because of the association of ReA with HLA-B27, one would not predict a pathological role for a T-cell population which displays MHC-independent cytolysis of infected target cells. However, HLA-B27, while not directly involved in γδ T cell recognition of infected targets may, as discussed earlier,[31–33] indirectly modulate the activities of the γδ T cells. Such interference with γδ T cells may have consequences for the αβ T-cell subset. For example, murine studies have demonstrated that, from the earliest stages of infection, γδ T cells can influence the cytokine milieu determining whether a T helper 1 (Th1) or Th2 response predominates.[40,41] It would be of interest to determine whether the *Yersinia*-responsive γδ T cells from ReA individuals display a different cytokine response than those in healthy individuals.

In conclusion, the results of the present study indicate that both normal PBMC and SFMC can mount a potent *in vitro* response to *Yersinia enterocolitica*. The responsive cells express predominantly the γδ TCR (Vγ9Vδ2) and such cells can kill infected targets in an MHC-independent manner. We have yet to determine whether such γδ T cells can play a pathological or beneficial role in ReA.

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## REFERENCES

1. Lanier L.L. (1995) Unusual lymphocytes – γδ T cells and NK cells. *The Immunologist* **3,** 182.
2. Schild H., Mavaddat N., Litzenberger C. *et al.* (1994) The nature of major histocompatibility complex recognition by γδ T cells. *Cell* **76,** 29.
3. Sciammas R., Johnson R.M., Sperling A.I. *et al.* (1994) Unique antigen recognition of a herpesvirus-specific TCR γδ cell. *J Immunol* **152,** 5392.
4. Tanaka Y., Sano S., Nieves E. *et al.* (1994) Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci USA* **91,** 8175.
5. Constant P., Davodeau F., Peyrat M.A. *et al.* (1994) Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* **164,** 267.
6. Tanaka Y., Morita C.T., Tanaka Y., Nieves E., Brenner M.B. & Bloom B.B. (1995) Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature* **375,** 155.
7. Morita C.T., Beckman E.M., Bukowski J.F. *et al.* (1995) Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. *Immunity* **3,** 495.
8. Balk S.P., Ebert E.C., Blumenthal R.L. *et al.* (1991) Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. *Science* **253,** 1411.
9. Faure F., Jitsikawa S., Miossec C. & Hergend T. (1990) CD1c as a target recognition structure for human T lymphocytes: analysis with peripheral blood γδ cells. *Eur J Immunol* **20,** 703.
10. Porcelli S., Brenner M.B., Greenstein J.L., Balk S.P., Terhorst C. & Bleicher P.A. (1989) Recognition of cluster differentiation 1 antigens by human $CD4^-$ $CD8^-$ cytolytic T lymphocytes. *Nature* **341,** 447.
11. Beckman E.M. & Brenner M.B. (1995) MHC class I-like, class II-like and CD1 molecules: distinct roles in immunity. *Immunol Today* **16,** 349.
12. Beckman E.M., Porcelli S.A., Morita C.T., Behar S.M., Furlong S.T. & Brenner M.B. (1994) Recognition of a lipid antigen by CD1-restricted $\alpha\beta^+$ T cells. *Nature* **372,** 615.
13. Sieling P.A., Chatterjee D., Porcelli S.A. *et al.* (1995) CD1-restircted T cell recognition of microbial antigens. *Science* **269,** 227.
14. Haas W., Pereira P. & Tonegawa S. (1993) Gamma delta cells. *Annu Rev Immunol* **11,** 637.

