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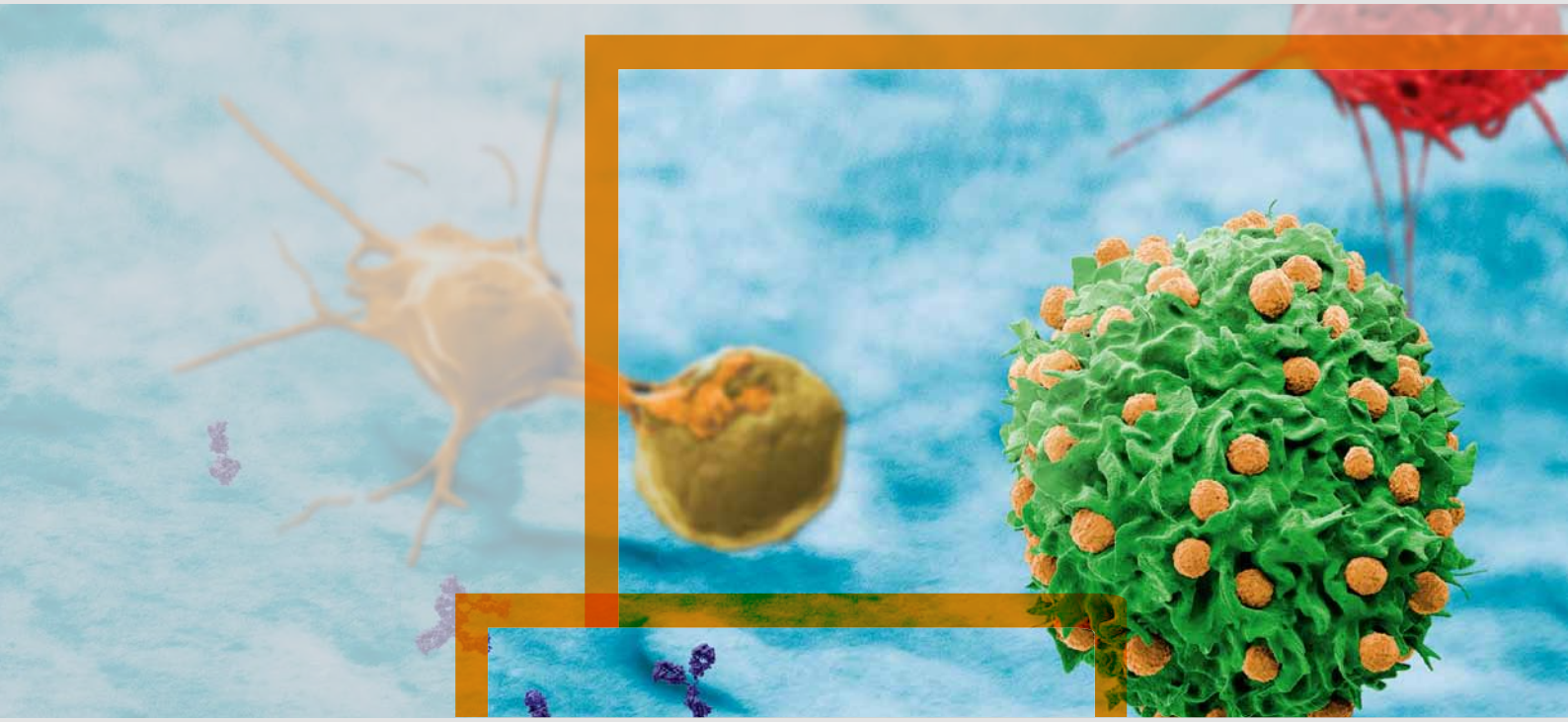

Highly purified peripheral blood γ/δ T cells isolated by MACS® Technology respond to NOD2 ligand

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Introduction

$CD3^+$γ/δ T cells display characteristics of the adaptive and the innate immune system[1]. The dominant subset of γ/δ T cells expresses a Vγ9Vδ2 T cell receptor (TCR), which recognizes phosphorylated intermediates of the bacterial non-mevalonate isoprenoid biosynthesis pathway. Synthetic phosphoantigens such as bromohydrin pyrophosphate (BrHPP) are capable of inducing large-scale Vγ9Vδ2 T cell expansion, which is independent of processing and presentation of such molecules by classical MHC molecules[1,2]. After TCR stimulation, γ/δ T cells rapidly release cytokines, such as IFN-γ and MIP-1α (CCL3), thereby activating other cells of the immune system[3]. Additionally, γ/δ T cells possess features of innate immune cells, such as antigen-presenting capacity and express pattern recognition receptors (PRR), including Toll-like receptors (TLR)[4,5]. TLR recognize a broad variety of structurally conserved molecules derived from microbes. TLR ligands have been shown to costimulate TCR-activated γ/δ T cells by enhancing production of cytokines and chemokines[6,7]. Besides TLR, another class of PRR, the nucleotide-binding leucine-rich repeat receptors (NLR), was detected in γ/δ T cell lines. However, these studies did not address whether the NLR nucleotide-binding oligomerization domain (NOD) 2 was expressed in freshly isolated γ/δ T cells[8].

