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Human CD4$^-$ CD8$^-$ $\alpha\beta^+$ T-cell receptor T cells recognize different mycobacteria strains in the context of CD1b

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SUMMARY

Double-negative $\alpha\beta^+$ T-cell receptor (TCR) human T cells have been reported to recognize antigen in the context of the HLA class I-like (Ib) CD1 complex. In particular, the CD1b molecule has been shown to act as the element of genetic restriction for antigens derived from *Mycobacterium tuberculosis*. The stenotopic nature of these major histocompatibility complex (MHC) class Ib molecules raised the question of whether the antigenic moiety recognized by CD4$^-$ CD8$^-$ $\alpha\beta^+$ TCR T cells was shared by different mycobacteria. We demonstrate here that a CD4$^-$ CD8$^-$ $\alpha\beta^+$ TCR T-cell line and three clones raised against *M. tuberculosis* proliferated following stimulation with soluble extracts from organisms of the *M. tuberculosis* complex, *M. leprae* and 10 out of 16 tested isolates of *M. avium* complex; however, four species of weakly or non-pathogenic mycobacteria were not stimulatory. Furthermore, the *M. tuberculosis* soluble extract (MTSE)-derived, recognized antigenic moiety proved to be proteinase K resistant and to have a molecular weight greater than 5000 MW, thus it differed from the reported antigenic moiety recognized by CD4$^-$ CD8$^-$ $\gamma\delta^+$ TCR cells. Our results suggest that a common antigenic moiety, presented by CD1b molecules to CD4$^-$ CD8$^-$ $\alpha\beta^+$ TCR T cells, is shared by many mycobacterial species. Therefore they raise interest in the question of whether CD4$^-$ CD8$^-$ $\alpha\beta^+$ TCR T cells, elicited by *M. tuberculosis*, may play a role in the natural history of other mycobacterial infections.

INTRODUCTION

T lymphocytes recognize antigens in the context of the highly polymorphic major histocompatibility complex (MHC) class I and II molecules.[1] This polymorphism is necessary in order to accommodate the large number of possible antigenic peptides, although any single MHC molecule can bind many peptides.[2,3] MHC allele-specific peptide sequence motifs have been identified for both MHC class I and class II epitopes.[4–6] While the majority of epitopes have a MHC-restricted binding profile, certain epitopes are able to bind promiscuously several MHC molecules.[7]

The epitopes presented by MHC class II and class I molecules are normally recognized by either CD4$^+$ or CD8$^+$ $\alpha\beta^+$ T-cell receptor (TCR) T lymphocytes. However, a small percentage of mature human $\alpha\beta^+$ TCR T cells do not express the CD4 or CD8 markers,[8,9] here after referred to as double-negative (DN) $\alpha\beta^+$ TCR T cells, and display some peculiar characteristics. In particular, these T cells seem to have a restricted TCR repertoire, common to many individuals,[10–12] and they recognize some, yet undefined, antigens of bacterial origin in the context of the MHC class Ib molecules CD1a and b,[12,13] which are not genetically polymorphic.[14,15] This feature, together with their restricted TCR usage, constitutes a unique example of stenotopic T-cell recognition.

In order to test whether DN $\alpha\beta^+$ TCR T cells may recognize shared antigenic moieties expressed by several mycobacteria, we have established a CD1b-restricted DN $\alpha\beta^+$ TCR T-cell line and clones specific for *Mycobacterium tuberculosis*. Further analysis has shown that the line and clones respond to different mycobacterial species. A first characterization of the recognized antigenic moiety showed that it is resistant to proteinase K treatment and that it has a molecular weight (MW) greater than 5000. *Mycobacterium*-specific DN $\gamma\delta^+$ T cells have been described previously,[16,17] and more recently the recognized antigen has been further characterized.[18,19] The nature of this antigen, in particular its molecular weight, which differs compared to that recognized by DN $\alpha\beta^+$ T cells, suggests that $\gamma\delta$ and $\alpha\beta$ T cells recognize two different moieties. Our results indicate that DN $\alpha\beta^+$ TCR T cells, although a small subset of T lymphocytes, may have a role in the natural history of infections with pathogenic mycobacteria. However, the molecular nature of the antigen recognized by DN $\alpha\beta^+$ TCR T cells must await further investigation.

