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Accumulation of a potent γδ T-cell stimulator after deletion of the *lytB* gene in *Escherichia coli*

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SUMMARY

Activation of human Vγ9/Vδ2 T cells by many pathogens depends on the presence of small phosphorylated non-peptide compounds derived from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid biosynthesis. We here demonstrate that in *Escherichia coli* mutants deficient in *lytB*, an essential gene of the MEP pathway, a potent Vγ9/Vδ2 T-cell activator accumulates by a factor of approximately 150 compared to wild-type *E. coli*. The compound responsible for the strong immunogenicity of this *E. coli* mutant was subsequently characterized and identified as a small pyrophosphorylated metabolite, with a molecular mass of 262 Da, derived from the MEP pathway. Stimulation of human peripheral blood mononuclear cells (PBMC) with extracts prepared from the *lytB*-deficient *E. coli* mutant led to upregulation of T-cell activation markers on the surface of Vγ9/Vδ2 T cells as well as proliferation and expansion of Vγ9/Vδ2 T cells. This response was dependent on costimulatory growth factors, such as interleukin (IL)-2, IL-15 and IL-21. Significant levels of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) were secreted in the presence of IL-2 and IL-15, but not in the presence of IL-21, demonstrating that proliferating phosphoantigen-reactive Vγ9/Vδ2 T cells do not necessarily produce proinflammatory cytokines.

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Abbreviations: DOXP, 1-deoxy-D-xylulose 5-phosphate; Dxr, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; ESI, electrospray ionization; FBPP, 3-formyl-1-butyl pyrophosphate; HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IFN-γ, interferon-γ; IL, interleukin; IPP, isopentenyl pyrophosphate; LMW, low molecular weight; ME, 2-*C*-methyl-D-erythritol; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MS, mass spectrometry; TNF-α, tumour necrosis factor-α.

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INTRODUCTION

γδ T cells play a crucial role in the immune response to microbial pathogens, many of which are capable of establishing chronic and debilitating diseases, such as tuberculosis and malaria.[1,2] In humans, activation of Vγ9/Vδ2 T cells by most pathogenic micro-organisms depends on compounds derived from the 2-*C*-methyl-D-erythritol 4-phosphate (MEP)[1] pathway of isoprenoid biosynthesis.[3,4] Beside the chloroplasts of algae and higher plants, this pathway is utilized by many bacteria,[5,6] as well as apicomplexan protozoa such as *Plasmodium falciparum*,[7] but is apparently absent in all vertebrates.[8–11] Thus, the unconventional γδ T-cell reactivity to common metabolites ensures a quick and efficient immune response to a broad range of evolutionarily distant pathogens that may otherwise escape classical major histocompatibility complex-restricted mechanisms.[12] While isopentenyl pyrophosphate (IPP), the precursor of all isoprenoids, was the

first ligand described for the Vγ9/Vδ2 T-cell receptor (TCR),[13–15] the natural amounts of IPP in bacteria do not reach the minimum required for inducing T-cell activation.[3] Fournié and co-workers showed that a mycobacterial molecule likely to be 3-formyl-1-butyl pyrophosphate (FBPP) is approximately 1000-fold more active than IPP;[16,17] because of its structural similarity to IPP, FBPP was suggested to be an intermediate of the MEP pathway.[17] However, the terminal steps of the MEP pathway still remain to be elucidated, with 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate (MEcPP) being the last metabolite in the reaction sequence known so far, and evidence that FBPP is a natural intermediate of IPP biosynthesis is missing.[18]

Most recently, a role for the genes *dxr*, *gcpE* and *lytB* in the MEP pathway has been demonstrated by us and others.[19–23] We constructed *Escherichia coli* mutant strains utilizing exogenously provided mevalonate for producing IPP, owing to complementation with a plasmid that provides the yeast enzymes of the classical mevalonate pathway of isoprenoid synthesis. Consequently, mutants deficient in the genes *dxr*, *gcpE* and *lytB*, respectively, are viable when the culture medium is supplemented with mevalonate.[20,21] Low-molecular-weight (LMW) extracts prepared from Δ*dxr* and Δ*gcpE* mutants have a significantly reduced capacity to stimulate Vγ9/Vδ2 T cells, compared to the parent *E. coli* strain,[4] thus proving the importance of the MEP pathway for biosynthesis of the γδ T-cell activator. In striking contrast, we show here that in Δ*lytB* bacteria, the Vγ9/Vδ2 T cell-stimulating molecule is greatly accumulated.

MATERIALS AND METHODS

Bacteria and plasmids

Bacterial strains *E. coli* Δ*gcpE* and *E. coli* Δ*lytB* with precise in-frame deletions of *gcpE* and *lytB*, respectively, were derived from the wild-type *E. coli* K-12 strain, DSM no. 498, ATCC 23716.[20,21] All mutant and wild-type (WT) strains harboured plasmid pSC-MVA with a synthetic operon to express *Saccharomyces cerevisiae ERG12* (mevalonate kinase), *ERG8* (phosphomevalonate kinase) and *ERG19* (mevalonate pyrophosphate decarboxylase).[20] Bacteria were grown in Standard 1 medium (Merck, Darmstadt, Germany) in the presence of 150 μg/ml ampicillin (Sigma, Taufkirchen, Germany) and 200 μM mevalonate; a stock solution of 1 M mevalonate was prepared by hydrolysing mevalonolactone (Fluka, Taufkirchen, Germany) with 1 M KOH at 37° for 30 min.

Synthesis of ^{14}C-labelled MEP

For enzymatic synthesis of [2-^{14}C]1-deoxy-D-xylulose 5-phosphate (DOXP), 0·8 mg of pyruvate (Sigma), 1·4 mg of [2-^{14}C]pyruvate (585 MBq/mmol; NEN Life Science Products, Zaventem, Belgium) and 5·5 mg of D-fructose-1,6-bisphosphate (Sigma) were incubated with 0·44 U rabbit aldolase (D-fructose-1,6-bisphosphate-D-glyceraldehyde-3-phosphate-lyase; Sigma), 22·4 U rabbit triose phosphate isomerase (D-glyceraldehyde-3-phosphate-ketol-isomerase; Sigma) and 40 μg of recombinant *E. coli* DOXP synthase.[24] [2-^{14}C]DOXP was precipitated with 100 mM $BaCl_2$ and 80% ethanol, and subsequently transformed into [2-^{14}C]MEP in the presence of 25 mM NADPH (Sigma) using 300 μg of recombinant *E. coli* DOXP reductoisomerase (Dxr).[7] Samples were then passed over a porous graphitized carbon column (Hypercarb; Thermo Hypersil, Runcorn, UK). Finally, [2-^{14}C]MEP was purified on a MonoQ column (Amersham Pharmacia, Freiburg, Germany), using 20 mM Tris–HCl, pH 8·0, and rising concentrations of NaCl, on a Gynkotech HPLC device (Gynkotech, Germering, Germany). This procedure yielded ≈1·6 mg of [2-^{14}C]MEP with an activity of ≈344 MBq/mmol (data not shown).

