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Citation

Ammann, S., Fuchs, S., Martin-Martin, L., Castro, C. N., Spielberger, B., Klemann, C., ... Ehl, S. (2020). Functional flow cytometry of monocytes for routine diagnosis of innate primary immunodeficiencies, *145*(1), 434-437. doi:10.1016/j.jaci.2019.09.002

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Note: To cite this publication please use the final published version (if applicable).

Functional flow cytometry of monocytes for routine diagnosis of innate primary immunodeficiencies



To the Editor:

Flow cytometry is valuable for diagnosing primary immunodeficiencies (PIDs). In addition to analyzing the presence or absence of immune cells, it can provide functional information on, for example, protein expression or signaling pathways. This is useful for PID screening or for validating genetic variants of uncertain significance (VUS). Although numerous tests are available for the diagnosis of PIDs affecting adaptive immunity, clinically validated functional assays for innate immune defects are limited.¹

Monocytes represent archetypical leukocytes of the innate immune system expressing receptors that sense pathogen-associated molecular patterns and cytokines.² In particular, monocytes stimulated through Toll-like receptors or intracellular nucleotide-binding oligomerization domain (NOD) receptors respond through immediate and copious TNF production (Fig 1, A).³ This response is effectively attenuated by IL-10.⁴ Moreover, monocytes rapidly establish antiviral resistance on type I interferon stimulation (Fig 1, A). However, determination of cytokine levels based on ELISAs or viral titers by using plaque assays in supernatants of stimulated cells is time-consuming, and bulk PBMC assays do not control for the number of responsive cells in individual preparations. Therefore these tests are not widely used in diagnostic laboratories.

Functional monocyte flow cytometry is an attractive alternative. Here we adapted standard experimental monocyte flow cytometric assays and validated their use in routine diagnostic workflows for relevant PIDs.

Cell preparation from 16 fresh, 186 shipped (within 24 hours), or 68 thawed PBMC samples involved monocyte enrichment by means of plate adhesion (Fig 1, B), as detailed in the Methods section in this article's Online Repository at www.jacionline.org. For the diagnosis of patients with defects in Toll-like receptor and NOD2 signaling pathways, we measured TNF production in response to 200 ng/mL LPS or 200 ng/mL L18-MDP compared with medium. Quality requirements were that less than 10% of monocytes (CD14⁺HLA-DR⁺) stained positive for TNF after incubation with medium and more than 60% were positive after LPS stimulation. Δ TNF represents the difference between percentages of TNF-producing cells after incubation with stimulus versus cells incubated in medium. Δ TNF of greater than 40% after LPS and greater than 10% after L18-MDP stimulation were elaborated as cutoff value across healthy donors (Fig 1, C and D). Overall, we screened 86 patients with severe invasive bacterial infections. Two patients had absent LPS responses (Δ TNF < 10%), whereas the L18-MDP response was normal. One carried homozygous mutations in IL-1 receptor-associated kinase 4 (*IRAK4*; Q293*), and the other carried homozygous mutations in *MYD88* (E52del). One patient with an LPS response of 37% had no further follow-up.

Furthermore, we used the assay in 135 patients with early-onset inflammatory bowel disease or with suspected X-linked inhibitor of apoptosis (XIAP) deficiency based on a clinical diagnosis of hemophagocytic lymphohistiocytosis or splenomegaly and episodes of fever, cytopenia, or both.⁵ Thirty patients had an L18-MDP response of less than 10% with normal LPS responses. Twenty-two (66%) of these had mutations in *BIRC4* (XIAP),

including 2 female subjects with severely skewed X-inactivation (Fig 1, D, black symbols).⁵⁻⁷ Notably, this included 4 patients with normal intracellular XIAP expression. Of the remaining 8 patients with reduced L18-MDP, 6 had *NOD2* mutations, 1 had neither XIAP nor *NOD2* mutations, and 1 had only negative test results for XIAP. Three patients with inflammatory bowel disease had *NOD2* variants but normal L18-MDP responses.