Our studies revealed that highly purified γ/δ T cells express NOD2 mRNA and NOD2 protein[9]. Furthermore, we investigated a possible function of NOD2 in freshly isolated γ/δ T cells by stimulating the cells with the minimally bioactive motif muramyl dipeptide (MDP) that mimics bacterial peptidoglycan.

Materials and methods

Cell isolation

Human γ/δ T cells were depleted from PBMCs using the Anti-TCRγ/δ MicroBead Kit (Miltenyi Biotec). For depletion of human $CD14^+$ monocytes, CD14 MicroBeads (Miltenyi Biotec) were used. Cell separation was performed using LS Columns (Miltenyi Biotec). After depletion, the proportion of residual γ/δ T cells or monocytes was <0.1%.

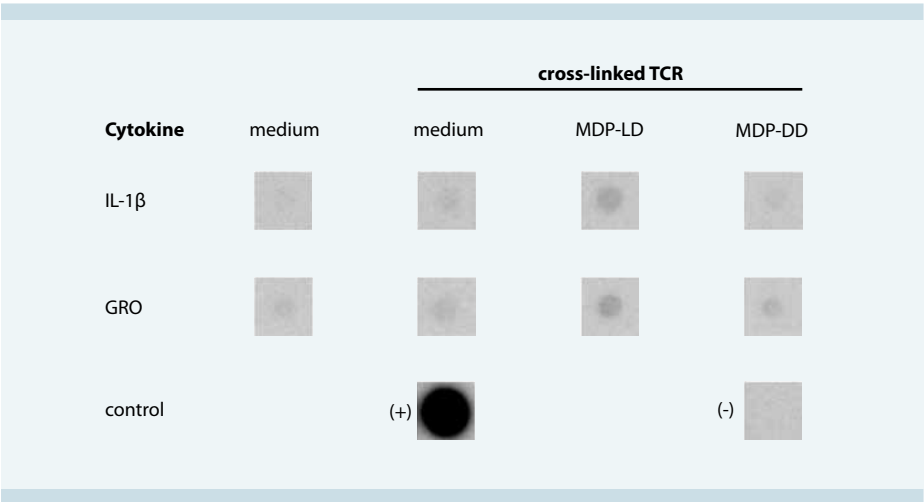

Figure 1 **Production of IL-1β and GRO in response to TCR cross-linking and MDP-LD.** γ/δ T cells were purified by positive selection with MACS Technology. 4×10^6 cells per well (24-well plate) were cultured in the absence or presence of rabbit anti-mouse Ig (cross-linked TCR) and MDP-LD or MDP-DD as indicated. Culture supernatants were analyzed for cytokines after 24 h. Positive and negative controls were included in the array.

To enrich γ/δ T cells or monocytes from PBMCs, the Anti-TCRγ/δ MicroBead Kit or CD14 MicroBeads were used. PBMCs were pre-treated with FcR Blocking Reagent (Miltenyi Biotec) to avoid non-specific binding of antibodies (Abs) to FcR-bearing cells. The purity of the positively selected cells ranged between 83 and 97% when only one LS Column was used for positive selection. This pre-enrichment of γ/δ T cells by MACS® Technology was necessary to avoid time-consuming flow sorting of PBMCs. To definitely ensure the depletion of residual monocytes in the enriched γ/δ T cell fraction and vice versa, a further purification step was done by sorting with a FACSAria® cell sorter (BD® Biosciences) or by using another LS Column. The purity of the cells was then >99%. For further details, please refer to Marischen *et al.*[9].