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Abbreviations: DN, double negative; MTSE, *Mycobacterium tuberculosis* soluble extract.

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MATERIALS AND METHODS

Antigens

Soluble extracts from *M. tuberculosis* (MTSE), *M. bovis*, *M. avium intracellulare* complex (14 different clinical isolates or strains), *M. avium ss. paratuberculosis* (two different isolates), *M. scrofulaceum*, *M. smegmatis*, *M. xenopi*, *M. kansasii* and *M. leprae* were prepared as described previously.[20] Ultrafiltrates were prepared by centrifugation of MTSE at 2000 *g* with Ultrafree-MC filter units (low-binding cellulose membrane, 5000 MW cut-off; Millipore, Eschborn, Germany).[17] Aliquots of the original preparation, as well as from the filtrates, were digested with proteinase K. Proteinase K (Sigma, St Louis, MO) was dissolved at a concentration of 2·5 mg/ml in 0·85% NaCl. The enzyme was mixed 1 : 2·5 (v/v) with the aliquots for a final enzyme concentration of 0·7 mg/ml and incubated for 30 min at 60°, followed by 10 min at 70° to inactivate the enzyme.[21]

Culture supernatants (CSN) were obtained at two different time-points from *M. tuberculosis* (strain H37Rv). CSN1 was fractionated by the modified Seibert precipitation technique into six fractions, F0–F5.[22] Four recombinant antigens of *M. tuberculosis*, 16 000, 19 000, 38 000 and 65 000 MW, were also tested.[23]

Monoclonal antibodies used in this study

Anti-CD4/anti-CD8 (Simultest; Leu-3a/Leu-2a), anti-CD14/anti-CD45 (Leucogate) and anti-CD3 were purchased from Becton Dickinson (San José, CA); ascites containing monoclonal antibodies anti-CD1a, b and c, respectively, were a gift from Dr L. Boumsell (Paris, France); anti-TCR$\alpha\beta$ (BMA 031) was a gift from Dr R. Kerrle (Behringwerke, Marburg, Germany).

Flow cytometry analysis

Fluorescence analysis was carried out by incubating 5×10^4 to 1×10^5 cells with the optimal concentration of the appropriate antibody in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) and 0·2% sodium azide, at 4° for 45 min. For indirect immunostaining a second layer was added containing fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) at a dilution of 1 : 100. Prior to analysis, cells were washed and fixed in 3% formaldehyde. A FACStar Plus (Becton Dickinson) was used to analyse the stained cells and for sorting.

Preparation of monocytes and induction for CD1 expression

Monocytes were isolated from leucocyte concentrates from healthy donors either by counterflow centrifugal elutriation or by plastic adherence. After isolating the peripheral blood mononuclear cells (PBMC) by Ficoll–Hypaque gradient separation, cells were adhered for 1–1·5 hr at 37° in RPMI-1640 (Gibco BRL, Paisley, UK) supplemented with 2·5% FCS, and then detached with 0·02% EDTA (disodium salt) in 0·85% saline for 5–10 min at 37°. Typically isolated monocyte populations were 60–70% CD14 positive and CD1a, b, c negative. Monocytes were incubated for 60 hr in RPMI-1640 supplemented with 5% FCS, 100 U/ml granulocyte–macrophage colony-stimulating factor (GM-CSF; Behringwerke) and 10 ng/ml interleukin-4 (IL-4; Sandoz, Basel, Switzerland). After this the monocytes stained positive for CD1a, b and c and expressed low levels of CD14, as previously reported.[12,13] The monocytes were then irradiated (5000 rads) and used as antigen-presenting cells.

Preparation of T-cell lines and clones

PBMC were obtained from a healthy donor by Ficoll–Hypaque gradient separation and frozen until used. Cells were then thawed and washed twice in cold RPMI-1640 supplemented with 2·5% FCS.

For establishing a $CD4^+$ T-cell line, 2×10^6 cells were cultured with MTSE 5 μg/ml in complete medium (RPMI-1640 supplemented with 10% human serum, 100 U/ml penicillin and 100 μg/ml streptomycin). Human recombinant IL-2 (20 ng/ml; Takeda Chemicals Industry, Osaka, Japan) was added after 48 hr and every 3–4 days. After 2 weeks the line was restimulated on irradiated autologous PBMC, and MTSE 5 μg/ml and IL-2 (20 ng/ml) was added as described above.