For diagnosis of an impaired response to IL-10, we modified the assay using preincubation with IL-10, which inhibits LPS-induced TNF production (Fig 1, A). After 4 hours of cell plating and gentle removal of nonadherent cells, IL-10 was added at concentrations of 0, 1, 5, or 20 ng/mL for 16 hours. Cells were stimulated with LPS (10 ng/mL) and GolgiPlug for an additional 2.5 hours. The relative reduction in TNF production by IL-10 preincubation was calculated. A reduction of greater than 35% in cells preincubated with 20 ng/mL IL-10 was required in the healthy control subjects for a valid analysis. We analyzed 30 patients with early-onset inflammatory bowel disease. In 5 patients IL-10 induced less than 35% reduction in the TNF response. They were sequenced for mutations in *IL10RA* and *IL10RB*. Two patients with reductions of less than 5% were found to have *IL10RB* deficiency, whereas in 1 patient (reduction of 28%) only a heterozygous *IL10RA* mutation was identified, and no mutation was identified in the remaining 2 patients (reduction of 31% and 33%, respectively; Fig 2, A and B).

To identify patients with type I interferon-signaling pathway defects, we used a flow cytometry-based assay of virus control in monocytes.⁸ In this assay PBMCs were incubated overnight with increasing IFN- α concentrations (0.01-10 U/mL). Medium was then substituted with medium containing recombinant vesicular stomatitis virus (VSV) expressing green fluorescent protein (GFP [VSV-GFP]; multiplicity of infection = 3.25) for 5 hours.⁹ Cells were then stained for CD14 before flow cytometry. The fraction of GFP⁺ monocytes was determined, and more than 80% of GFP⁺ cells after incubation with medium in the control group were required for a valid analysis. The reduction of the GFP⁺ fraction of IFN- α -stimulated cells compared with medium-incubated cells was taken to measure type I interferon activity. A consistent reduction in numbers of GFP⁺ cells to less than 20% of the medium control value at the greatest IFN- α dosage was confirmed in healthy donors.

We analyzed 14 patients with severe or recurrent viral infections, including 1 patient with severe measles and rubella-induced deafness after MMRV vaccination and 2 with a VUS in tyrosine kinase 2 (*TYK2*). The test result was abnormal in 4 patients. One was given a diagnosis of compound heterozygous signal transducer and activator of transcription 2 (*STAT2*) deficiency, and 1 was given a diagnosis of homozygous *STAT1* deficiency. Functional *TYK2* deficiency was confirmed in the other 2 patients (Fig 2, D and E).

Overall, all 3 monocyte flow cytometric assays provided robust and reproducible results with fresh, shipped (<24 hours), and thawed samples (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). They identified patients with loss-of-function mutations in *IL10RA*, *BIRC4*, *NOD2*, *IRAK4*, *MyD88*, *TYK2*, *STAT1*, and *STAT2*. Whether the tests reliably discriminate subjects with monoallelic mutations remains to be fully explored. We have also successfully used the IL-10 inhibition assay to validate loss-of-function mutations in *STAT3* (data not shown), whereas it did not allow validation of *STAT3* gain-of-function mutations. We expect that the IFN- α assay detects