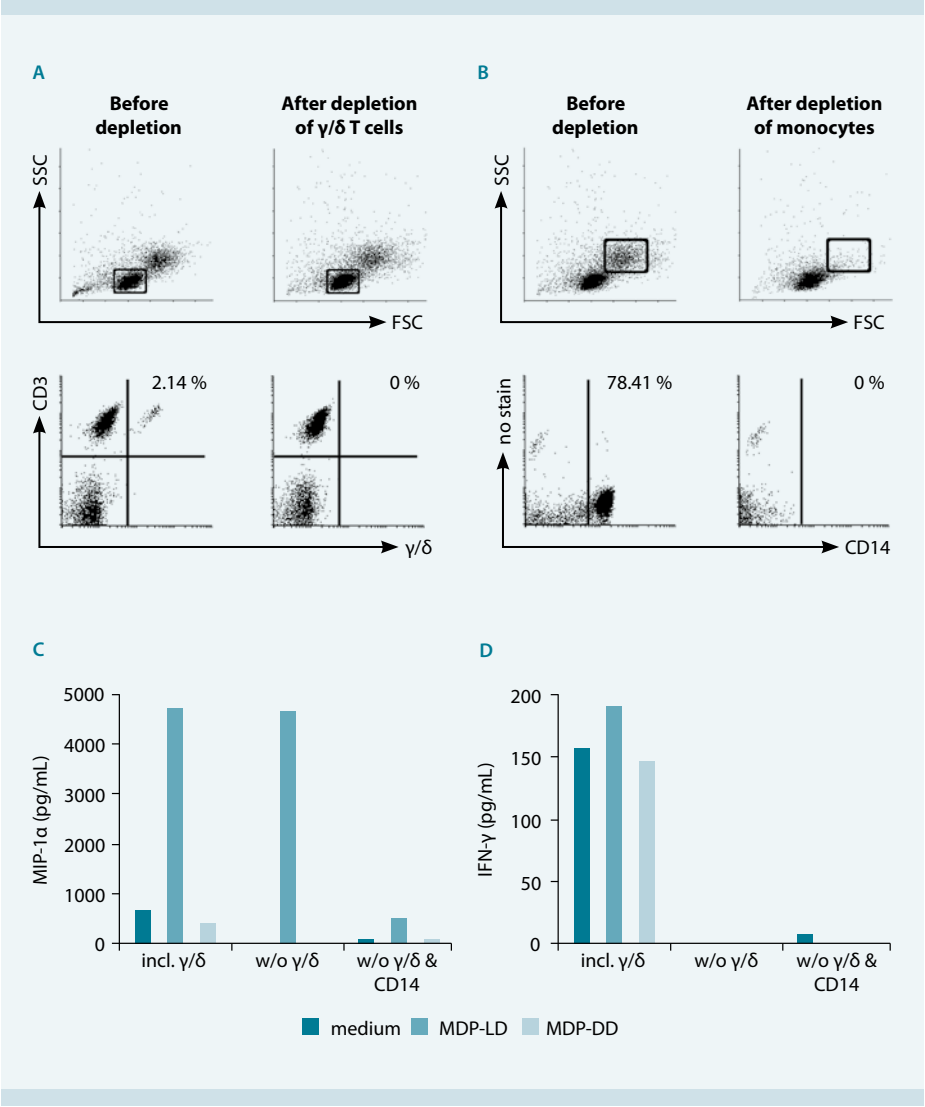

Figure 2 MIP-1α and IFN-γ production by PBMCs depleted or not of γ/δ T cells and monocytes. (A, B) Gates were set on lymphocytes (A, upper dot plots) or monocytes (B, upper dot plots) to determine γ/δ T cells or monocytes among PBMCs, respectively, before and after depletion of the appropriate cell population. The frequencies of $CD3^+TCR\gamma/\delta^+$ T cells (A, lower dot plots) or $CD14^+$ monocytes (B, lower dot plots) among PBMCs of one representative donor before and after depletion of γ/δ T cells or monocytes, respectively, are shown. (C, D) Undepleted PBMCs (incl. γ/δ), PBMCs depleted of γ/δ T cells (w/o γ/δ), and PBMCs depleted of both γ/δ T cells and monocytes (w/o γ/δ & CD14) were stimulated with BrHPP and rIL-2 in the absence (medium) or presence of active MDP-LD or inactive MDP-DD as indicated. 1.5×10^5 cells per well (96-well plates) were used throughout the experiment. Concentrations of MIP-1α (C) and IFN-γ (D) in the supernatants were determined by ELISA after 24 h. Results of fig. 2D were reproduced from Marischen *et al.* (ref. 9) with the permission of John Wiley and Sons. Results of one representative experiment out of four are shown in figure 2 C and D.