T cells were cloned as described previously.[8] Briefly, 2 weeks after the last stimulation cells were washed twice and cloned by limiting dilution (0·3 cell/well) in Terasaki plates, with irradiated (4500 rads) heterologous PBMC (5×10^5 cells/ml), IL-2 (20 ng/ml) and phytohaemagglutinin (PHA, 1 : 1000; Difco, Detroit, MI). Clones were transferred every other week in fresh complete medium supplemented with IL-2 (20 ng/ml), PHA (1 : 1000) and irradiated (4500 rads) heterologous PBMC. IL-2 (20 ng/ml) was added every 3–4 days.

For establishing a DN T-cell line, cells were incubated at 4° for 45 min with optimal concentrations of anti-CD4–FITC/anti-CD8–phycoerythrin (PE) (Simultest) in RPMI-1640 supplemented with 1% FCS. The cells were then washed twice with RPMI-1640/2·5% FCS. DN cells were sorted with a FACStar (Becton Dickinson). The purified $CD4^-$ $CD8^-$ T cells were cultured and expanded by stimulation with MTSE 5 μg/ml and heterologous irradiated (5000 rads) monocytes, stimulated as described above. Recombinant IL-2 (20 ng/ml) was added after 48 hr and then every 3–4 days. After 2 weeks cells were restimulated as described. After 5 weeks of culture cells were stained for CD4 and CD8 expression and subsequently resorted. After 12 weeks of stimulation with antigen, T cells were subsequently propagated with PHA (1 : 1000), recombinant IL-2 and heterologous irradiated (4500 rads) PBMC. IL-2 was again added every 3–4 days. The MTSE-specific DN T-cell line was cloned and propagated as described above.

Proliferation assays

Proliferation assays of PBMC were performed with 5×10^4 cells over 6 days, in triplicate, in the presence or absence of antigens in 200 μl of complete medium, in 96-well round-bottomed microtitre plates. [^{3}H]thymidine (0·5 μCi; Amersham Int., Amersham, UK) was added for the final 8 hr. The assay was then harvested onto glass fibre filters.

Proliferation assays of T-cell lines and clones were performed by incubating cells for 3 days in triplicate in complete medium in 96-well round-bottomed microtitre plates. Briefly, 10^4 T cells (clone or line) were washed twice and added to irradiated 3×10^4 antigen-presenting cells, in the presence or absence of different mycobacterial antigens at a concentration of 5 μg/ml, and anti-CD1 antibodies (1 : 500 dilution of ascites). [^{3}H]thymidine (0·5 μCi) was added for the final 8 hr of the

Table 1. Proliferation assays of *M. tuberculosis*-specific PK CD4⁺ clones 12, 13 and 25

	Proliferation (c.p.m.)		
	Exp. 1	Exp. 2	Exp. 3
Antigen-presenting cells	Autologous irradiated PBMC	Autologous irradiated monocytes	Heterologous irradiated PBMC
Clone 12			
Medium	383 ± 122	133 ± 35	23 ± 4
IL-2 (20 ng/ml)	66 522 ± 6434	16 138 ± 7983	11 751 ± 1705
MTSE (5 μg/ml)	36 611 ± 1765	79 864 ± 2920	25 ± 13
Clone 13			
Medium	242 ± 64	154 ± 45	19 ± 7
IL-2 (20 ng/ml)	28 651 ± 4366	11 766 ± 1530	9003 ± 2141
MTSE (5 μg/ml)	20 278 ± 1882	60 257 ± 4998	45 ± 50
Clone 25			
Medium	641 ± 12	115 ± 30	22 ± 2
IL-2 (20 ng/ml)	79 603 ± 405	15 176 ± 1615	19 324 ± 589
MTSE (5 μg/ml)	24 189 ± 3324	60 890 ± 4105	23 ± 6

T cells (10^4) were cultured for 3 days with the indicated antigen-presenting cells in the presence of IL-2 or MTSE. [^{3}H]thymidine incorporation (c.p.m.) is expressed as arithmetic mean ± SEM of triplicate cultures.

assay, which was then harvested onto glass fibre filters. [^{3}H]thymidine incorporation was assessed by standard liquid scintillation counting in a LKB Betaplate counter (Pharmacia, Turku, Finland).

