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Rapid Loss of Dendritic Cell and Monocyte Responses to TLR Ligands following Venipuncture

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Abstract

Blood samples from multiple sites are collected in multicenter trials, and frequently shipped to centralized laboratories for processing and comparable experimental evaluation. It is therefore of crucial interest to assess the preservation of immune cell functions after overnight shipment of whole blood. Here we evaluated the ability of pDCs, mDCs and monocytes to respond to TLR ligands at multiple timepoints following venipuncture as compared to immediate processing. Our results demonstrate a profound impairment of APC function, in particular of IFN- α production of pDCs, if whole blood was processed later than 6 hours after venipuncture. Overnight shipment or extended rest of whole blood before processing therefore severely compromises the ability of APCs to respond to TLR ligands, and this has to be taken into consideration when designing multicenter trials.

Keywords

Innate Immunity; Antigen Presenting Cells; Dendritic Cells; Monocytes; Toll Like Receptors; Vaccine Trials

Introduction

The activation of innate signaling pathways is considered crucial for an effective vaccine response, as the innate immune system is responsible for shaping the quality and function of the subsequent adaptive immune response (Iwasaki and Medzhitov, 2004; Pulendran and Ahmed, 2006; Querec et al., 2006). Adjuvants are an important component of non-live vaccines as many efficient subunit vaccines do not display any significant immunogenicity without the addition of such adjuvants (Kwissa et al., 2007). The addition of TLR ligands increases the immunogenicity of peptide/protein based, DNA based and tumor cell based vaccines (Liu et al., 1998; Cho et al., 2000; Heckelsmiller et al., 2002; Kochling et al., 2003; Lore et al., 2003; Wille-Reece et al., 2005; Wille-Reece et al., 2006). It has furthermore been demonstrated that some live vaccines, which display strong immunogenicity without adjuvants, elicit their

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effects though the activation of innate pattern recognition receptors, activating TLRs directly themselves, and therefore serve as auto-adjuvants (Querec et al., 2006; Kwissa et al., 2007).

Due to this important role of the innate immune system in modulating vaccine immunogenicity, studies of the ability of innate immune cells to respond to stimulation with TLR ligands are warranted and represent an expanding area of immunological research in multicenter trials in humans (Pulendran and Ahmed, 2006). Given that shipment of samples from collection sites to processing sites may vary, it is therefore crucial to evaluate the impact of time since venipuncture on the ability of innate immune cells to respond to TLR ligands. While previous studies have demonstrated that delays of more than 6 hours between venipuncture and blood processing (Bull et al., 2007) or overnight rest and/or overnight shipment (Weinberg et al., 1998; Betensky et al., 2000; Bull et al., 2007) reduces the ability of antigen-specific T cells to respond to antigenic stimulation *ex vivo*, little is known regarding the impact of delays in blood processing on the function of APCs. Here we demonstrate that the ability of dendritic cells and monocytes to respond to stimulation with TLR ligands can be significantly altered by time, and that it is therefore crucial to control for the time between venipuncture and blood processing when evaluating the innate immune function.

Materials and Methods

Study Subjects

Blood samples from healthy study participants enrolled at Massachusetts General Hospital (MGH) were included in the studies. The MGH Institutional Review Board approved the study, and each subject gave informed consent for participation in the study.

Stimulation of PBMC by HIV-1-derived ssRNA and cytokine detection

Blood was drawn in acid citrate dextrose (ACD) tubes and PBMCs were separated from whole blood by Ficoll-Hypaque (Histopaque 1077, Sigma Aldrich) density gradient centrifugation after 0hr, 6hrs, 12hrs and 24hrs of venipuncture. One and one half million PBMCs/ml were stimulated with 1µg/ml CL097 (Invivogen) or 5µM ODN2216 (Invivogen). Unstimulated cells served as negative controls. 5µg/ml Brefeldin A (Sigma) was added to each tube in order to inhibit cellular cytokine release. Stimulations for all assays were conducted at 37°C and under 5% CO₂ conditions.