Activation of cells

PBMCs and γ/δ T cell- or monocyte-depleted PBMCs were stimulated with the γ/δ T cell–specific antigen BrHPP (200 nM, Innate Pharma®) in the presence of rIL-2 (Novartis). Positively selected γ/δ T cells or monocytes were cultured in uncoated wells or wells coated with 1 µg/mL rabbit anti-mouse Ig, which binds to anti-TCRγ/δ mAb present on the γ/δ T cells or CD14 mAb present on monocytes after labeling with MACS MicroBeads. Additionally, all cell cultures were incubated with 10 µg/mL MDP-LD isomer (tlrl-mdp, Invivogen®) or as a control with inactive MDP-DD isomer (tlrl-mdpc; Invivogen) for 24 h without rIL-2 in serum-free X-VIVO™15 medium (Lonza®). For detailed culture conditions see reference 9.

Cytokine analysis

Cytokines were analyzed in cell culture supernatants by using the RayBio® Human Cytokine Antibody Array VI & 6.1 Map and VII & 7.1 Map (Hoelzel Diagnostic), which allows simultaneous detection of 2×60 cytokines and chemokines. Signals were detected by chemiluminescence, followed by semiquantitative analysis with the AIDA software (Raytest). To determine the intensity, local background was subtracted from each value and normalized against the positive controls of each membrane. IFN-γ and MIP-1α were detected by DuoSet® sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

Flow cytometry

To determine the proportion of γ/δ T cells among PBMCs, cells were labeled with anti-CD3 mAb and anti-TCRγ/δ mAb. Monocytes among PBMCs were analyzed after staining with anti-CD14 mAb. Antibodies were obtained from BD Biosciences.

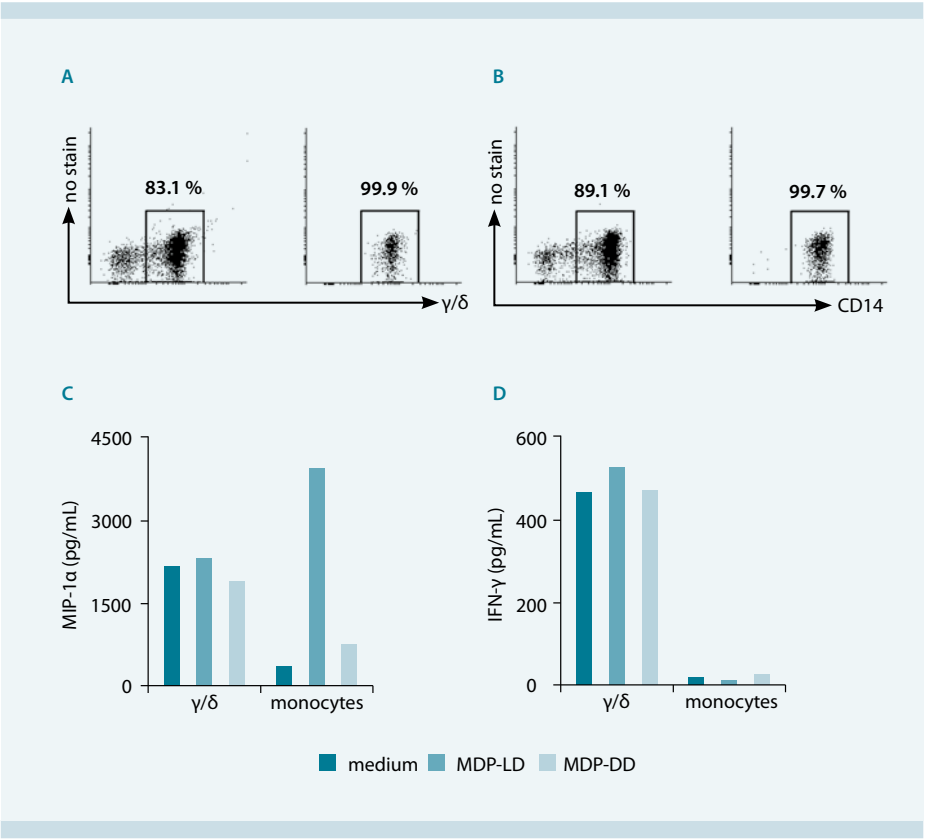

Figure 3 **Effects of MDP-LD on highly purified monocytes and γ/δ T cells.** (A, B) The purities of γ/δ T cells (A) or CD14⁺ monocytes (B) were determined after MACS Separation with one LS Column (A and B, left dot plots) and after subsequent flow sorting (A and B, right dot plots), shown for one representative donor. (C, D) Highly purified γ/δ T cells (1.5×10^5) were stimulated through the TCR via immobilized rabbit anti-mouse Ab in the presence or absence of MDP-LD or MDP-DD, whereas monocytes (1.5×10^5) were cultured in medium or activated by MDP-LD or MDP-DD as indicated. Concentrations of MIP-1α (C) and IFN-γ (D) in the supernatants were analyzed by ELISA after 24 h. Results of figure 3D were reproduced from Marischen *et al.* (ref. 9) with the permission of John Wiley and Sons.