RESULTS

Establishment and characterization of CD4⁺ and DN αβ⁺ TCR line and clones specific for *M. tuberculosis*

CD4⁺ T-cell lines and clones specific for MTSE were established from the PBMC of a donor, PK, previously shown to be reactive to this antigen (data not shown). The data demonstrating their antigen specificity, requirement for autologous antigen-presenting cells and phenotype are shown in Table 1 and Fig. 1a.

The DN αβ⁺ TCR T-cell line was established as previously described.[13] Figure 1b, c shows the phenotypic characterization of this cell line, which maintained this stable phenotype as proved by repeated flow cytometry. All the DN clones described in this report were also CD4⁻ CD8⁻ αβ⁺ TCR (data not shown).

The DN αβ⁺ TCR T-cell line, as well as all the DN T-cell clones, proliferated in response to MTSE only if monocytes (autologous or heterologous) expressing CD1 molecules were used as antigen-presenting cells (Table 2). Hence, antigen-presenting cells not treated with GM-CSF and IL-4 did not present antigen (Table 2). However, unstimulated autologous mono-

Table 2. Proliferation assays of DN T cells in the presence of different APC

	Proliferation (c.p.m.)		
	Exp. 1	Exp. 2	Exp. 3
T cells	DN line PK 1	DN clone PK 8	DN clone PK 8
Antigen-presenting cells	Autologous monocytes	Heterologous monocytes	Heterologous monocytes
CD1 expression	+	+	−
Medium	556 ± 56	85 ± 14	91 ± 27
IL-2 (20 ng/ml)	31 609 ± 1331	3234 ± 159	6346 ± 886
MTSE (5 μg/ml)	3908 ± 1035	1856 ± 88	76 ± 7
Antigen-presenting cells only	158 ± 53	68 ± 22	52 ± 12

DN T cells responded to MTSE presented by autologous CD1-expressing irradiated monocytes (Exp. 1). DN T cells also responded to MTSE presented by heterologous, irradiated monocytes expressing CD1 (Exp. 2); no response was seen if monocytes were not expressing CD1 (Exp. 3).

T lymphocytes (10^4) from line PK DN I and clone PK DN 8 were incubated for 3 days with the indicated antigen-presenting cells (3×10^4) in the presence of no antigen, IL-2 or MTSE. Experiments 2 and 3 were done on the same day and the same source of monocytes was used. The results are expressed as the arithmetic mean of [^{3}H]thymidine incorporation ± SEM of cultures set up in triplicate (c.p.m.).

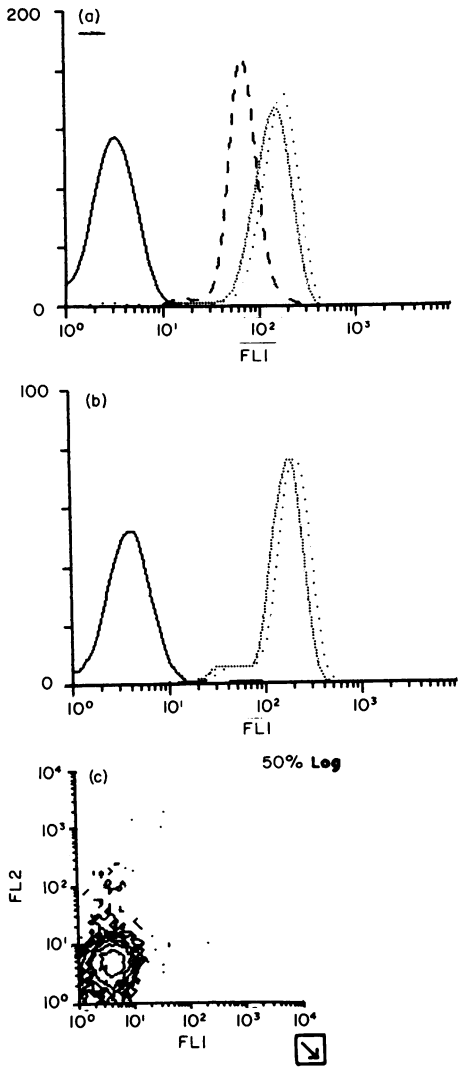

Figure 1. Flow cytometry analysis of the MTSE-specific $CD4^+$ clone PK 25 and T-cell line PK DN I. (a) PK clone 25: solid line, negative control; dotted line, anti-CD3; broken line, BMA 031 ($\alpha\beta$ TCR); dashed line, anti-CD4. (b) PK DN I: solid line, negative control; dotted line, anti-CD3; broken line, BMA 031 ($\alpha\beta$ TCR). (c) PK DN I: FL1, anti-CD4–FITC (Leu-3a); FL2, anti-CD8–PE (Leu-2a).