Intracellular cytokine content of monocytes and plasmacytoid dendritic cells (pDCs) was determined following 20hrs of stimulation. PBMCs were washed in PBS and resuspended in about 100µl PBS. Subsequently PBMCs were stained for 20 min for with the following surface markers: anti-CD3-Alexa700, anti-CD19-Alexa700, anti-CD56-Alexa700, anti-CD11c-PE, anti-CD14-APCCy7 and anti-CD123-PECy5. Following another wash and resuspension in approx 100µl PBS, PBMCs were fixed for 20 min with 100µl Fix&Perm Reagent A (Caltag). Following surface fixation, cells were washed again in PBS, resuspended in about 100µl PBS, and incubated for 20 min with 100µl Fix&Perm Reagent B (Caltag) and the antibodies for intracellular cytokine staining (anti-IFN-α-FITC (PBL), anti-IL-12-APC, and anti-TNF-α-PECy-7 (all antibodies BD Biosciences unless indicated otherwise)). All samples were acquired on an LSR II (BD). The frequency of cytokine-producing pDCs, myeloid dendritic cells (mDCs) and monocytes was determined by subsequent analysis using Flow Jo software (version 8.5.2, Treestar, Inc). pDCs were defined as CD3^{neg}CD19^{neg}CD56^{neg}CD14^{neg}CD11c^{neg}CD123^{pos} cells, mDCs as CD3^{neg}CD19^{neg}CD56^{neg}CD14^{neg}CD123^{neg}CD11c^{pos} and monocytes were defined as CD3^{neg}CD19^{neg}CD56^{neg}CD14^{pos} cells.

Statistics

Paired *t*-tests were applied to calculate statistical significance of differences within individuals between 2 different time points. One way repeated measures ANOVA was used to determine statistical significance of differences between several processing time points.

Results

Significant decline of the ability of pDCs to respond to TLR stimulation over time following venipuncture

pDCs play an important role in bridging innate and adaptive immunity. Thus, the assessment of their responses is key for the testing and evaluation of immune stimulatory potentials of new vaccine candidates. In this study we tested the ability of pDCs to respond to TLR7/8 and TLR9 ligands over time by comparing the function of pDCs in PBMCs isolated from whole blood immediately after venipuncture to the function of pDCs in PBMCs isolated from whole blood after 6, 12 and 24hr delays following venipuncture. These timepoints are reflective of those that commonly occur in multicenter trials involving shipment of blood from collection sites to centralized processing facilities.

pDCs in PBMCs that were isolated from whole blood immediately following venipuncture (0hr= baseline) showed strong induction of IFN- α production following 20hrs of stimulation with the TLR7/8 ligand CL097 (Figure 1A, 1B and 1C) or the TLR9 ligand ODN2216 (supplemental Figure 1). The mean percent of IFN- α pDCs was 23.4% in response to CL097 (Figure 1B) and 4.3% in response to ODN2216 (supplemental Figure 1). However, this ability to produce IFN- α decreased significantly for pDCs in PBMCs isolated from blood collected from the same venipuncture, but processed at a later time point (Figure 1B and 1C). The mean percentage of pDCs producing IFN- α in response to CL097 significantly decreased from 23.4% at 0hr to 11.1% after 6hrs ($p=0.03$, paired *t*-test), 6.3% after 12hrs ($p=0.009$) and 1.8% after 24hrs ($p=0.02$). This represented a greater than 50% reduction of IFN- α pDCs as early as 6hrs after venipuncture (Figure 1C).

A similar trend was observed in the pDC IFN- α response to the TLR9 agonist ODN2216 (mean percentage of pDCs producing IFN- α at 0hr: 4.3%, at 6hrs 1.6%, at 12hrs 0.4% and at 24hrs: 0.5%). The loss of IFN- α producing pDCs in response to ODN2216 after a processing delay of 24hrs represented a decline greater than 85%, but this decline did not reach statistical significance (supplemental Figure 1).