Metabolic ^{14}C labelling of metabolites derived from the MEP pathway

A fresh overnight culture of Δ*gcpE* and Δ*lytB* mutants, respectively, was diluted 1 : 100 in 2 ml of medium supplemented with 100 μM mevalonate and 100 μg/ml ampicillin. As MEP cannot be taken up by *E. coli*, [2-^{14}C]MEP was treated with 2 U shrimp alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) at 37° for 30 min, and the resulting product, [2-^{14}C]-methyl-D-erythritol ([2-^{14}C]ME), was added to the cultures at a concentration of 50 μM (corresponding to *c*. 17·1 kBq/ml). Fosmidomycin, a potent inhibitor of the Dxr enzyme,[7] was used at 100 μM to block the endogenous biosynthesis of MEP and consequently to ensure an optimum incorporation of [2-^{14}C]ME into metabolites produced via the MEP pathway. After incubation at 37° for 6 hr, bacterial cells were pelleted, resuspended in 200 μl of 5 mM Tris–HCl, pH 7·5, and lysed by repeated freeze–thaw cycles in the presence of 2 mg of lysozyme (Merck). Cell debris was pelleted and the supernatants were loaded onto Microcon YM-3 filters (Millipore, Eschborn, Germany). The flow-through was lyophilized, resuspended in 3 μl of H_2O and analysed on high performance thin layer chromatography silica gel plates (Kieselgel 60; Merck) developed with propanol : ethylacetate : H_2O (6 : 1 : 3). Radioactive spots were visualized by autoradiography after exposure for 4 weeks.

γδ T-cell stimulation assays

Bacteria were harvested from fresh liquid cultures at an OD_{600} of ≈0·8 and sonicated in a 1 : 10 volume of phosphate-buffered saline (PBS). LMW fractions were obtained using Centriprep 3-kDa filters (Millipore). Blood samples from healthy donors were kindly provided by the Institut für Klinische Immunologie und Transfusionsmedizin (Klinikum der Justus-Liebig-Universität Giessen, Giessen, Germany). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood by density centrifugation over LSM medium (ICN Biomedicals, Eschwege, Germany). PBMC (2×10^5/well) were seeded in RPMI-1640 (200 μl) supplemented with 25 mM HEPES, 2 mM L-glutamine, 25 μg/ml

gentamycin (all from Life Technologies, Karlsruhe, Germany) and 10% human AB serum (Bayerisches Rotes Kreuz, Augsburg, Germany). For routine assays, recombinant human interleukin-2 (IL-2) (Life Technologies) was used at 10 U/ml for short-term cultures (18–72 hr), and at 100 U/ml for longer culture periods. For comparison between different growth factors, IL-2, IL-15 (both Promocell, Heidelberg, Germany), or IL-21[25] was added at a concentration of 0·1–10 ng/ml. LMW preparations were added at dilutions of 1 in 200 (corresponding to $\approx 1{\cdot}0 \times 10^7$ bacteria/well) to 1 in 5×10^6 ($\approx$400 bacteria/well). Cells incubated in medium alone, and cells stimulated with 10 μM IPP (Sigma), served as negative and positive controls, repectively. Cells were harvested at different time-points and analysed for γδ T-cell activation and γδ T-cell outgrowth on an Epics XL flow cytometer supported with Expo32 software (Beckman Coulter, Krefeld, Germany), using the following Beckman Coulter monoclonal antibodies (mAbs): CD3-PC5 (UCHT1), Vγ9-fluorescein isothiocyanate (FITC) (Immu360), Vδ2-FITC (Immu389), CD25-phycoerythrin (PE) (B1·49·9), CD69-PE (TP1·55·3), CD94-PE (HP-3B1), and HLA-DR-PE (Immu357), together with the appropriate isotype controls. Gates were set on total lymphocytes and on $CD3^+$ lymphocytes, and results obtained from 10 to 30 000 gated events were expressed as percentage of $V\gamma9^+$ cells among $CD3^+$ lymphocytes on day 6, and of $CD69^+$ (day 1), $CD25^+$ (day 3), $CD94^+$ (day 6), or $HLA\text{-}DR^+$ (day 6) cells among $V\gamma9^+$ $CD3^+$ lymphocytes. For cytokine analysis, culture supernatants were taken after 18 and 72 hr, and tested for tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), respectively, using the human OptEIA sets (Becton-Dickinson, Hamburg, Germany).

Chromatography and mass spectrometry

Cultures of Δ*gcpE* and Δ*lytB* mutants, respectively, were grown in 100 ml of Standard 1 medium to an OD_{600} of ≈0·8. LMW extracts were prepared as described above (except that 20 mM ammonium formiate, pH 8·0, was used instead of PBS) and passed over an Isolute C18 reverse-phase (RP) column (International Sorbent Technology, Mid Glamorgan, UK). The flow-through was then loaded onto a Source 15Q anion-exchange column (Amersham Pharmacia) and fractionated using a Waters HPLC device coupled to a PDA 996 photodiode array detector (Waters, Eschborn, Germany), scanning 1 spectrum/second between λ210 and 500 nm with a 1·2-nm resolution. Buffers used were: A, 20 mM ammonium formiate, pH 8·0; B, 500 mM ammonium formiate, pH 8·0; C, 1 M ammonium formiate; and chromatography was performed with a flow rate of 10 ml/min as follows: wash, 30 min 100% A; elution, linear gradient from 100% A to 100% B in 30 min; 10 min 100% C; equilibration, 10 min 100% A. Fractions collected were first tested in standard γδ T-cell activation assays at a dilution of 1 in 400 000, using pools consisting of four fractions each. The individual fractions from the only pool inducing upregulation of CD69 expression within 18 hr were then tested at a dilution of 1 in 400 000. The active sample, Δ*lytB* #20, and the corresponding negative control, Δ*gcpE* #20, were analysed by electrospray ionization orthogonal-time-of-flight mass spectrometry (ESI-o-ToF MS) (Mariner; PE Biosystems, Framingham, MA) equipped with a nano-ESI-source (Protana, Odense, Denmark). Sample solutions (3 μl) were filled in gold-coated nanospray capillaries and sprayed at flow rates of 10–30 nl/min. The voltage of the nanospray needle was set to 900 V; the temperature of the heated transfer capillary was 200°C. Spectra were taken in the negative ion mode and averaged over 10 scans of 3 seconds each. Structural analysis by MS-MS of selected ions was performed using a quadruple ion trap MS (LCQ; Finnigan MAT, San Jose, CA) with a relative collision energy of 20–30%.

Statistical analysis

Data were expressed as mean value ± standard error of the mean (SEM). Statistical analysis was performed using a two-tailed Student's *t*-test for unequal variance, with differences considered statistically significant at a *P*-value of $< 0{\cdot}05$. R^2 values were calculated using Microsoft Excel 2000 software.