Results and discussion

MDP enhanced cytokine secretion in purified γ/δ T cells

γ/δ T cells were isolated from PBMCs from three healthy donors by positive selection using an LS Column. The purity of the cells was between 83 and 97%. Purified γ/δ T cells were cultured in medium or stimulated by TCR cross-linking (via rabbit anti-mouse Ig) in the absence or presence of MDP-LD or MDP-DD for 24 h. IL-1β and GRO production were measured by a human cytokine Ab array. Figure 1 shows that MDP-LD induced secretion of IL-1β and GRO by the purified TCR-stimulated γ/δ T cells, whereas MDP-DD had no effect. However, IL-1β and GRO are produced mainly by monocytes, and not by γ/δ T cells. This suggests that residual monocytes were present in the γ/δ T cell population, which could be responsible for production of these cytokines. Similar to our results, Lancioni and colleagues observed that CD4⁺ T cells with a purity of 97% differ from highly purified CD4⁺ T cells with regard to their responses to LPS.[10] Therefore, we examined in more detail γ/δ T cell– or monocyte-depleted PBMCs and highly purified, freshly isolated γ/δ T cells or monocytes.

Abrogation of TCR/MDP-LP–induced IFN-γ production after depletion of γ/δ T cells from PBMCs

PBMCs were completely depleted of γ/δ T cells (fig. 2A) or monocytes (fig. 2B) by MACS Technology. Undepleted PBMCs or PBMCs depleted of γ/δ T cells or PBMCs depleted of both γ/δ T cells and monocytes were stimulated with BrHPP in the absence or presence of MDP-LD or MDP-DD for 24 h (fig. 2C, D). In these experiments, we analyzed the cytokine MIP-1α, which can be secreted by both γ/δ T cells and monocytes, and IFN-γ, which is exclusively produced by T cells. We observed that MDP-LP, but not inactive MDP-DD, enhanced MIP-1α production in undepleted PBMCs, but also in PBMCs depleted of γ/δ T cells. The depletion of monocytes almost abolished the secretion of MIP-1α suggesting that MDP-LP–reactive monocytes were the major producers of MIP-1α. Moreover, MDP-LD, but not MDP-DD, enhanced IFN-γ secretion in BrHPP-stimulated PBMCs, which was abrogated after γ/δ T cell depletion (fig. 2D). These experiments indicate a moderate direct costimulatory effect of MDP-LD on γ/δ T cells, which was investigated in further experiments using highly purified γ/δ T cells from 18 healthy donors (see ref. 9).

MDP-LD directly increases IFN-γ production in highly purified γ/δ T cells

From the same PBMCs shown in figure 2 as well as from 3 additional healthy donors (see ref. 9), γ/δ T cells and CD14⁺ monocytes were isolated by a combination of MACS Separation and flow sorting or by two consecutive MACS Separations. We obtained highly purified cells from both separation procedures. The data for cells purified by the combination of MACS Separation and flow sorting from one representative donor are shown in figure 3A and B. Highly purified monocytes stimulated with MDP-LD produced higher levels of MIP-1α than the control or cells incubated with inactive MDP-DD (fig. 3C). Freshly isolated, highly purified γ/δ T cells from the same donor secreted IFN-γ in response to TCR cross-linking. IFN-γ secretion was slightly enhanced in the presence of MDP-LD, but not with MDP-DD (fig. 3D). Purified monocytes did not produce IFN-γ under these culture conditions (fig. 3D). Similar results were obtained after two consecutive MACS Separations as shown in figure 6 of reference 9.

Conclusion

- MACS Separation followed by flow sorting as well as two consecutive MACS Separations permit the isolation of highly purified γ/δ T cells or CD14⁺ monocytes from PBMCs.
- The highly purified cell populations allow functional studies on innate receptors, such

as NOD2, expressed by freshly isolated γ/δ T cells.

- In order to obtain PBMCs devoid of γ/δ T cells and CD14$^+$ monocytes, these cells were completely removed by MACS Separation.
- The biologically active MDP-LD isomer, but not the inactive MDP-DD isomer, enhanced IFN-γ production by TCR-stimulated, freshly isolated, highly purified γ/δ T cells, further underscoring a role of γ/δ T cells in anti-bacterial immunity.

Acknowledgment

We thank John Wiley and Sons for the kind permission to reproduce figures from Marischen *et al.* (ref. 9).

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