The Median Fluorescent Intensity (MFI) of IFN- α in response to CL097 (gated on all CD123 + pDCs) was significantly reduced after 6hrs (430 at 0hr vs. 272 at 6hrs, paired *t*-test $p=0.03$), but then remained more stable after this initial reduction (222.8 at 12hrs and 247.5 at 24hrs, Figure 1F and 1G). Despite the lack of further reductions the MFI remained significantly lower for all comparisons to baseline ($p<0.05$ for all comparisons). When the MFI of only the IFN- α pDCs was measured the decline was significant only after a 24hrs delay of blood processing after venipuncture (mean MFI of IFN- α +pDCs at baseline vs. mean MFI of IFN- α +pDCs at 24hrs 10003 vs. 4055, $p=0.04$, paired *t*-test, data not shown). Of note, the frequency of pDCs did not change during this study period (mean 0.3% at 0hr; 0.6% at 6hrs, 0.6% at 12hrs and 0.4% at 24hrs, $p>0.2$ for all comparisons (paired *t*-test for CL097 stimulated pDCs)).

The ability of pDCs to produce TNF- α in response to stimulation with CL097 also decreased significantly with increasing time intervals between venipuncture and blood processing from a mean of 30% TNF- α pDCs at baseline (0hr) to 27.8% at 6hrs ($p=0.2$; paired *t*-test), 20.5% at 12hrs ($p=0.04$) and 16.9% at 24hrs ($p=0.04$) (Figure 1A, 1D and 1E). However, this decline was less rapid and less pronounced than for IFN- α (Figure 1E). There was also a trend for the

MFI of TNF- α (gated on all CD123+ pDCs) to decrease, however, this did not reach statistical significance. ($p > 0.05$ in paired t -tests for all comparisons to 0hr, Figure 1H and 1I).

The percentage of pDCs producing TNF- α in response to the TLR9 ligand ODN2216 also declined over time. The mean percentage of TNF- α producing pDCs was at baseline 15.2%, at 6hrs 8.9%, at 12hrs 4.5% and at 24hrs 5.1%. Although this decline represented a greater than 65% reduction as compared to baseline, this did not reach statistical significance for all comparisons (supplemental Figure 1).

Taken together these data demonstrate that the ability of pDCs to respond with cytokine secretion to TLR7/8 ligand stimulation decreases rapidly following venipuncture, and that IFN- α production is affected the earliest, shifting the cytokine profile of pDCs towards TNF- α production when blood is not immediately processed after venipuncture.

Significant decline of the ability of mDCs to respond to TLR stimulation over time following venipuncture

In parallel to the studies described above for pDCs, the ability of mDCs to produce IL-12 and/or TNF- α was assessed by multicolor flow cytometry following 20hrs of stimulation with either the TLR7/8 ligand CL097 or the TLR9 ligand ODN2216 (Figure 2A). A high percentage of mDCs in PBMCs isolated immediately after venipuncture produced TNF- α (mean of 41.4% of mDCs) and a smaller percentage produced IL-12 (mean 7.8%) in response to CL097 (Figure 2A, 2B and 2D). Compared to these responses detected in immediately processed samples, mDCs showed decreasing ability to produce cytokines with delayed processing of blood (Figure 2A to 2E). TNF- α production of mDC in response to CL097 declined from a mean of 41.4% at baseline (0hr) to a mean of 36.7% at 6hrs ($p = 0.23$, paired t -test), 28.5% at 12hrs ($p = 0.03$) and 22.2% at 24hrs ($p = 0.04$). This represented a more than 25% decrease in the percentage of TNF- α + mDCs at 12hrs (Figure 1E). Similarly, the percentage of mDCs responding with IL-12 production to TLR7/8 ligand CL097 decreased over time. This decline reached significance after 24hrs (mean of 7.8% IL-12+ mDCs at 0hr, 5.8% at 6hrs ($p = 0.35$, compared to 0hr, paired t -test), 3.7% at 12hrs ($p = 0.06$) and 3.1% at 24hrs ($p = 0.04$), representing a 50% reduction in IL-12+ mDCs at 12hrs (Figure 2C). Furthermore, the mean MFI for TNF- α production, but not the mean MFI of IL-12 production (both gated on all CD11c + mDCs), declined by more than 50% over time, but this decline also did not reach statistical significance (Figure 2F to 2I). When the MFI of TNF- α + mDCs only, or the MFI of IL-12+ mDCs only was measured, this did not decline significantly for neither of the comparisons to baseline ($p \geq 0.2$ for all comparisons of MFIs of TNF- α + mDCs and IL-12+ mDCs compared to baseline using paired t -test). The frequency of mDCs did not decline during this study period (3.7% at 0hr; 6.2% at 6hrs, 9% at 12hrs and 10% at 24hrs, for all comparisons), but rather tended to increase. This increase however did not reach statistical significance ($p > 0.5$ for all comparisons with baseline).