RESULTS

E. coli Δ*lytB* mutants accumulate a metabolite that is derived from methyl-D-erythritol

In recent studies, we demonstrated that the enzymes encoded by the *E. coli* genes *gcpE* and *lytB* are essential for isoprenoid synthesis via the MEP pathway.[20,21] As the final biochemical steps in this pathway still remain to be unveiled, labelling experiments were performed in order to gain a more detailed insight into the function of the LytB and GcpE proteins. Thus, Δ*lytB* and Δ*gcpE* mutants were grown in medium supplemented with ^{14}C-labelled ME. Separation of the corresponding LMW extracts by thin-layer chromatography revealed that in Δ*gcpE* mutants, most of the [2-^{14}C]ME was not metabolized (Fig. 1). In contrast, in the Δ*lytB* mutant there was an additional spot indicating the accumulation of an unknown metabolite with reduced chromatographic mobility. Unfortunately, the amounts of labelled material were too limited to allow quantification of radioactivity by liquid scintillation or further characterization by mass spectrometry (data not shown).

Disruption of the *lytB* gene leads to a >100-fold accumulation of a γδ T-cell stimulating molecule

From previous work we know that a compound derived from the MEP pathway represents the natural activator for phosphoantigen-reactive human γδ T cells.[3,4] Thus, we speculated that the metabolite accumulating in Δ*lytB* bacteria (Fig. 1) might in fact be this highly immunogenic substance. Here, in γδ T-cell stimulation assays, LMW extracts prepared from WT bacteria induced expansion of Vγ9/Vδ2 T cells at dilutions down to 1 : 12 500, corresponding to $1{\cdot}6 \times 10^5$ bacteria/well (Fig. 2); in accordance with our previous observations,[4] LMW extracts from Δ*gcpE* mutants were less active than those from WT *E. coli*. However, in striking contrast, Δ*lytB* mutants exhibited

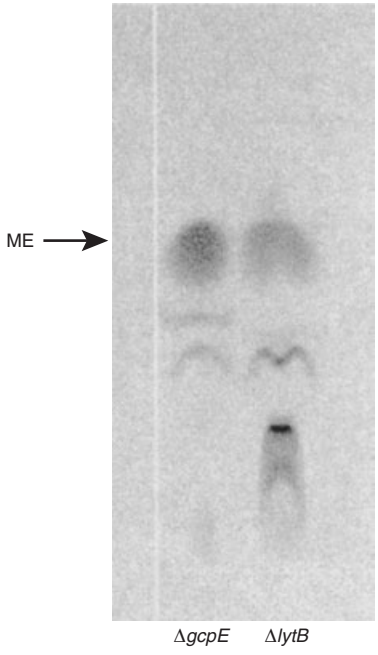


Figure 1. (a) Thin-layer chromatography analysis of *Escherichia coli* Δ*gcpE* and Δ*lytB* grown in the presence of [2-^{14}C]2-*C*-methyl-D-erythritol (ME). Low molecular weight preparations were examined on an HPTLC silica gel plate, and radioactive spots were visualized by autoradiography.

a γδ T-cell activity that was considerably above that of the WT bacteria; significant outgrowth of Vγ9/Vδ2 T cells could be detected at dilutions down to 1 : 312 500, corresponding to $6{\cdot}4 \times 10^3$ bacteria/well. The relative increase in γδ T cells detected did reflect a true expansion of this population, as no substantial death of αβ T cells occurred (data not shown), which is in accordance with similar observations from other groups.[2,17,26–29]

We then further investigated the LMW extracts prepared from Δ*lytB* bacteria, by measuring immunological parameters correlated with γδ T-cell activation by phosphoantigens. Thus, at a dilution of 1 : 62 500, the Δ*lytB* extract did not only stimulate proliferation of γδ T cells, but also induced upregulation of the activation markers CD25, CD69 and HLA-DR, and the C-type lectin CD94 on the surface of Vγ9/Vδ2 T cells, as well as secretion of TNF-α and IFN-γ by PMBC (Fig. 3). This activity was not present in extracts from WT or Δ*gcpE* bacteria tested at the same dilution.

Using serial dilutions of an IPP standard, we obtained positive correlations between antigen concentration and γδ T-cell activation, measured as γδ T-cell expansion and expression of CD25 and CD69 (Fig. 4). These calibration curves enabled us to estimate the activity of the bacterial extracts tested and express it as IPP equivalents; importantly, all three analyses performed gave very similar levels (Table 1). Thus, LMW extracts from WT *E. coli* had a γδ T-cell stimulatory potential comparable to an IPP concentration of ≈3 mM, whereas the activity of the Δ*gcpE*

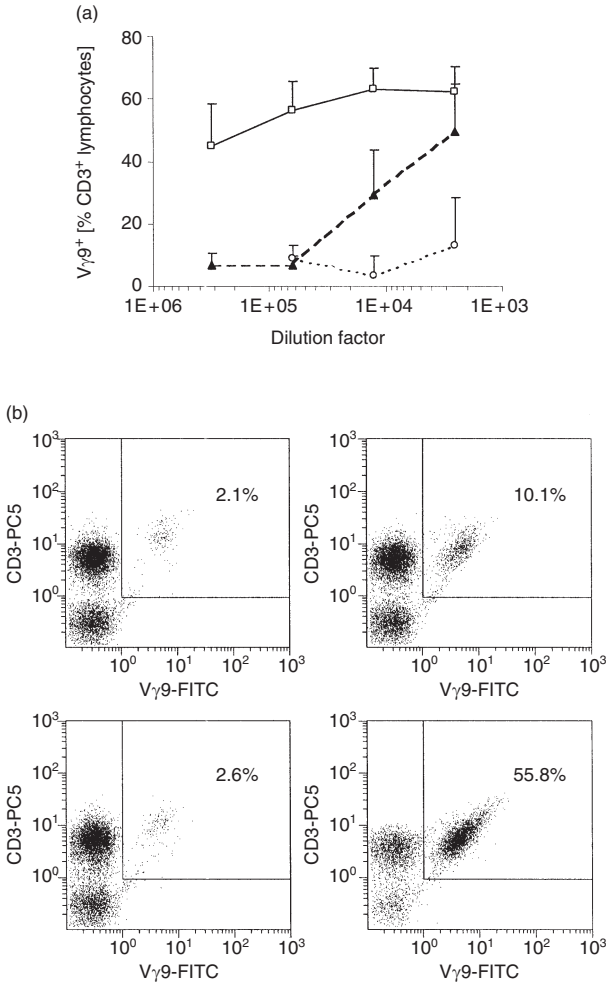


Figure 2. Disruption of the *lytB* gene increases the γδ T-cell stimulatory potential of *Escherichia coli*. (a) Vγ9$^+$ T-cell outgrowth in the presence of bacterial extracts. Peripheral blood mononuclear cells (PBMC) were incubated over a period of 6 days with medium alone, or with low molecular weight extracts prepared from wild-type *E. coli* (black triangles), Δ*gcpE* (open circles), and Δ*lytB* (open squares), at different dilutions. Data shown represent mean values + standard error of the mean from three donors analysed. (b) Flow cytometry analysis. PBMC were incubated with medium alone (top left panel), or with LMW extracts prepared from WT *E. coli* (top right panel), Δ*gcpE* (bottom left panel), or Δ*lytB* (bottom right panel), at a dilution of 1 : 12 500. Panels shown are representative data obtained from one blood donor.

mutant was significantly lower, equivalent to ≈0·8 mM. Strikingly, LMW extracts from bacteria deficient in *lytB* were greater than 100-fold more active than extracts from the WT bacteria, containing an antigenic compound that was as potent as ≈450 mM IPP.