cytes presented the same MTSE preparation efficiently to $CD4^+$ clones (Table 1). The proliferative response of DN $\alpha\beta^+$ TCR T cells to MTSE could be blocked using an anti-CD1b monoclonal antibody, but not by anti-CD1a or anti-CD1c monoclonal antibodies (Fig. 2), thus demonstrating that CD1b is the element of genetic restriction for the MTSE-specific DN $\alpha\beta^+$ TCR T cells. DN $\alpha\beta^+$ TCR T cells did not recognize $CD1b^+$ monocytes in the absence of MTSE, as shown in Fig. 2 (medium).

Proliferative response of DN T lymphocytes to different mycobacterial species

As shown in Fig. 3, the DN $\alpha\beta$ TCR^+ T-cell line proliferated,

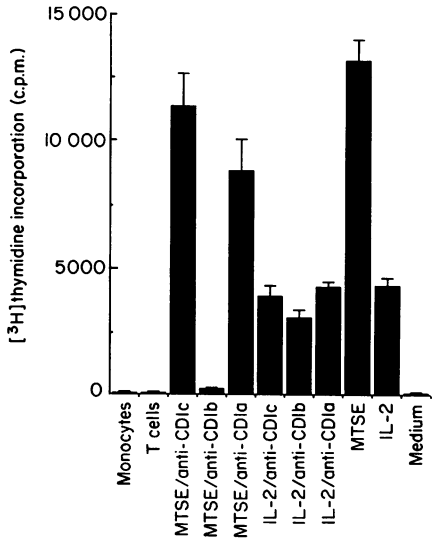

Figure 2. Blocking of the proliferative response of PK DN clone 16 to MTSE with anti-CD1b monoclonal antibody. T cells (10^4) were cultured for 3 days with CD1-expressing heterologous irradiated monocytes (3 ± 10^4) and MTSE (5 μg/ml) or IL-2 (20 ng/ml) in the presence or absence of anti-CD1 monoclonal antibody (anti-CD1a, anti-CD1b and anti-CD1c, respectively, each at a dilution of 1 : 500). As a control, T cells were challenged with $CD1^+$ monocytes in the absence of antigen (medium). The results are expressed as the arithmetic mean of [^{3}H]thymidine incorporation ± SEM; the experiments were performed in triplicates.

though to a different extent, in the presence of 15 out of the 25 tested preparations from several mycobacterial species. The recognized soluble extracts were derived from *M. tuberculosis*, *M. bovis*, several isolates of the *M. avium* complex including *M. paratuberculosis*, and *M. leprae*. However, *M. scrofulaceum*, *M. smegmatis*, *M. xenopi* and *M. kansasii* were not recognized. The recognition profile of three tested DN T-cell clones was found to be similar to that found for the T-cell line (data not shown).

Further characterization of the recognized antigenic moiety

To define further the nature of the antigenic moiety recognized by DN T cells, six 'Seibert fractions', prepared by ethanol–acetic acid precipitation from the culture supernatant, and four recombinant antigens from *M. tuberculosis* were used to challenge DN $\alpha\beta^+$ TCR T cells. No proliferative response was observed against any of the recombinant antigens (data not shown). However, the first four of the six Seibert fractions induced a significant proliferative response, indicating that they contained the recognized antigenic moiety (Fig. 4).

DN $\alpha\beta^+$ T cells were also challenged with preparations of MTSE that were separated by size, and treated with proteinase K. As shown in Fig. 5 only the preparations with a molecular weight greater than 5000 were recognized. There was no difference in the proliferative response when the MTSE preparation was proteinase treated, suggesting that the antigenic moiety might not be a protein. The reactivity of $CD4^+$ T cells specific for MTSE was abolished by proteinase K treatment, indicating that the enzymatic treatment was effective (data not shown).