15. KRONENBERG M. (1994) Antigens recognized by $\gamma\delta$ T cells. *Curr Opin Immunol* **6,** 64.
16. MOMBAERTS P., ARNOLDI J., RUSS F., TONEGAWA S. & KAUFMANN S.H. (1993) Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. *Nature* **365,** 53.
17. KAUFMAN S.H.E. (1995) Immunity to intracellular bacteria and protozoa. *The Immunologist* **3,** 221.
18. LADEL C.H., BLUM C., DREHER A., REIFENBERG K. & KAUFMANN S.H.E. (1995) Protective role of $\gamma\delta$ T cells and $\alpha\beta$ T cells in tuberculosis. *Eur J Immunol* **25,** 2877.
19. BURMESTER G.R., DASER A., KAMRADT T. *et al.* (1995) Immunology of reactive arthritides. *Annu Rev Immunol* **13,** 229.
20. HERMANN E., YU D.T.Y., MEYER ZUM BUSCHENFELDE K.-H. & FLEISCHER B. (1993) HLA-B27-restricted CD8 T cells derived from synovial fluids of patients with reactive arthritis and ankylosing spondylitis. *Lancet* **342,** 646.
21. MERTZ A., DASER A., BRAUN J., BATSFORD S. & SIEPER J. (1994) The evolutionarily conserved ribosomal proteins L2 and L23 of *Yersinia enterocolitica* O:3 are major targets antigens in *Yersinia*-triggered reactive arthritis. *Arthritis Rheum* **37,** S224.
22. LAHESMAA R., SHANAFELT M.C., STEINMAN L. & PELTZ G. (1994) Immunopathogenesis of human inflammatory arthritis: lessons from Lyme and reactive arthritis. *J Infect Dis* **170,** 978.
23. GASTON J.H.S., DEANE K.H., JECOCK R. & PEARCE J. (1996) Identification of two *Chlamydia trachomatis* antigens recognized by synovial fluid T cells from chlamydia-induced reactive arthritis. *J Rheumatol* **23,** 130.
24. PROBST P., HERMANN E., MEYER ZUM BUSCHENFELDE F.H. & FLEISCHER B. (1993) Multiclonal synovial T cell response to *Yersinia enterocolitica* in reactive arthritis: the Yersinia 61 kDa heat shock protein is not the major target antigen. *J Infect Dis* **162,** 385.
25. PROBST P., HERMANN E., MEYER ZUM BUSCHENFELDE K.H. & FLEISCHER B. (1993) Identification of the *Yersinia enterocolitica* urease beta subunit as a target for human synovial T lymphocytes in reactive arthritis. *Infect Immun* **61,** 4507.
26. GOODALL J.C., HENWOOD J., BACON P.A. & GASTON J.S.H. (1995) Marked conservation of the CDR3 of the $\beta$ chain of TCRs recognising a mycobacterial heat shock protein with strong sequence similarity to human heat shock protein 60. *J Immunol* **155,** 2329.
27. PANCHAMOORTHY G., MCLEAN J., MODLIN R.J. *et al.* (1991) A predominance of the T cell receptor V$\gamma$2V$\delta$2 subset in human mycobacteria-responsive T cells suggests germline encoded recognition. *J Immunol* **147,** 3360.
28. DE LIBERO G., CASORATI G., GIACHINO C. *et al.* (1991) Selection by two powerful antigens may account for the presence of the major population of human peripheral $\gamma\delta$ T cells. *J Exp Med* **173,** 1311.
29. HARA T., MIZUNO Y., TAKAKI K. *et al.* (1992) Predominant activation and expansion of V$\gamma$9-bearing $\gamma\delta$ T cells *in vivo* as well as *in vitro* in *Salmonella* infection. *J Clin Invest* **90,** 204.
30. PEYRAT M.A., DAVODEAU F., HOUDE I. *et al.* (1995) Repertoire analysis of human peripheral blood lymphocytes using a human V$\delta$3 region-specific monoclonal antibody – characterization of dual T cell receptor (TCR) $\delta$ chain expressors and $\alpha\beta$ T cells expressing V$\delta$3J$\alpha$C-encoded TCR chains. *J Immunol* **155,** 3060.
31. NAKAJIMA H., TAMIYAMA H. & TAKIGUCHI M. (1995) Inhibition of $\gamma\delta$ T cell recognition by receptors for MHC class I molecules. *J Immunol* **155,** 4139.
32. KAPASI K. & INMAN R.D. (1992) HLA-B27 expression modulates gram-negative bacterial invasion into transfected L cells. *J Immunol* **148,** 3554.
33. KAPASI K. & INMAN R.D. (1994) ME-1 epitope of HLA-B27 confers class I-mediated modulation of gram-negative bacterial invasion. *J Immunol* **153,** 833.
34. DAVIS M.M. & CHIEN Y.-h. (1995) Issues concerning the nature of antigen recognition by $\alpha\beta$ and $\gamma\delta$ T cell receptors. *Immunol Today* **16,** 316.
35. ROCK E.P., SIBBALD P.R., DAVIS M.M. & CHIEN Y.-h. (1994) CDR3 length in antigen-specific immune receptors. *J Exp Med* **179,** 323.
36. DAVODEAU F., PEYRAT M.A., HALLET M.M., HOUDE I., VIE H. & BONNEVILLE M. (1993) Peripheral selection of antigen receptor junctional features in a major human $\gamma\delta$ subset. *Eur J Immunol* **23,** 804.
37. BREIT T.M., WOLVERS-TETTERO L.M. & VAN DONGEN J.J.M. (1994) Unique selection determinant in polyclonal V$\delta$2-J$\delta$1 junctional regions of human peripheral $\gamma\delta$ T lymphocytes. *J Immunol* **152,** 2860.
38. HERMANN E., LOHSE A.W., MAYET W.H. *et al.* (1992) Stimulation of synovial fluid mononuclear cells with the human 65-kD heat shock protein or with live enterobacteria leads to preferential expansion of TCR-$\gamma\delta^+$ lymphocytes. *Clin Exp Immunol* **89,** 427.
39. HERMANN E., ACKERMANN B., DUCHMANN R. & MEYER ZUM BUSCHENFELDE K.H. (1995) Synovial fluid MHC-unrestricted $\gamma\delta$ T lymphocytes contribute to antibacterial and anti-self cytotoxicity in the spondylarthropathies. *Clin Exp Rheum* **13,** 187.
40. FERRICK D.A., SCHRENZEL M.D., MULVANIA T., HSIEH B., FERLIN W.G. & LEPPER W. (1995) Differential production of interferon-gamma and interleukin-4 in response to Th1-stimulating and Th2-stimulating pathogens by gamma-delta T cells *in vivo*. *Nature* **373,** 255.
41. HSIEH B., SCHRENZEL M.D., MULVANIA T., LEPPER H.D., DIMOLFETTOLANDON L. & FERRICK D.A. (1996) *In vivo* cytokine production in murine listeriosis – evidence for immunoregulation by $\gamma\delta^+$ T cells. *J Immunol* **156,** 232.