The γδ T-cell stimulating molecule can be purified by anion-exchange chromatography

In order to further characterize the immunogenic molecule accumulating in Δ*lytB* mutants, the 3-kDa LMW filtrate

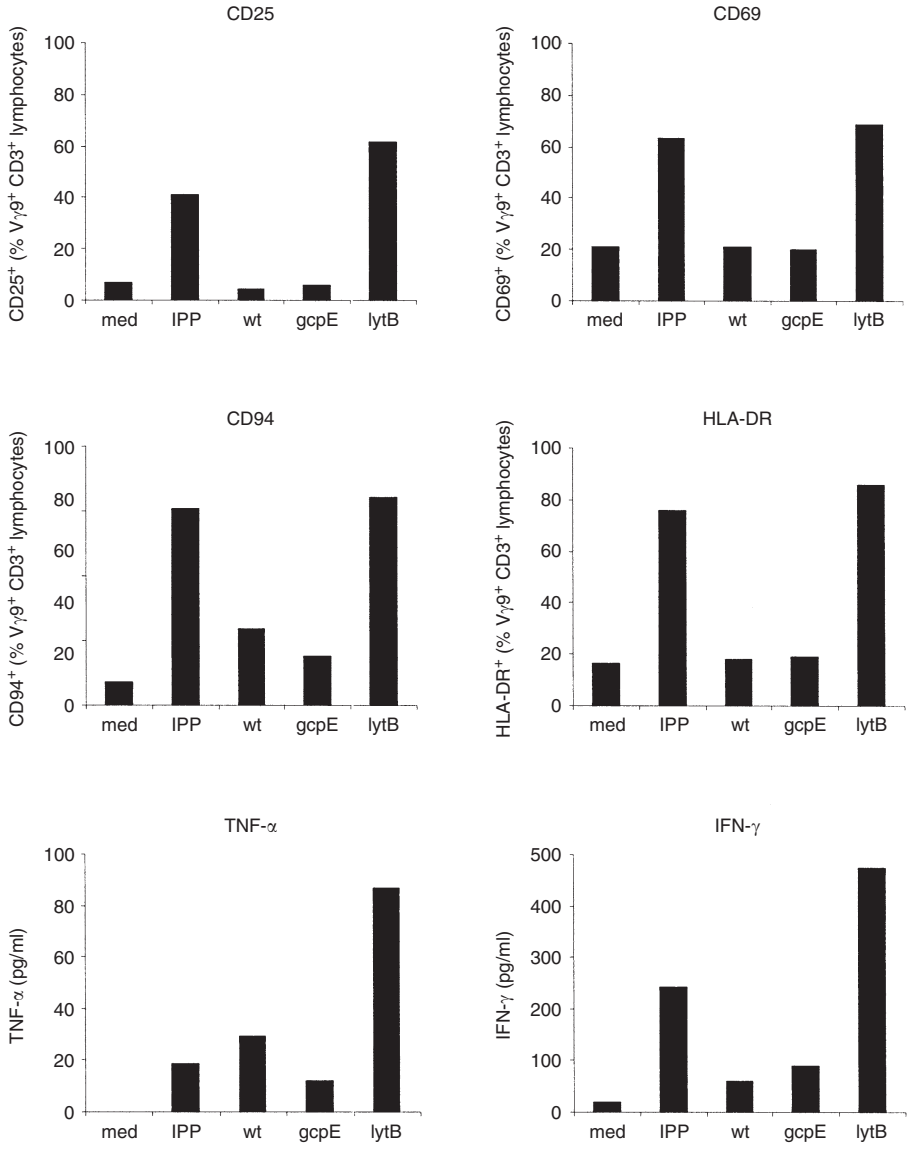


Figure 3. Effect of Δ*lytB* low molecular weight extract on a range of immunological parameters. Peripheral blood mononuclear cells (PBMC) were incubated with medium alone, with isopentenyl pyrophosphate (IPP) at 2 μM, or with LMW extract prepared from wild-type (WT) *Escherichia coli*, Δ*gcpE*, or Δ*lytB*, at a dilution of 1 : 62 500. Data represent results from one donor, for expression of CD25 (day 3), CD69 (day 1), CD94 (day 6) and human leucocyte antigen (HLA)-DR (day 6) on $V\gamma9^+$ T cells, and secretion of tumour necrosis factor (TNF-α) (day 1) and interferon-γ (IFN-γ) (day 3) by PBMC. Note: the pattern shown is typical for a number of blood donors analysed ($n = 3$–6); however, with absolute levels of CD94 and HLA-DR expression, as well as absolute amounts of cytokines secreted, greater biological variation was found between individuals than in the data for γδ T-cell expansion and expression of CD25 or CD69. Also, for some donors changes in CD94 and HLA-DR expression in the presence of serial dilutions of phosphoantigens was inconsistent despite good dose-dependent responses, as judged by CD25 and CD69 expression, thus making CD25 and CD69 the more reproducible activation markers tested.

was passed over a C18 reversed-phase column and fractionated subsequently by anion-exchange chromatography, using ammonium formiate as eluent. An LMW preparation from Δ*gcpE* bacteria was subjected to the same protocol and served as a control. Unexpectedly, there was no significant difference in the absorbance profiles between the Δ*gcpE* and Δ*lytB* mutants, measured between 210 and 500 nm (Fig. 5, and data not shown). Consequently, all fractions collected were then tested in pools for their γδ T-cell reactivity. No sample obtained from Δ*gcpE* bacteria stimulated γδ T cells above background levels. In contrast, a peak of bioactivity was recovered in the pool consisting of fractions 19–22, whereas all other samples collected, as well as the flow-through of the column and the wash fractions, remained negative (Fig. 5). Testing the individual fractions of the positive pool showed that the γδ T-cell-stimulating molecule was eluted in fraction no. 20, at an ammonium formiate concentration of ≈250 mM.