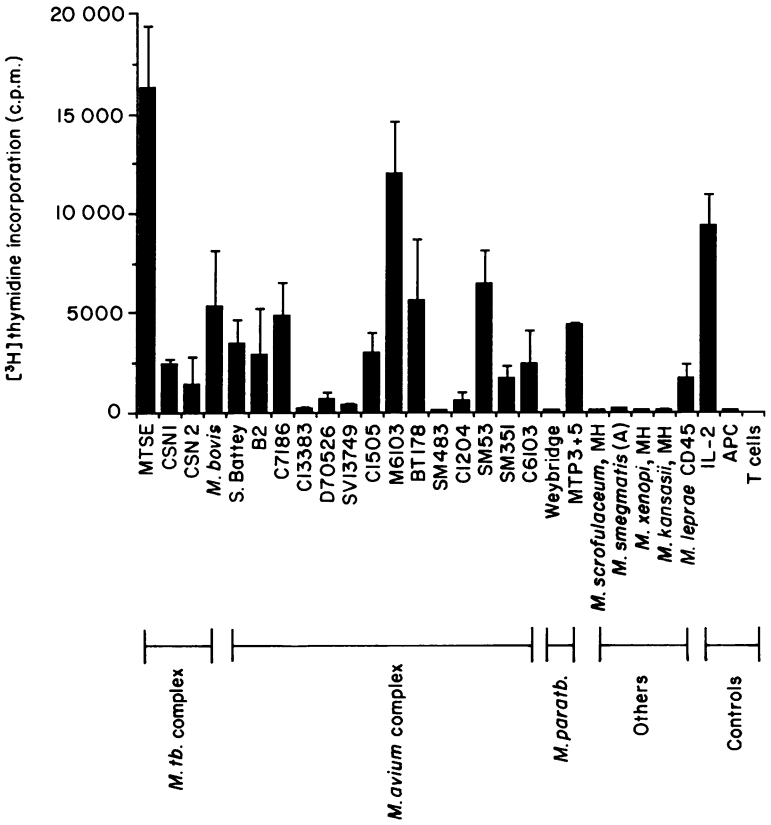

Figure 3. Proliferative response of line PK DN I to 25 antigen preparations from different mycobacteria. T cells (10^4) were cultured in triplicates for 3 days with irradiated, heterologous, CD1-expressing monocytes (3×10^4) and the indicated antigens. The results show the mean [^{3}H]thymidine incorporation ± SEM. The same results were obtained in different experiments with the line as well as with the DN T-cell clones. APC, antigen-presenting cells.

Recognition by CD4⁺ T cells of mycobacterial antigens

A panel of CD4⁺ T-cell clones raised against MTSE was also tested for their potency to respond to mycobacterial extracts by proliferation. The results showed that all of the tested mycobacterial species were recognized, though to a

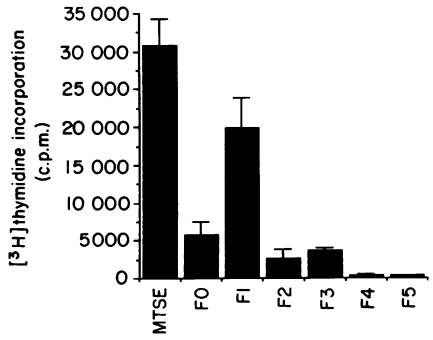

Figure 4. The proliferative response of DN line PK DN I was tested in the presence of six Seibert fractions of culture supernatant recombinant antigens 1 and 4 from *M. tuberculosis*. T cells (10^4) were cultured for 3 days with heterologous, CD1-expressing irradiated monocytes (3×10^4) with the indicated Seibert fractions F0–F5 and MTSE (5 μg/ml). The results are expressed as the arithmetic mean of [^{3}H]thymidine incorporation ± SEM of cultures set up in triplicates. Similar results were obtained several times with the same line as well as with PK DN clones 16 and 17.