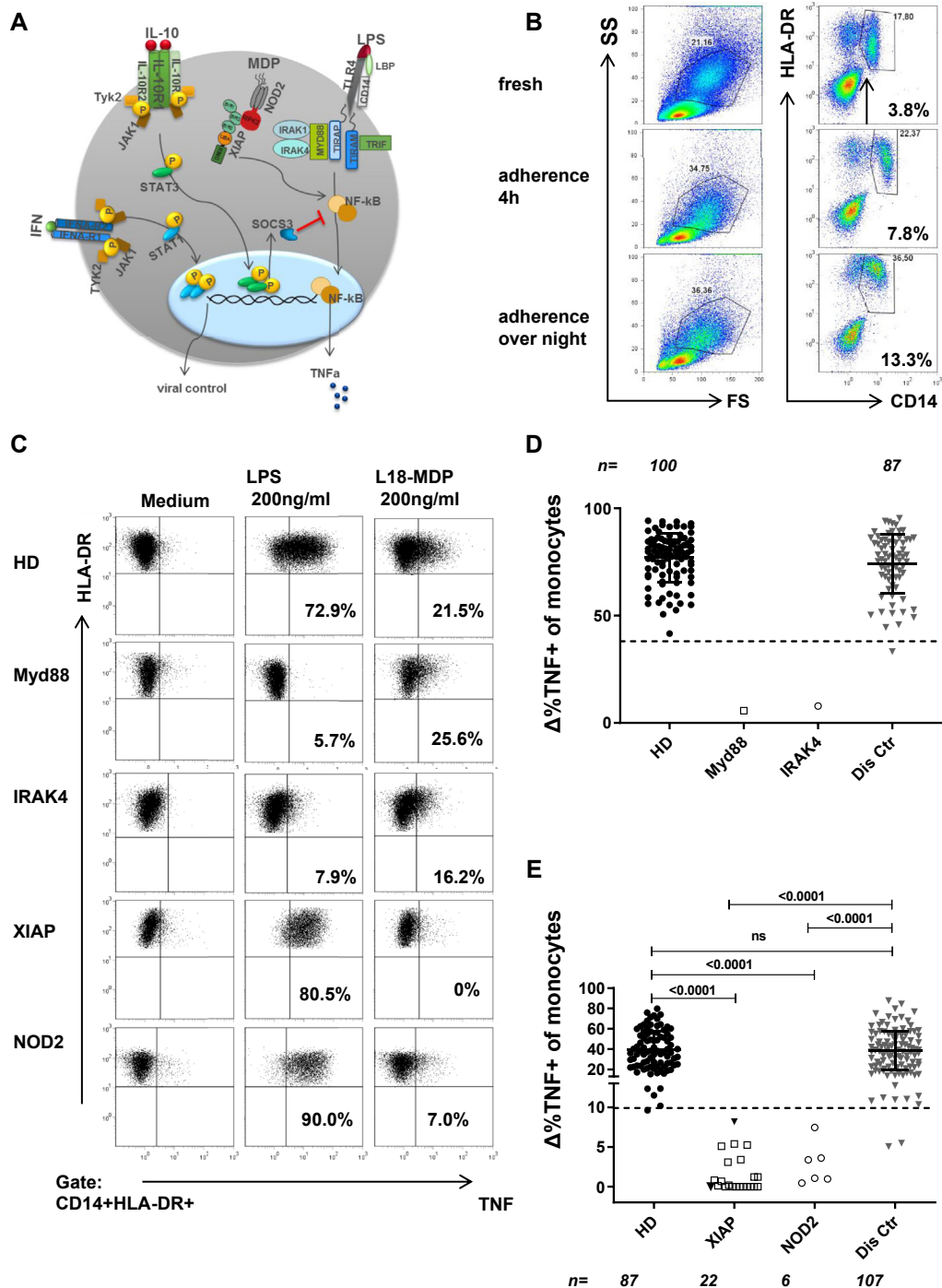


FIG 1. A, Monocyte signaling pathways targeted by diagnostic assays. **B**, Monocyte enrichment by means of plate adherence before stimulation assays. **C**, Enriched monocytes were incubated with medium, LPS (200 ng/mL), or L18-MDP (200 ng/mL), and the fraction of TNF-producing CD14⁺HLA-DR⁺ cells was determined by using flow cytometry. **D**, Δ TNF response (Percentage TNF⁺stimulated cells – Percentage TNF⁺medium-incubated cells) to LPS in 87 patients with invasive bacterial infections and healthy donors (HD; upper panel), patients given a diagnosis of MYD88 or IRAK4 deficiency, and patients with similar clinical presentation, but no mutation was detected if investigated (Dis Ctr). Response to L18-MDP in healthy donors, 20 XIAP-deficient male and 2 symptomatic female XIAP carriers (closed triangles), 6 patients with a diagnosis of NOD2 deficiency, and 107 patients presenting with early-onset inflammatory bowel disease (EO-IBD) or suspected XIAP deficiency, but no mutation was detected if investigated (Dis Ctr; lower panel). Statistical analysis was performed with the Mann-Whitney U test. FS, Forward scatter; HD, healthy donor; IL-10R, IL-10 receptor; JAK, Janus kinase; LBP, LPS-binding protein; Myd88, myeloid differentiation response gene-88; NF- κ B, nuclear factor κ B; ns, not significant; SOCS3, suppressor of cytokine signaling 3; SS, side scatter; TLR, Toll-like receptor.