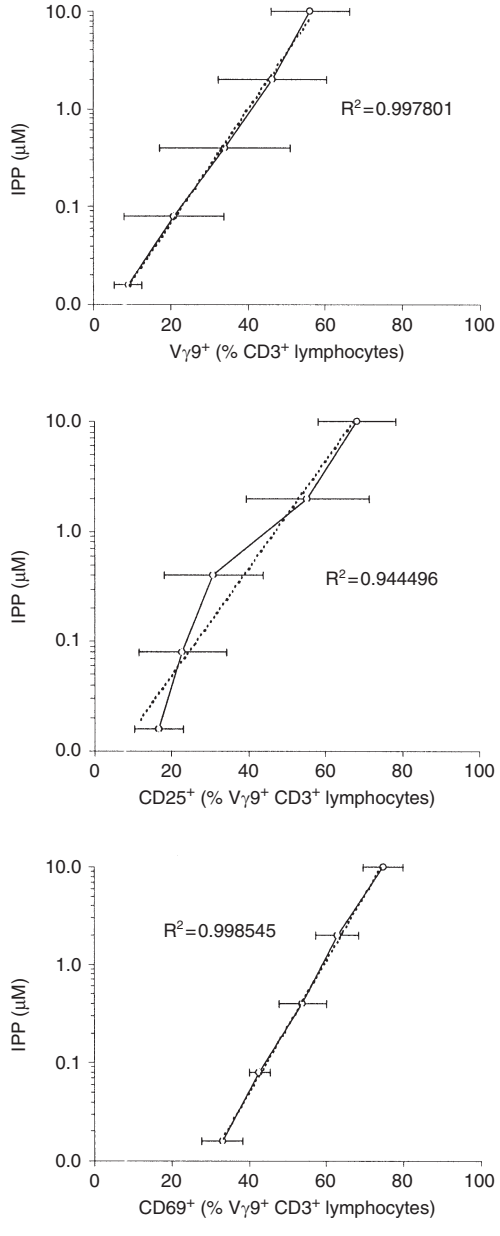


Figure 4. Dose–response to isopentenyl pyrophosphate (IPP). Peripheral blood mononuclear cells were incubated with medium alone, or with IPP at different concentrations. The data represent the mean value ± SEM from three donors analysed, for $V\gamma9^+$ T-cell outgrowth, and expression of CD25 and CD69 on $V\gamma9^+$ T cells. Trend lines and R^2 values were calculated using Microsoft Excel software.

Titration of fraction no. 20 against an IPP standard gave a bioactivity corresponding to ≈2500 mM IPP (Table 2); the side fraction no. 21 still had ≈500 mM IPP bioactivity (data not shown).

Mass spectrometry analysis identifies a molecule of M_r 262

To obtain structural information of the bioactive metabolite, ESI-MS analysis was performed on the active fraction

Table 1. Immunological activity of extracts from different strains of *Escherichia coli*¶

Equivalent to IPP (mM)	WT	Δ*gcpE*	Δ*lytB*
Expt. 1†	4·04	0·69	462·58
Expt. 2‡	2·54	0·85	475·11
Expt. 3§	2·66	0·79	394·64
Mean ± SEM	3·08 ± 0·48	0·78 ± 0·05*	444·11 ± 25·00*

¶Calibration curves were obtained using serial dilutions of an isopentenyl pyrophosphate (IPP) standard (Fig. 4). Bioactivity of the bacterial extracts analysed is expressed as IPP equivalents from three experiments performed.
†Outgrowth of $V\gamma9^+$ $CD3^+$ T cells, day 6 ($n=3$).
‡Upregulation of CD25 on $V\gamma9^+$ $CD3^+$ T cells, day 3 ($n=3$).
§Upregulation of CD69 on $V\gamma9^+$ $CD3^+$ T cells, day 1 ($n=3$).
*$P<0{\cdot}05$, using a two-tailed Student's *t*-test for unequal variance.
SEM, standard error of the mean; WT, wild type.

derived from Δ*lytB* bacteria, Δ*lytB* #20, and the corresponding fraction from Δ*gcpE* mutants, Δ*gcpE* #20. In the negative ion spectrum of Δ*lytB* #20, the most abundant peak was at *m/z* 261 $[M\text{-}H]^-$; two other peaks at *m/z* 381 $[M + NaHPO_4]^-$ and *m/z* 523 $[2M\text{-}H]^-$ could be correlated to this molecule (Fig. 6). These signals were not detectable in fraction Δ*gcpE* #20, where the most prominent peak was at *m/z* 277, with common impurity peaks (e.g. *m/z* 216·9 and *m/z* 1192·2) at comparable intensities as in fraction Δ*lytB* #20; most of the residual UV absorbance of fractions Δ*lytB* #20 and Δ*gcpE* #20 could be ascribed to the ion at *m/z* 1192·2 (data not shown). Negative mode ion trap MS/MS from *m/z* 261 yielded ions at *m/z* 243 (corresponding to H_2O loss) and *m/z* 159 (corresponding to a pyrophosphate anion, $HP_2O_6^-$) (data not shown). Subsequent large-scale purification of the γδ T-cell activator from a 1-l culture of *E. coli* Δ*lytB* mutants by anion-exchange chromatography, and its structural characterization by ^1H, ^{13}C and ^{31}P nuclear magnetic resonance spectroscopy and nuclear Overhauser effect spectroscopy analysis, as (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) has been described elsewhere.[30]

Δ*lytB* #20-driven γδ T-cell activation does not result in secretion of proinflammatory cytokines in the presence of IL-21

Apart from their reactivity towards small pyrophosphorylated molecules, Vγ9/Vδ2 T cells also depend on cytokines in the local microenvironment, a fact that may bias the readouts of bioactivity assays. Consequently, we investigated the influence of different interleukins on the γδ T-cell response towards the novel activator present in fraction Δ*lytB* #20. IL-2 and IL-15 are clearly the most important growth factors known for γδ T cells, both of which synergize with IL-12.[26] IL-21 is a novel cytokine that is closely related to IL-2 and IL-15, and produced by activated $CD4^+$ T cells but not by $CD19^+$ B cells or $CD14^+$ monocytes.[25] Here, all three cytokines, IL-2, IL-15 and