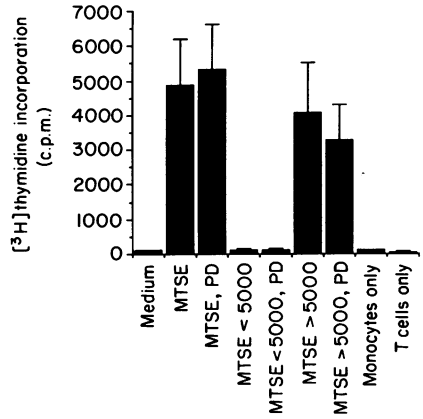

Figure 5. PK DN I recognizes proteinase K-treated MTSE of molecular weight greater than 5000. T cells (10^4) were cultured for 3 days with 3×10^4 heterologous, CD1-expressing, irradiated monocytes in the presence of the following antigens at 5 μg/ml: MTSE, proteinase-treated MTSE (MTSE, PD), ultrafiltrate of MTSE < 5000 MW with or without proteinase treatment (MTSE < 5000; MTSE < 5000, PD), ultrafiltrate of MTSE > 5000 MW with or without proteinase treatment (MTSE > 5000; MTSE > 5000, PD), or in the absence of any antigen (Medium). Cultures were set up in triplicates and the results are expressed as the arithmetic mean of [^{3}H]thymidine incorporation ± SEM.

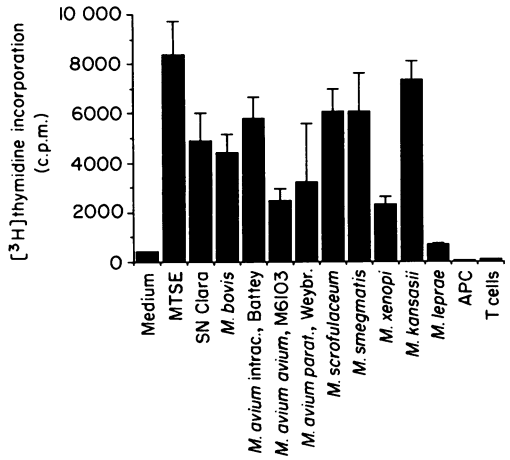

Figure 6. Proliferative response of $CD4^+$ clone 23 to antigens from different mycobacteria. Cloned T cells (10^4) and 3×10^4 irradiated autologous PBMC were cultured with the antigens (5 μg/ml) indicated. Results are the arithmetic means of [^{3}H]thymidine incorporation ± SEM of a representative experiment set up in triplicate; counts in the presence of IL-2 were 31 606 ± 1810 c.p.m.

different extent, except *M. leprae* which failed to stimulate any of the tested $CD4^+$ T-cell clones. Although we do not know whether an unusual mycobacterial antigen is recognized by our $CD4^+$ T-cell clones, the same preparation of *M. leprae* was able to stimulate $CD4^- CD8^- \alpha\beta^+$ TCR T cells. It is of particular interest that stimulation of the $CD4^+$ T cells was achieved with extracts from four mycobacterial species (*M. scrofulaceum*, *M. xenopi*, *M. kansasii* and *M. smegmatis*) which failed to stimulate any of the DN T cells. Representative results obtained with PK $CD4^+$ clone 23 are shown in Fig. 6.

DISCUSSION

DN $\alpha\beta^+$ TCR T cells have been shown to recognize antigen presented by the non-classical MHC molecules CD1;[12,13] in particular CD1b has been shown to act as antigen-presenting molecule for *M. tuberculosis*.[13] However, the possible cross-reactivity of DN $\alpha\beta^+$ TCR T cells restricted by CD1b with other species of mycobacteria has so far not been investigated. This question was addressed in our study using DN $\alpha\beta^+$ TCR MTSE-specific T-cell lines and clones raised from a healthy donor.

As a source of antigen-presenting cells, autologous monocytes as well as monocytes obtained from different individuals allowed antigen recognition if they were pretreated with IL-4 and GM-CSF. This combination of cytokines has been reported to induce CD1 molecules on monocytes.[12,13] Antibody blocking experiments proved that the mycobacterial antigen was recognized in the context of CD1b. Furthermore, no direct recognition of CD1b by MTSE-specific, DN $\alpha\beta^+$ TCR T cells was observed.

The DN T-cell line reacted consistently with extracts from organisms of *M. tuberculosis* complex, the majority of *M. avium* complex isolates and with *M. leprae*, but no reaction could be shown with *M. scrofulaceum*, *M. smegmatis*, *M. xenopi* and *M. kansasii*. The reactivity profile of the T-cell line was also confirmed with all the three tested DN T-cell clones (data not shown). The four mycobacterial species, which did not induce DN T-cell proliferation, were not void of antigen, since they were stimulatory for $CD4^+$ clones raised from the same donor.