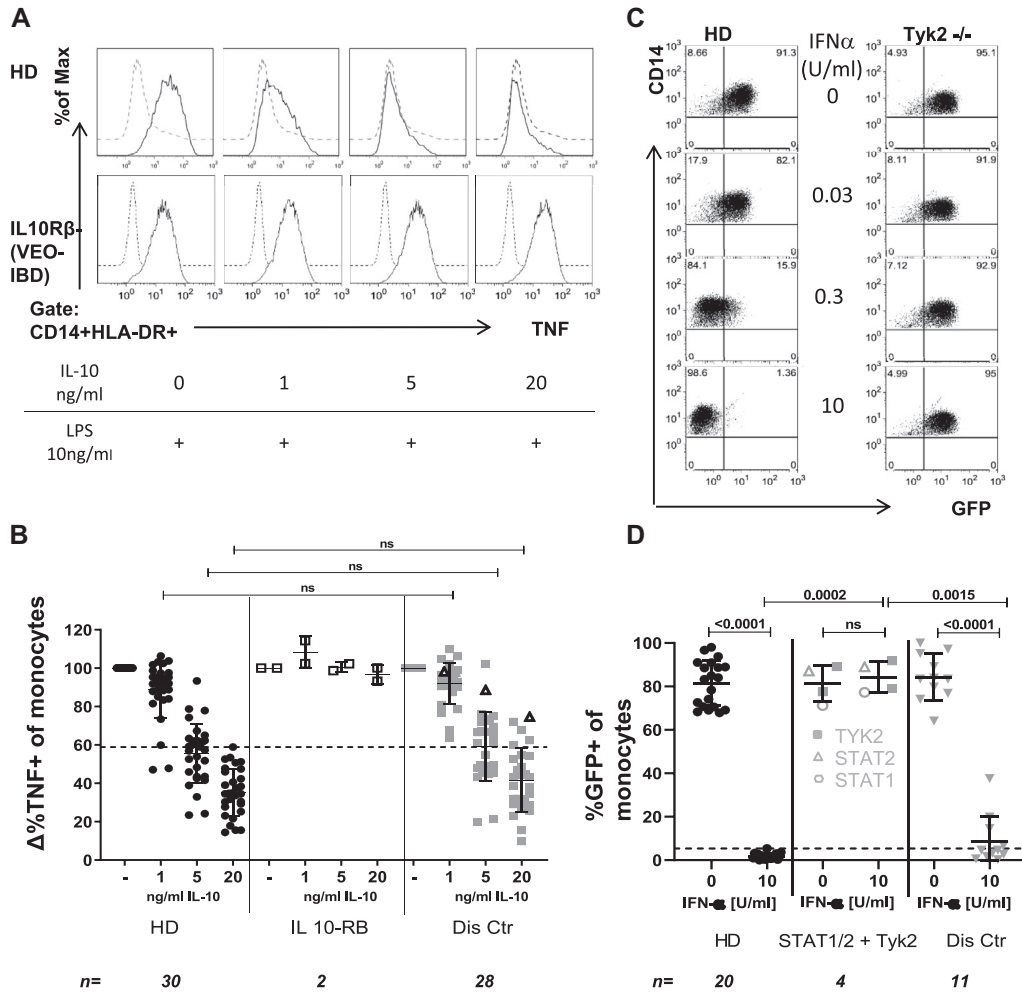


FIG 2. A, Enriched monocytes were preincubated overnight with medium and IL-10 (0, 1, 5, or 20 ng/mL) and then stimulated with LPS (10 ng/mL). The fraction of TNF-producing CD14⁺HLA-DR⁺ cells was determined by using flow cytometry. Histograms show overlays of medium-incubated (*dotted line*) and stimulated cells (*solid line*). B, ΔTNF (Percentage TNF⁺stimulated cells – % TNF⁺medium-incubated cells) results of healthy donors, 2 patients given a diagnosis of *IL10RB* deficiency, 28 patients screened for EO-IBD (*Dis Ctr*) including 1 with a heterozygous *IL10RA* mutation (*open triangle*). C, Enriched monocytes preincubated with IFN-α (0, 0.03, 0.3, and 10 U/mL) overnight were infected with VSV-GFP. Virus control was determined based on loss of GFP expression in CD14⁺ monocytes by using flow cytometry. D, Results obtained in healthy donors; patients with a diagnosis of *TYK2* (2), *STAT1*, and *STAT2* deficiency; and 10 patients presenting with susceptibility to viruses, but no mutation was detected if investigated (*Dis Ctr*). Statistical analysis was performed by using the Mann-Whitney *U* test. HD, Healthy donors; ns, not significant.

IFN-α/β receptor deficiency and the L18-MDP assay detects linear ubiquitin chain assembly complex and receptor interacting serine/threonine kinase 2 deficiency, but this requires validation.

Functional tests interrogating the integrity of immune-signaling pathways currently have several advantages over first-line genetics. First is a turnaround time of 24 to 48 hours, which is relevant for immediate treatment decisions. In most places it still takes longer to obtain validated genetic results. Second is an immediate functional result, whereas for VUS, the functional effect requires confirmation. Third is the ability to interrogate the full pathway, potentially including molecules thus far not associated with PIDs and therefore not detected by using candidate gene or clinical whole-exome sequencing analysis. Fourth is lower cost for material, personnel, and data processing. The obvious disadvantage is that functional results require genetic confirmation, but this

becomes easier if performed pathway oriented. Moreover, considering the robustness of the assays presented here, immediate clinical decisions, including prophylaxis, specific treatment, and hematopoietic stem cell transplantation donor search, can already be made based on the functional results.