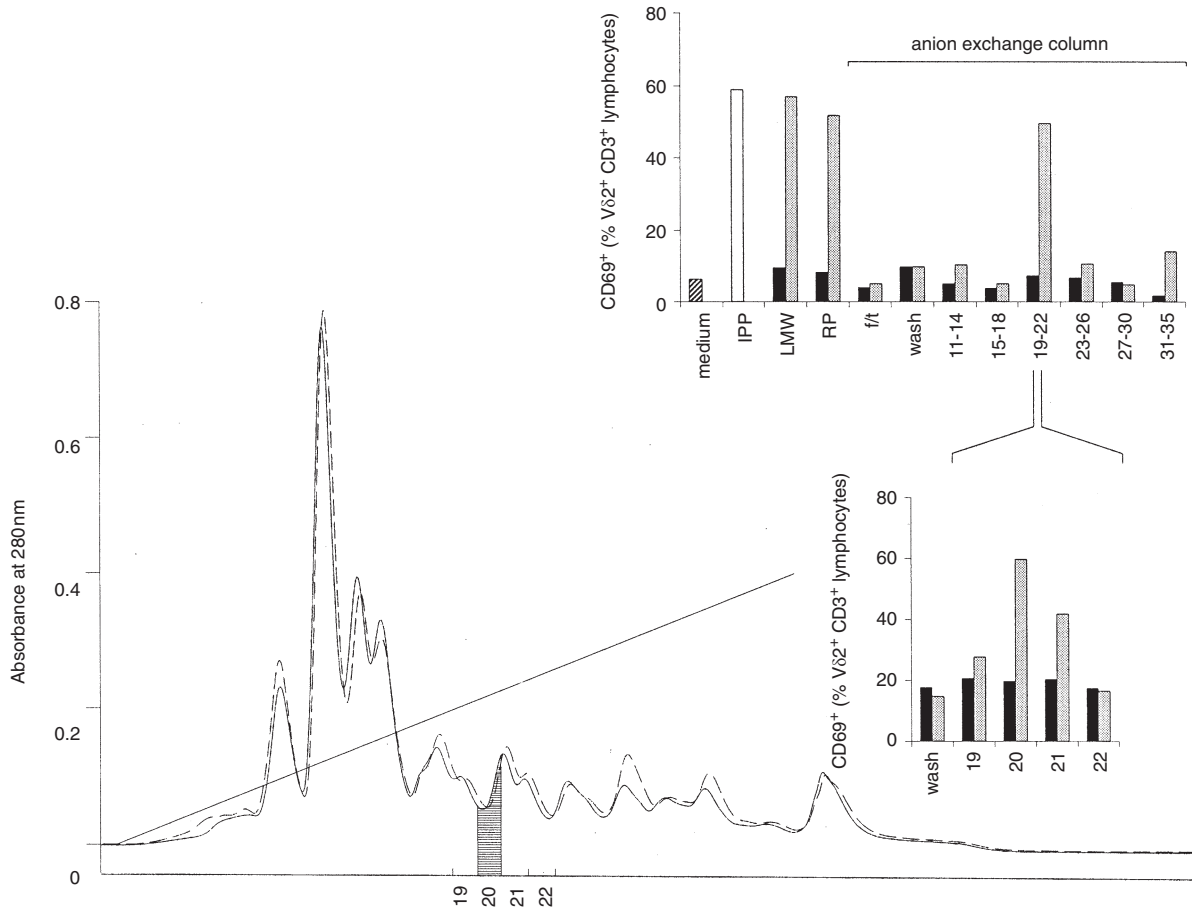


Figure 5. Fractionation of bacterial extracts by anion-exchange chromatography. Δ*gcpE* and Δ*lytB* low molecular weight (LMW) extracts, respectively, were passed over a reverse phase (RP) column and fractionated on a Source 15Q column, using an ammonium formiate gradient (20–500 mM). Absorbance at 280 nm is shown as a solid line for Δ*lytB* and a dashed line for Δ*gcpE*. Fractions collected were first tested in pools consisting of four each; fractions 19 to 22 were subsequently tested individually for their ability to upregulate CD69 expression on $V\delta2^+$ T cells. Samples tested were: medium control; isopentenyl pyrophosphate (IPP) as positive control, 10 μM; 3-kDa LMW filtrate, 1 in 50 000 (LMW) extract; LMW extract after RP column chromatography (=sample loaded onto a Source 15Q column), 1 in 50 000 (RP); Source 15Q flow-through, 1 in 50 000 (f/t); Source 15Q wash fraction, 1 in 50 000; fractions 11–35, in pools or individually, 1 in 400 000. Solid bars, Δ*gcpE*; hatched bars, Δ*lytB*.

IL-21, costimulated the expansion of Vγ9/Vδ2 T cells among $CD3^+$ T cells in the presence of fraction Δ*lytB* #20, while neither antigen nor cytokines alone had a significant proliferative effect (Fig. 7). Moreover, IL-21 increased the Δ*lytB* #20-driven upregulation of CD25 and CD69 on Vγ9/Vδ2 T cells, similarly to IL-2 and IL-15, although an effect on CD25 and CD69 expression in the absence of antigenic stimulation was barely detectable, in contrast to the other cytokines tested. Addition of IL-21 to PBMC cultures enhanced the IL-2- or IL-15-induced upregulation of CD69 expression, both in the presence or absence of antigenic stimulation; the additive effect on CD25 expression and γδ T-cell proliferation was less pronounced (data not shown). While IL-2 and IL-15 costimulated the Δ*lytB* #20-induced synthesis of both TNF-α and IFN-γ by PBMC, IL-21 did not enhance the Δ*lytB* #20-driven TNF-α production, and maximum IL-21 induced IFN-γ levels were much lower than those achieved in the presence of IL-2 or IL-15.

DISCUSSION

Over the past few years, Vγ9/Vδ2 T cells have been shown to react towards many pathogenic bacteria, such as *Brucella*, *Coxiella*, *Ehrlichia*, *E. coli*, *Francisella*, *Legionella*, *Listeria*, *Mycobacterium*, *Pseudomonas*, *Salmonella* and *Yersinia*, as well as to the protozoan parasites *Plasmodium* and *Toxoplasma*.[12,31,32] Although some bacteria lacking the alternative MEP pathway of isoprenoid synthesis, such as *Staphylococcus* or *Streptococcus*, may be capable of stimulating Vγ9/Vδ2 T cells via superantigen recognition sites,[33,34] recent studies have established a general link between Vγ9/Vδ2 T-cell reactivity[35] and the presence of the

MEP pathway.[3,4] Accordingly, from our current data it is now clear that the LytB enzyme[21] plays a crucial role in the metabolism of the natural Vγ9/Vδ2 TCR ligand. Stimulation of human PBMC with minute quantities of Δ*lytB* LMW preparations led to a drastic upregulation of the known T-cell activation markers CD25,[26] CD69[26] and HLA-DR,[36] as well as the killer inhibitory receptor, CD94,[37] on the surface of Vγ9/Vδ2 T cells, to proliferation and expansion of Vγ9/Vδ2 T cells,[4] and to secretion of the proinflammatory cytokines IFN-γ and TNF-α.[27,38,39]

Recently, a molecule of M_r 262 (TUBag1) was identified in *Mycobacterium fortuitum*. Data from ion-trap ESI-MS and comparative photodiode array absorption analysis of TUBag1, together with NMR spectroscopy signals that were obtained using the corresponding γ-UTP and γ-TTP phosphodiesters, TUBag3 and TUBag4, suggested that this mycobacterial γδ T-cell activator is FBPP.[13,16,17] As the most potent natural Vγ9/Vδ2 T-cell stimulator identified to date, this compound is active in the low nM range, whereas most other natural and synthetic substances (such as IPP and other prenyl pyrophosphates, alkylphosphates, alkylamines, and aminobisphosphonates) have bioactivities between 1 and 100 μM.[13,15,28,29,40–42] Only now, some synthetic FBPP derivatives such as halohydrin pyrophosphates with bioactivities similar to, or even above, the naturally occurring γδ T-cell activator, have been described.[43–46] However, the connection between FBPP and the MEP pathway of isoprenoid synthesis has only been deduced indirectly from the close structural similarity between FBPP and IPP.[17] A mass of 262 atomic units as well as fragments at *m/z* 243 and *m/z* 159, as found in our present study, are in accordance with the chemical structure of FBPP;[17] however, related molecules such as 3,4-epoxy-3-methyl-1-butyl pyrophosphate (EpoxPP), may give identical fragmentation patterns,[44] thereby making the MS-based approach insufficient to support any proposed structure.[45] Indeed, recent ^{1}H, ^{13}C and ^{31}P NMR spectroscopy studies on the purified substance have identified the

Table 2. Immunological activity of fraction Δ*lytB* #20*

Dilution factor	CD69 expression (% $V\gamma9^{+}$ $CD3^{+}$)†	Bioactivity (mM IPP)‡
Medium control	29·8 ± 8·4	–
$6{\cdot}25 \times 10^{7}$	29·9 ± 12·4	–
$1{\cdot}25 \times 10^{7}$	35·1 ± 7·0	3759·5
$2{\cdot}5 \times 10^{6}$	40·4 ± 1·4	1823·8
$5{\cdot}0 \times 10^{5}$	52·2 ± 0·2	2592·5
$1{\cdot}0 \times 10^{5}$	63·0 ± 1·3	3104·2
$2{\cdot}0 \times 10^{4}$	66·2 ± 0·6	1065·9
Mean ± SEM§	–	2469·2 ± 472·7

*A calibration curve was obtained using serial dilutions of an isopentenyl pyrophosphate (IPP) standard, ranging from 80 nM to 10 μM and yielding the formula (at $R^2 = 0{\cdot}99$): Bioactivity (μM IPP) = $0{\cdot}000869 \times e^{0{\cdot}167 \times (\text{CD69 expression level})}$.