To define further the nature of the antigen recognized, we tested the response of the DN T cells to six Seibert fractions, as well as to four recombinant antigens derived from *M. tuberculosis*. Reactivity was detected only against the first four fractions, each containing several constituents of different molecular weight,[22] but no responsiveness of $CD4^+$ T-cell clones was found to fractions −0 and −1 (data not shown), which were specifically recognized only by the DN $\alpha\beta^+$ TCR T cells. Although the precise constituents of the Seibert fractions are not known, the presence of the antigenic moiety in the first two fractions could be a useful basis for the further characterization of the recognized antigen moiety. No proliferative response was seen to any of the four tested recombinant antigens.

In addition, aliquots of MTSE were prepared as described in the Materials and Methods, and treated with proteinase K. DN $\alpha\beta^+$ TCR T cells showed a proliferative response only to the preparation with a size greater than 5000 MW. Furthermore, proteinase treatment did not abolish the antigenicity of the preparation. In contrast, the antigenicity for MTSE-specific $CD4^+$ T cells was destroyed by proteinase K. These data are highly suggestive that the DN $\alpha\beta^+$ TCR T cells do not recognize proteins, although further experiments are required to define its molecular nature.

Interestingly, antigens derived from *M. tuberculosis* were also recognized by DN $\gamma\delta^+$ TCR T cells. However, they differed in molecular weight (1000–3000 MW)[16–19] from the antigen recognized by DN $\alpha\beta^+$ TCR T cells, as shown by us and elsewhere.[13]

The function of DN $\alpha\beta^+$ TCR T cells has yet to be clarified, since they have only recently been identified and studied;[8–12,24–26] thus it is still difficult to define their role, if any, *in vivo*. Some reports indicate that these cells may act as natural suppressors,[27,28] but further characterization is required to understand their significance.

Some features of the DN T cells indicate that they have been activated *in vivo* upon antigenic challenge. In particular their phenotype *in vivo*, prior to *in vitro* stimulation, with expression of HLA-DR and CD45RO, is suggestive of a previous antigenic engagement.[29] This is further supported by the restricted TCR usage indicative of an *in vivo* selection of DN $\alpha\beta^+$ TCR T cells,[10–12,29] and by their persistence *in vivo* for a period of several years.[12]

Little is known about the *in vivo* expression of CD1 molecules in pathological conditions. The normal tissue distribution of these molecules includes dendritic dermal cells, Langerhans' cells, mantel zone in lymphoid organs and monocytoid cells, and induction of CD1b has been reported during chronic inflammation.[14] This is not surprising since a combination of IL-4 and GM-CSF, normally detected in an inflammatory process, is a potent inducer of CD1 expression *in vitro*.

The stenotopy of the CD1 molecules (i.e. their lack of polymorphism) suggests that they may bind only a limited spectrum of antigenic moieties, most likely a set of conserved epitopes from important pathogens.[30] In our study we demonstrate

that antigens derived from pathogenic mycobacteria (*M. tuberculosis* complex, *M. avium* complex and *M. leprae*) can be recognized in the context of the CD1b molecule. Since we do not know the molecular nature of the recognized antigen we cannot speculate whether single or cross-reactive moieties are shared among the different species and strains. Nevertheless it seems significant that four rarely or non-pathogenic mycobacterial species were not recognised by DN T cells.

The lack of recognition of MTSE by a panel of DN $\alpha\beta^+$ TCR T-cell clones, raised from the same individual and expressing different TCR,[10] indicates that the TCR is directly involved in MTSE recognition. These experiments also suggest that the MTSE preparation is not acting as a mitogen. Its activity as a superantigen seems to be ruled out by the requirement of antigen-processing, as shown by Porcelli *et al.*[13]

Once the role of DN $\alpha\beta^+$ TCR T cells has been clarified, the CD1b-restricted antigenic moieties recognized by these cells may become ideal candidates for the development of subunit vaccines. Thus further studies will be required to eventually clarify the function and relevance of these cells in human pathological conditions. Recently, since submission of this manuscript, Beckman *et al.*[31] have reported that DN $\alpha\beta^+$ TCR T cells were stimulated by mycolic acid, proving that a non-protein antigen is recognized by these cells.

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