We thank all the physicians who contributed to the study, including Peter Hasselblatt, Anna Gschöpf and the AD team, Bodo Grimbacher, Claire Canning, and Cathal Steele. We acknowledge the support of Martin Schwemmler (Freiburg, Germany) for the VSV-GFP assay.

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Supported by the Deutsche Forschungsgemeinschaft (SFB1160,TPA01) and the Jeff Modell Foundation.

Disclosure of potential conflict of interest: S.E. has received consulting fees from UCB and SOBI but not in relation to this study. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online September 14, 2019.
<https://doi.org/10.1016/j.jaci.2019.09.002>

TNF induces production of type 2 cytokines in human group 2 innate lymphoid cells



To the Editor:

It is well recognized that group 2 innate lymphoid cells (ILC2s) play a key role in both innate immunity to protect against parasitic pathogens and pathologic processes for many type 2 inflammatory diseases, including asthma and chronic rhinosinusitis with nasal polyps (CRSwNP).^{1,2} ILC2-mediated inflammation is mainly triggered through production of type 2 cytokines, including IL-5 and IL-13. Although the epithelium-derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) are well appreciated to contribute to ILC2-mediated production of type 2 cytokines, recent studies found that members of the TNF superfamily (TNFSF), including TNFSF11 (receptor activator of nuclear factor κ B [NF- κ B; RANK] ligand), TNFSF15 (TNF-like cytokine 1A), and TNFSF18 (glucocorticoid-induced TNF receptor [TNFR]–related protein [GITR] ligand), are also able to induce IL-5 and IL-13 in ILC2s through activation of their respective receptors: TNFR superfamily 11A (TNFRSF11A [RANK]), TNFRSF25 (death receptor 3) and TNFRSF18 (GITR).¹⁻⁵ Nonetheless, whether TNF itself (also known as TNF- α) is able to activate ILC2s and whether the TNFRs TNFRSF1A (TNFRI) and TNFRSF1B (TNFRII) are expressed on ILC2s has not been investigated. Many TNFRSFs, including RANK, GITR, and death receptor 3, share the NF- κ B signaling pathway through activation of TNFR-associated factor family members, and TNFRII has similar signaling pathways to them.² The aim of this study was to examine whether TNFRII is expressed on ILC2s and whether TNF induces production of type 2 cytokines in ILC2s.

We first measured mRNA for TNFRII in human ILC2s. We sorted human ILC2s from peripheral blood of healthy subjects and from nasal polyps (NPs; a type 2 inflammatory manifestation) collected from patients with CRSwNP during sinus surgery. We also used human peripheral blood monocytes and the Jurkat cell line as positive and negative controls of TNFRII, respectively. Real-time quantitative RT-PCR was performed by using a TaqMan method, and detailed methods are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org. We found that TNFRII mRNA was similarly expressed on both blood and NP ILC2s at about 14% of the level on monocytes and was significantly greater than the Jurkat cell line ([Fig 1, A](#)).

We then determined cell-surface expression of TNFRII on ILC2s by using flow cytometry. Gating strategies and antibodies identifying human ILC2s are shown in [Fig E1](#) and [Table E1](#) in this article's Online Repository at www.jacionline.org. We found that TNFRII was expressed on ILC2s from both blood and NPs, and levels were significantly greater than those on Jurkat cells ([Fig 1, B and C](#)).

We next cultured human ILC2s sorted from blood and NP tissue in RPMI 1640 supplemented with 25 IU/mL IL-2 and 10% FBS and investigated the effect of TNF. We found that TNF time- and dose-dependently induced production of IL-5 and IL-13, although IL-5 protein was only detected at 96 hours after stimulation in human blood ILC2s ([Fig 2, A](#), and see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org). Previously, we found that ILC2s are activated in NP tissue *in vivo*, and therefore ILC2s sorted from NP tissue release IL-5 and IL-13

METHODS**Supplementary information**

All assays were performed with PBMCs from patients and a travel control isolated from whole blood after a shipment time of less than 24 hours by using Ficoll density centrifugation. Overall, 16 fresh, 186 shipped, and 68 thawed PBMC samples were analyzed, and a similar range of values was obtained with samples from healthy donors investigated under these 3 conditions. After PBMC isolation, we determined the fraction of CD14⁺HLA-DR⁺ cells among live nucleated cells using flow cytometry with CD45 (PB; BD Biosciences, San Jose, Calif), CD14 (allophycocyanin [APC]; BD Biosciences), and HLA-DR (Pe-Cy7; BD Biosciences).