†Upregulation of CD69 expression was monitored after incubation of peripheral blood mononuclear cells for 18 hr in the presence of serial dilutions of fraction Δ*lytB* #20 ($n = 3$).

‡Bioactivity of fraction Δ*lytB* #20 was calculated for each dilution using the formula: $\text{Bioactivity}_{\Delta lytB\ \#20}$ (mM IPP) = (1/1000 × [bioactivity (μM IPP) × dilution factor]).

§Mean bioactivity from the five dilutions tested that resulted in upregulation of CD69 expression above the background value. An analogous experiment measuring γδ T-cell expansion after 6 days resulted in a bioactivity value of 2487·6 ± 911·4 mM IPP.

SEM, standard error of the mean.

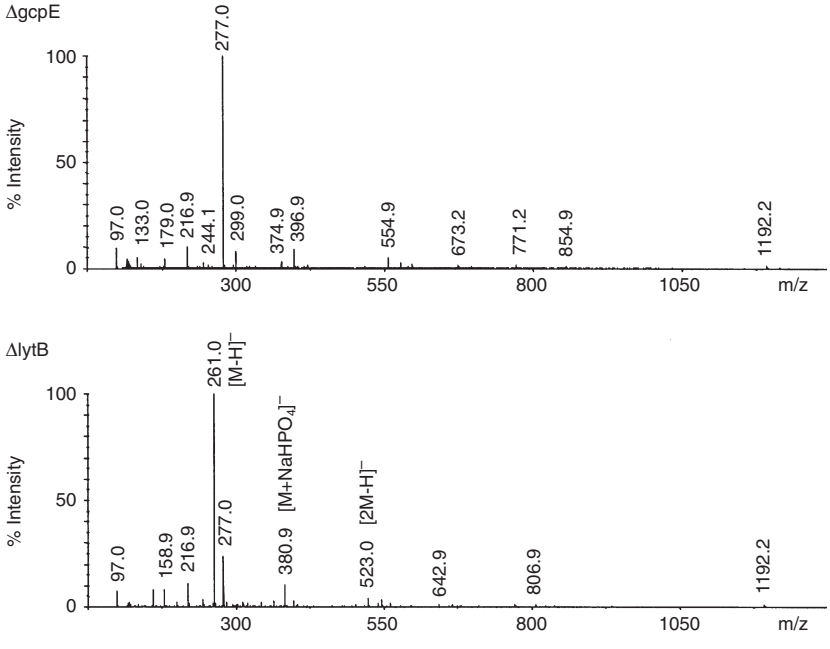
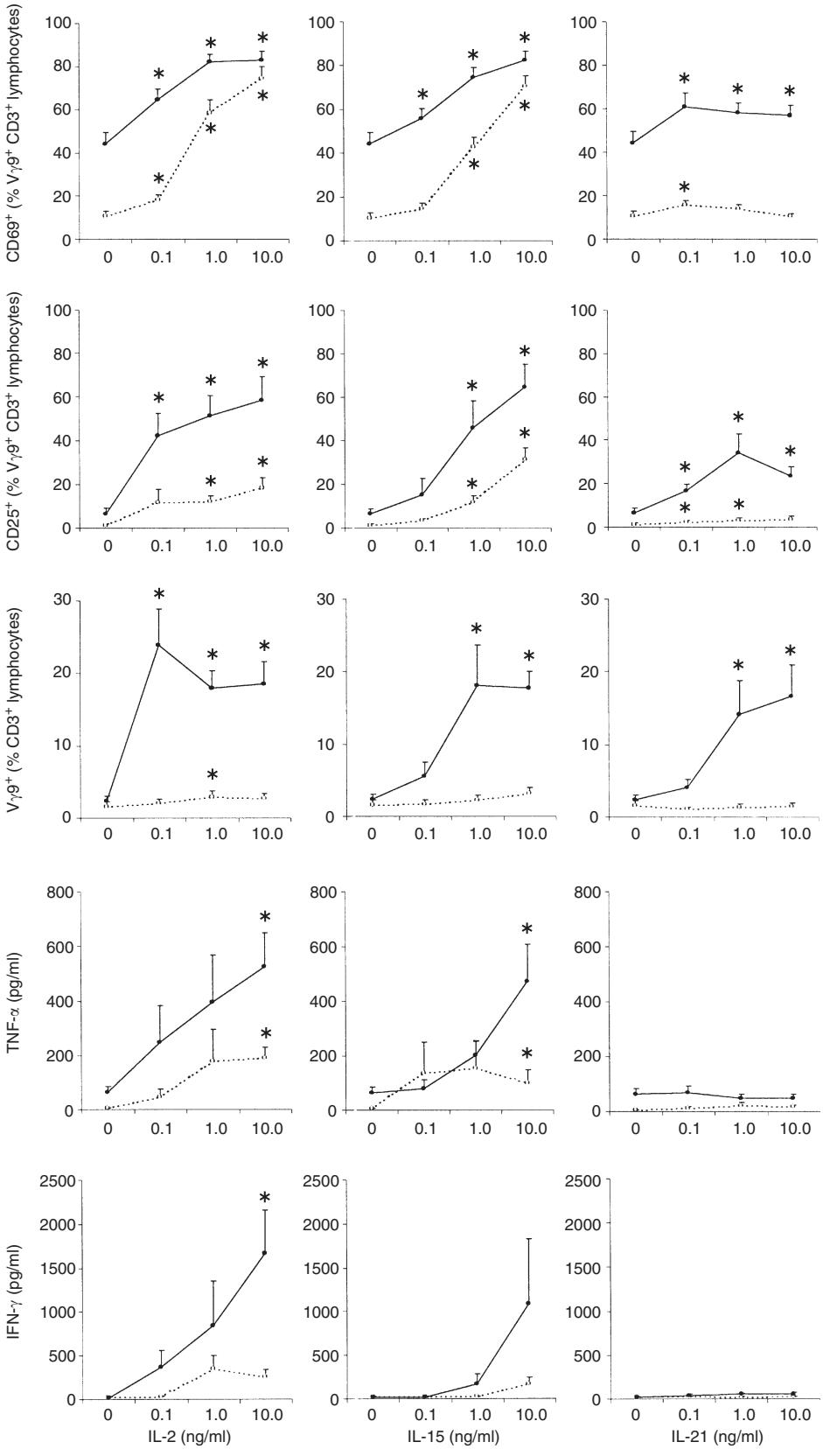


Figure 6. Negative mode electrospray ionization-mass spectrometry (ESI-MS) analysis of fractions Δ*gcpE* #20 and Δ*lytB* #20.