Taken together, these data show a significant reduction in the percentage of cytokine-producing mDCs in response to TLR stimulation when blood is not immediately processed after venipuncture, in line with the data for pDCs described above.

Significant decline of the ability of monocytes to respond to TLR stimulation over time following venipuncture

The activation of monocytes is an important component of an effective immune response, and expression levels of co-stimulatory molecules on monocytes can predict the magnitude of vaccine titers induced by the trivalent inactivated influenza vaccine (van Duin et al., 2007). We therefore also examined the ability of monocytes to produce cytokines in response to TLR ligands after immediate and delayed processing of whole blood. After incubation with the

TLR7/8 ligand CL097 monocytes produced significant amounts of TNF- α (Figure 3A). The mean percentage of TNF- α + monocytes in response to the TLR 7/8 ligand CL097 was 63% when PBMC were isolated from whole blood directly after venipuncture (baseline, 0hr). Although the number of monocytes increased over time (0.3% at 0hr, 1.3% at 6hrs, 1.5% at 12hrs and 1% at 24hrs), the production of TNF- α by monocytes already began to deteriorate when the isolation of PBMCs from whole blood was delayed for 6hrs (mean percentage of TNF- α + monocytes declined to 44% at 6hrs from 63% at baseline, $p=0.03$, paired t -test), and further declined with increasing time between venipuncture and isolation of PBMC to 30.4% at 12hrs ($p=0.001$) and 32.4% at 24hrs ($p=0.009$) (Figure 3B), representing a 50% reduction in TNF- α + monocytes by 12hrs (Figure 3C). Similarly, the MFI was already significantly reduced when blood was processed with a delay of 6hrs after venipuncture [mean MFI (gated on all CD14+monocytes) 0hr vs. 6hrs: 1224 vs. 704.4, paired t -test $p=0.01$] and further decreased with increasing time to processing (mean MFI 0hr vs. 12hrs: 1224 vs. 406, $p=0.0004$ and mean MFI 0hr vs. 24hrs: 1224 vs. 461, $p=0.003$, Figure 3D and 3E). When the MFI was measured on TNF- α + monocytes only, the reduction was not significant for either of the comparisons to baseline ($p>0.05$ for all comparisons to baseline using paired t -test). Taken together, these data show that the ability of monocytes to produce TNF- α is also significantly impaired with increasing delay of blood processing following venipuncture.

Discussion

The innate immune system has become a recent focus of many vaccine studies as effective vaccines have been demonstrated to activate pattern recognition receptors on antigen presenting cells (Querec et al., 2006). TLR signaling in antigen presenting cells can subsequently modulate the function of adaptive immune responses (Iwasaki and Medzhitov, 2004). The complexity of assays testing immune responses does not always allow them to be conducted at the site of blood draw and therefore larger clinical trials often involve shipment of samples from multiple trial sites to central processing laboratories. It is consequently of crucial interest to assess the impact of time from venipuncture to sample processing on innate immune function when evaluating samples from multiple clinical sites.