PBMCs were then plated in medium (Iscove modified Dulbecco medium [IMDM] + 10% FCS + 1% Penicillin/Streptomycin) in a 24-well plate depending on assay setup either for 4 hours or overnight, followed by gentle removal of nonadherent cells, to increase the monocyte fraction. To be able to analyze sufficient monocytes, we only plated PBMCs if they contained at least 1% of CD14⁺HLA-DR⁺ cells among live nucleated cells. At greater than this cutoff, we have not seen increased variability of TNF production as a function of monocyte count.

After the adhesion step, the attached cell fraction was left on the plate and incubated in medium containing stimulation substances (see below) and GolgiPlug (Fixation and Permeabilization Kit; BD Biosciences) in assays with TNF production as a readout. After incubation, cells were harvested with Accutase C (Sigma-Aldrich, St Louis, Mo) for 4 minutes at 37°C and transferred to ice, followed by cell resuspension with ice-cold fluorescence-activated cell sorting (FACS) buffer (PBS + 2% FCS). After a washing step, cells were stained for surface expression of HLA-DR (Pe-Cy7; BD Biosciences) and CD14 (APC; BD Biosciences) and fixed (Fixation and Permeabilization Kit; BD Biosciences). After permeabilization by washing twice with 1× Perm/Wash (Fixation and Permeabilization Kit; BD Biosciences), anti-TNF-phycoerythrin (PE; clone MAb11; BD Biosciences) was added for 30 minutes at 4°C, followed by washing with Perm/Wash and then with FACS buffer. Cells were acquired with a Navios flow cytometer (Beckman Coulter, Brea, Calif) and analyzed with Kaluza software (Beckman Coulter).

The adhesion step and the permeabilization protocol led to a significant change in the phenotype of the monocyte population (Fig E2). Using the markers HLA-DR, CD14, and CD16 to distinguish between nonclassical (HLA-DR⁺CD16⁺⁺CD14⁻), intermediate (HLA-DR⁺CD16⁺CD14⁺), and classical (HLA-DR⁺CD16⁻CD14⁺) monocytes, we detected loss of the nonclassical and intermediate phenotypes, and most cells have the classical phenotype. It has been described that nonclassical monocytes are the major TNF producers. Although their depletion leads to reduction of LPS-induced TNF production by PBMCs of about 28% (Belge et al^{E1}), this still leaves a robust response that can be well analyzed by using FACS. Notably, the goal of the assays is not to analyze different monocyte subsets and their function but to study the integrity of genetically disturbed signaling pathways. For this purpose, it is important to analyze cells that are comparable in their response pattern. Therefore the (protocol-caused) restriction of the analysis to classical monocytes is rather beneficial for the assay purpose.

To address the effect of *in vivo* monocyte activation on the LPS/L-18-MDP assay, we analyzed 3 patients with active primary hemophagocytic lymphohistiocytosis, EBV-induced secondary hemophagocytic lymphohistiocytosis (treated with steroids), and bacterial sepsis. Monocyte activation was confirmed by using CD14/CD16 staining. The response of these cells was similar to those of healthy donors donating blood on the same day (day controls) and well within the range of values observed in a pool of healthy donors. For the IL-10 inhibition assay, control subjects with disease all had active inflammatory bowel disease. Although not documented, we expect a variable extent of monocyte activation in these patients. Nevertheless, the results of these control subjects with disease (n = 28) were similar to those of healthy donors (n = 30). These limited observations suggest that monocyte activation does not significantly influence the performance of the investigated assays. More experience will be needed to confirm the robustness of these assays.