CD69+ (% Vγ9+ CD3+ lymphocytes)
CD25+ (% Vγ9+ CD3+ lymphocytes)
Vγ9+ (% CD3+ lymphocytes)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-2 (ng/ml)
IL-15 (ng/ml)
IL-21 (ng/ml)
0
0.1
1.0
10.0

γδ TCR ligand accumulating in *E. coli* Δ*lytB* mutants as HMB-PP, a novel compound derived from the MEP pathway that is ≈10^4 times more potent in activating Vγ9/Vδ2 T cells than IPP.[30] In contrast to FBPP, the chemical structure of HMB-PP is in accordance with incorporation studies using deuterium-labelled ME isotopomers,[18] and insinuates a very elegant mechanism for the still unclear biosynthetic reaction sequence leading from MEcPP to IPP (Fig. 8). However, at present we do not know whether the Vγ9/Vδ2 TCR ligand is the actual substrate for LytB or merely represents a side product of the MEP pathway, utilizing an intermediate produced by GcpE (or another yet-unidentified enzyme downstream of GcpE). Yet, recently Hecht *et al*. have detected HMB-PP in bacteria overexpressing the *gcpE* gene,[47] thus supporting our own conclusions.[30]

Finally, it is important to note that *E. coli* Δ*gcpE* mutants seem to accumulate another metabolite derived from the MEP pathway, detectable as a mass at *m*/*z* 277 eluted from the anion-exchange column under the same conditions as for the *m*/*z* 261 compound; this correspondence in the chromatographical behaviour may be a result of the close chemical similarity between the metabolites of the MEP pathway. The presence of a pyrophosphate group in the *m*/*z* 277 molecule was confirmed by ion trap MS/MS (U. Bahr and M. Hintz, unpublished). These findings suggest that in *E. coli* Δ*gcpE* there is an overproduction of MEcPP that may serve as the putative substrate for the GcpE enzyme catalysing the synthesis of the natural γδ TCR ligand, or its precursor.[47] This conclusion is corroborated by recent data indicating that MEcPP itself does not display any γδ T-cell activity.[48]

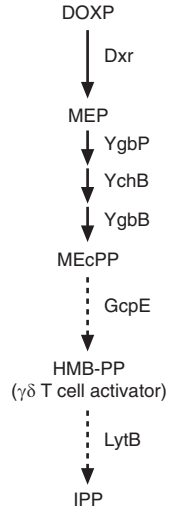


Figure 8. Proposed scheme for the final biosynthetic reactions of the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in *Escherichia coli*. Initially, 1-deoxy-D-xylulose 5-phosphate (DOXP) is converted into MEP by DOXP reductoisomerase (Dxr). Subsequently, the enzymes YgbP, YchB and YgbB catalyse the transformation of MEP into 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate (MEcPP), with a molecular mass (M_r) of 278. GcpE is involved in the next step(s) using MEcPP as substrate to produce the γδ T-cell activator, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) with M_r 262, whereas LytB mediates a/the subsequent reaction leading to IPP.

In addition to the molecular characterization of the γδ T-cell activator, we have identified IL-21 as a potent costimulator of human phosphoantigen-reactive Vγ9/Vδ2 T cells. This confirms that expansion of activated γδ T cells depends on additional growth factors which may derive from bystander $CD4^+$ T cells during an antigen-specific immune response to the pathogen, such as IL-2, IL-4 or IL-7, or from cells of the innate immune system at early stages of an infection when $CD4^+$ T cells have not yet been recruited, like IL-12 and IL-15.[26] The synergism between IL-21 and IL-2 or IL-15 is in accordance with previous findings showing that combinations of IL-21 and IL-2 or IL-15 have an additive effect on the lytic function of human natural killer cells; also, IL-21 potentiates the costimulation of murine T-cell proliferation by IL-2 or IL-15.[25,49] The biological implication for this seemingly redundant utilization of IL-21 as another Vγ9/Vδ2 T-cell costimulator remains to be elucidated, as both IL-2 and IL-21 are produced by activated $CD4^+$ T cells, and signalling of all three interleukins, IL-2, IL-15 and IL-21, via their respective receptors involves activation of STAT1, STAT3 and STAT5, as well as JAK1 and JAK3.[50,51] In this respect, it is intriguing that IL-21 has similar effects as IL-2 and IL-15 on the Δ*lytB* #20-driven γδ T-cell proliferation and CD25 and CD69 surface expression, but is far less active in stimulating the Δ*lytB* #20-induced secretion of IFN-γ and TNF-α by PBMC. These findings demonstrate that the production of proinflammatory cytokines, which has been considered a key function of Vγ9/Vδ2 T cells and is a widely used read-out in bioactivity assays,[27,38,39] is not necessarily associated with proliferating Vγ9/Vδ2 T cells. The immunology of IL-21 costimulated phosphoantigen-reactive Vγ9/Vδ2 T cells is well worth further attention.

Taken together, we have demonstrated that a pyrophosphorylated molecule of M_r 262 accounts for the strong immunogenicity of a mutant *E. coli* strain deficient in *lytB*.

Figure 7. Dependence of γδ T-cell activity on costimulation with interleukin (IL)-2, IL-15 and IL-21. Peripheral blood mononuclear cells were incubated with medium alone (open circles), or with Δ*lytB* #20 at a dilution of 1 in 100 000, corresponding to a bioactivity equivalent to 25 μM isopentenyl pyrophosphate (IPP) (solid circles), in the presence of different concentrations of the recombinant cytokines. Data shown represent the mean value + SEM for expression of CD69 and CD25 on $V\gamma9^+$ $CD3^+$ T cells, for outgrowth of $V\gamma9^+$ T cells among $CD3^+$ T cells, and for secretion of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (all $n=6$ except $n=3$ for IFN-γ). $^*P<0{\cdot}05$ compared to the corresponding medium controls, using a two-tailed Student's *t*-test for paired data.

As there is increasing evidence that Vγ9/Vδ2 T cells contribute to the immune response against some of the most harmful pathogens infecting humans, the unexpected accessibility of the natural Vγ9/Vδ2 TCR ligand in large quantities, and the identification of IL-21 as a potent growth factor, will further aid our understanding of the unconventional function of Vγ9/Vδ2 T cells in diseases such as tuberculosis, tularemia, malaria, or Crohn's disease.[52–55] The fact that in the presence of IL-21, Δ*lytB* #20-reactive Vγ9/Vδ2 T-cells proliferate without secreting significant levels of proinflammatory cytokines may be of considerable relevance for immunotherapeutic approaches in these infections.

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