Previous studies have addressed the impact of shipment and processing delays on CD8+ T cell function and have reported significant decreases in antigen-specific T cell function if blood was cryopreserved or evaluated later than 8hrs after venipuncture (Weinberg et al., 1998; Betensky et al., 2000; Bull et al., 2007) or if fresh blood was used in assays after overnight shipment or overnight rest of samples (Betensky et al., 2000). Furthermore, it has been shown that the function of APCs, and in particular pDCs, is severely compromised after cryopreservation (Ida et al., 2006). However, to our knowledge, no study exists to this date that addresses the consequences of time to processing for the function of freshly isolated APCs following TLR stimulation. Here we systematically assessed the impact of delays between venipuncture and cell processing on the ability of APCs to respond to stimulation with TLR ligands in healthy individuals. Our results demonstrate a progressive decline in the function of pDCs, mDCs and monocytes, as measured by their ability to produce cytokines in response to TLR stimulation, with increasing time between venipuncture and the isolation of PBMCs.

After 20hrs of stimulation with a TLR7/8 ligands innate immune cells showed significant intra-individual differences in cytokine responses, depending on how soon after venipuncture the blood was processed. Interestingly, pDC production of type one interferon seemed to be affected earlier and stronger (> 90% reduction over 24hrs) as compared to pDC TNF- α production (just over 40% reduction), not only diminishing the overall capacity of innate immune cells to produce cytokines but also altering the functional profile of pDCs with time since venipuncture. Similar to the reduction in percentage of IFN- α + pDCs, we observed a significant reduction in the IFN- α MFI of all pDCs (gated on all CD123+ pDCs) as early as

after 6hrs of processing delay. In contrast, the decline in the TNF- α MFI for pDCs (gated on all CD123+ pDCs) did not reach statistical significance. Another way of quantifying MFI is measuring the MFI of cytokine-positive cells only. When the MFIs for the IFN- α + pDCs was quantified over time (gated on the cytokine positive pDCs only), the MFI for IFN- α + pDCs was reduced significantly only after 24hrs, indicating that the reduction of the IFN- α MFI of all CD123+ pDCs was significantly driven by the reduction of the percentage of IFN- α producing pDCs, and less by a reduction of the cytokine production per pDC.

Similar to the observed reduction in cytokine production by pDCs with increasing time from venipuncture to cell processing, the ability of mDCs and monocytes to produce cytokines in response to TLR ligands decreased over time. A significant reduction in the percentage of TNF- α + and IL-12+ mDCs occurred after a delay between venipuncture and processing of 12hrs and 24hrs, respectively. The function of monocytes was significantly affected by delays in processing of 6hrs. The decrease in the ability of pDCs, mDCs and monocytes to respond to TLR stimulation by cytokine production was not accompanied by a reduction in the number of these individual cell subsets, suggesting a beginning apoptotic process impacting their function. This is in line with a previously published study demonstrating rapid spontaneous apoptosis of pDCs in culture, resulting in 40% of cells displaying signs of apoptosis figures after 4hrs (Grouard et al., 1997).

Conclusion

In conclusion, the results of this study demonstrate the importance of taking time to processing into account when comparing innate immune responses in fresh human blood, as delays in processing not only altered the magnitude, but also the quality of the innate response to TLR ligands. These findings are of great significance when comparing the *ex vivo* function of innate cells in multi center studies that involve shipping of whole blood.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Betensky RA, Connick E, Devers J, Landay AL, Nokta M, Plaeger S, Rosenblatt H, Schmitz JL, Valentine F, Wara D, Weinberg A, Lederman HM. Shipment impairs lymphocyte proliferative responses to microbial antigens. *Clin Diagn Lab Immunol* 2000;7:759–63. [PubMed: 10973450]
- Bull M, Lee D, Stucky J, Chiu YL, Rubin A, Horton H, McElrath MJ. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods* 2007;322:57–69. [PubMed: 17382342]
- Cho HJ, Takabayashi K, Cheng PM, Nguyen MD, Corr M, Tuck S, Raz E. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat Biotechnol* 2000;18:509–14. [PubMed: 10802617]
- Grouard G, Risoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* 1997;185:1101–11. [PubMed: 9091583]