Protocols**LPS/L18-MDP assay.**

- Depending on monocyte counts, plate 1 to 3 × 10⁶ PBMCs/well in 1 mL of IMDM/10% FCS (medium) in 3 wells of a 24 well-plate.
- Centrifuge at 30g for 3 minutes and rest overnight at 37°C.
- Remove nonadherent cells by means of gentle pipetting with a 1-mL pipette.
- Add 1 mL of stimulation medium with 1:1000 GolgiPlug (Fixation/Permeabilization Kit with BD GolgiPlug).
- Stimulation media
 - A. Medium (IMDM + 10% FCS)
 - B. LPS (final concentration, 200 ng/mL (Sigma-Aldrich))
 - C. L18-MDP (final concentration, 200 ng/mL; InvivoGen, San Diego, Calif)
- Resuspend gently with a 1-mL pipette.
- Centrifuge at 30g for 3 minutes, incubate for 2 hours at 37°C, and carefully take off supernatant.
- Add 200 μL of Accutase (Sigma-Aldrich), incubate for 4 minutes at 37°C, place on ice, add 1 mL of cold FACS buffer (PBS + 2% FCS), resuspend, and transfer to FACS tubes.
- Wash once with cold FACS buffer.
- Apply surface staining for 20 minutes at 4°C: anti-CD14-APC (BD Biosciences) and anti-HLA-DR-PC7 (BD Biosciences).
- Add 100 μL of Cytofix/Cytoperm (Fixation/Permeabilization Kit with BD GolgiPlug) and incubate for 15 minutes at 4°C.
- Wash 2 times with 1 mL of Perm/Wash (1:10 diluted with H₂O).
- Add 10 μL of anti-TNF-PE (BD Biosciences) for 30 minutes at 4°C.
- Wash once with 1 mL of Perm/Wash (1:10 diluted with H₂O).
- Wash once with FACS buffer.

IL-10-mediated inhibition assay.

- Depending on monocyte counts: plate 1 to 3 × 10⁶ PBMCs/well in 1 mL of IMDM/10% FCS (medium) in 3 wells of a 24-well plate.
- Incubate for 6 hours at 37°C.
- Remove nonadherent cells by means of gentle pipetting and add 500 μL of complete IMDM medium.
- Add IL-10 (R&D Systems, Minneapolis, Minn) for a final concentration of 1, 5, or 20 ng/mL.
- Resuspend gently with a 1-mL pipette; centrifuge plate at 30g for 3 minutes.
- Incubate overnight at 37°C.
- Add 10 ng/mL LPS + GolgiPlug (Fixation/Permeabilization Kit with BD GolgiPlug).
- Resuspend gently with a 1-mL pipette; centrifuge plate at 30g for 3 minutes.
- Incubate for 2.5 hours at 37°C.
- Take off supernatant, add 200 μL of Accutase (Sigma-Aldrich), and incubate for 4 minutes at 37°C.
- Harvest by resuspending (FACS buffer) into sterile FACS tubes on ice.
- Wash 1 time with FACS buffer (PBS + 2% FCS).
- Apply surface staining for 20 minutes at 4°C: CD14-APC and HLA-DR-PC7.
- Add 100 μL of Cytoperm/Cytofix Plug (Fixation/Permeabilization Kit with BD GolgiPlug) for 20 minutes at 4°C.
- Wash 2 times with 1 mL of Perm/Wash (1:10 diluted with H₂O).
- Add 10 μL of TNF-PE for 30 minutes at 4°C.
- Wash 1 time with 1 mL of Perm/Wash (1:10 diluted with H₂O).
- Wash 1 time with FACS buffer.

VSV-GFP assay.

- Plate 10⁶ PBMCs in 500 μL of IMDM/10% FCS in 6 wells of a 24 well-plate.
- Add 500 μL of stimulation medium containing 10/0.3/0.03/0 U/mL IFN-α as a final concentration.
- Incubate overnight at 37°C.
- Gently resuspend and remove 500 μL of medium, including nonadherent cells.

- Add 500 μ L of medium containing VSV-GFP (multiplicity of infection = 3.75).
- Incubate for 5 hours at 37°C on the rocker.
- Take off supernatant.
- Add 200 μ L of Accutase (Sigma-Aldrich) and incubate for 5 minutes at 37°C.
- Add 500 μ L of medium and transfer cells into FACS tubes.
- Wash once with FACS buffer (PBS + 2% FCS).
- Add 3 μ L of anti-CD14-APC for 20 minutes at 4°C.
- Add 500 μ L of Optilyse C (Beckman Coulter) for 10 minutes at room temperature.
- Add 500 μ L of PBS for 5 minutes at room temperature.

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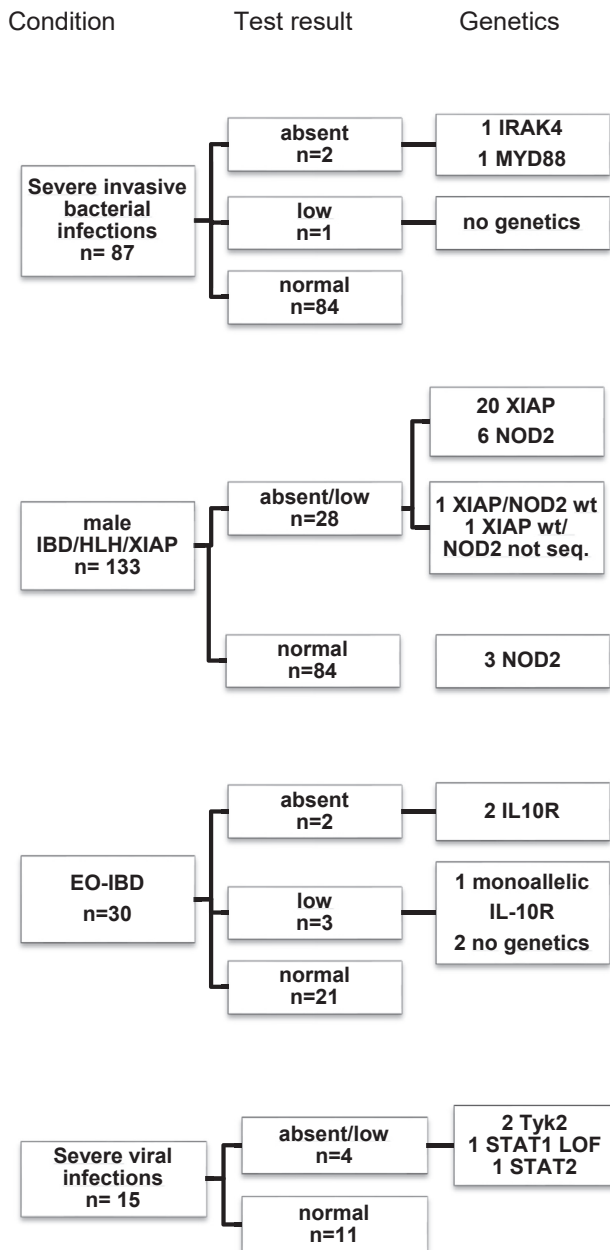


FIG E1. Summary of diagnoses obtained by means of functional monocyte screening with flow cytometry. *EO-IBD*, Early-onset inflammatory bowel disease; *HLH*, hemophagocytic lymphohistiocytosis; *IL-10R*, IL-10 receptor; *LOF*, loss of function; *wt*, wild-type.

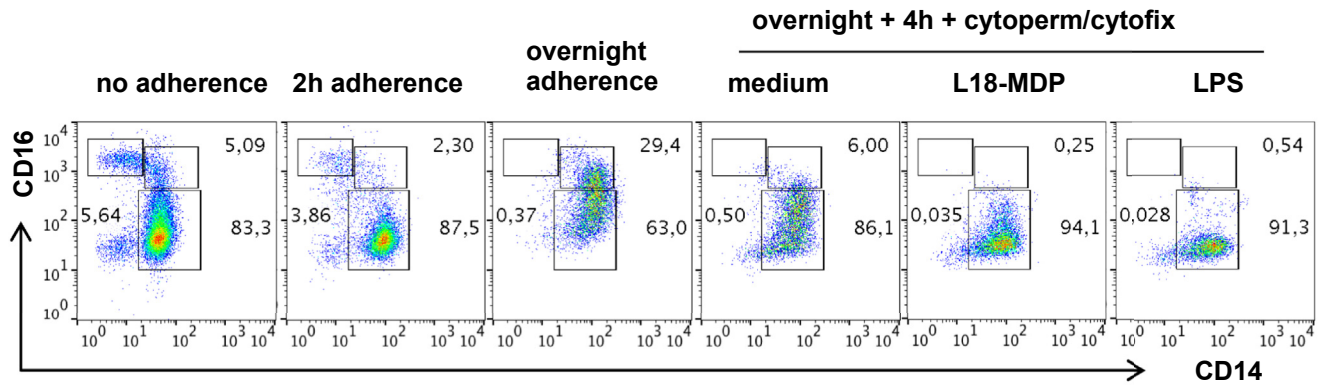


FIG E2. Monocyte phenotype after *in vitro* manipulation (healthy donor). Flow cytometry of monocytes (gated by using forward/side scatter, HLA-DR, and CD14 staining) using fresh PBMCs (panel 1), PBMCs plated in 24-well plates for 2 hours or overnight (panels 2 and 3), or PBMCs plated overnight, washed, and then incubated in medium alone (panel 4), medium containing L18-MDP (panel 5), or medium containing LPS (panel 6) for 4 hours and then treated with Cytotfix/Cytoperm buffer. *Dot plots* in panels 2 to 6 show CD14 and CD16 staining of the adherent cell fraction gated for monocytes.