- Heckelsmiller K, Beck S, Rall K, Sipos B, Schlamp A, Tuma E, Rothenfusser S, Endres S, Hartmann G. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. *Eur J Immunol* 2002;32:3235–45. [PubMed: 12555669]
- Ida JA, Shrestha N, Desai S, Pahwa S, Hanekom WA, Haslett PA. A whole blood assay to assess peripheral blood dendritic cell function in response to Toll-like receptor stimulation. *J Immunol Methods* 2006;310:86–99. [PubMed: 16455104]
- Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004;5:987–95. [PubMed: 15454922]
- Kochling J, Konig-Merediz SA, Striebeck R, Buchwald D, Korte A, Von Einsiedel HG, Sack F, Henze G, Seeger K, Wittig B, Schmidt M. Protection of mice against Philadelphia chromosome-positive acute lymphoblastic leukemia by cell-based vaccination using nonviral, minimalistic expression vectors and immunomodulatory oligonucleotides. *Clin Cancer Res* 2003;9:3142–9. [PubMed: 12912966]
- Kwissa M, Kasturi SP, Pulendran B. The science of adjuvants. *Expert Rev Vaccines* 2007;6:673–84. [PubMed: 17931149]
- Liu HM, Newbrough SE, Bhatia SK, Dahle CE, Krieg AM, Weiner GJ. Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. *Blood* 1998;92:3730–6. [PubMed: 9808567]
- Lore K, Betts MR, Brenchley JM, Kuruppu J, Khojasteh S, Perfetto S, Roederer M, Seder RA, Koup RA. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J Immunol* 2003;171:4320–8. [PubMed: 14530357]
- Pulendran B, Ahmed R. Translating innate immunity into immunological memory: implications for vaccine development. *Cell* 2006;124:849–63. [PubMed: 16497593]
- Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 2006;203:413–24. [PubMed: 16461338]
- van Duin D, Allore HG, Mohanty S, Ginter S, Newman FK, Belshe RB, Medzhitov R, Shaw AC. Prevacine determination of the expression of costimulatory B7 molecules in activated monocytes predicts influenza vaccine responses in young and older adults. *J Infect Dis* 2007;195:1590–7. [PubMed: 17471428]
- Weinberg A, Betensky RA, Zhang L, Ray G. Effect of shipment, storage, anticoagulant, and cell separation on lymphocyte proliferation assays for human immunodeficiency virus-infected patients. *Clin Diagn Lab Immunol* 1998;5:804–7. [PubMed: 9801338]
- Wille-Reece U, Flynn BJ, Lore K, Koup RA, Kedl RM, Mattapallil JJ, Weiss WR, Roederer M, Seder RA. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proc Natl Acad Sci U S A* 2005;102:15190–4. [PubMed: 16219698]
- Wille-Reece U, Flynn BJ, Lore K, Koup RA, Miles AP, Saul A, Kedl RM, Mattapallil JJ, Weiss WR, Roederer M, Seder RA. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 2006;203:1249–58. [PubMed: 16636134]

Figure 1. delayed processing of blood leads to loss of functional capacity of pDCs

Figure 1A shows representative Flow plots of CD123+ pDCs producing IFN- α (upper panel) or CD123+ pDCs producing TNF- α (lower panel) following 20hrs of stimulation with the TLR7/8 ligand CL097. Eight tubes of blood were drawn from each of four donors and two tubes of blood per donor were processed at different time points following venipuncture. The times specified on top of the upper panel indicate the time of venipuncture to processing. Figure 1B and 1D show the percent IFN- α (1B) and the percent TNF- α (1D) producing pDCs of all pDCs following 20hrs of stimulation with CL097. The different shades of the columns indicate the time from blood draw to processing (0h= baseline, 6h, 12h and 24h). The indicated p values represent the results of Repeated Measures Analysis of Variances (ANOVAs).

Figure 1C and 1E illustrate the mean percent IFN- α producing pDCs (1C) and the mean percent TNF- α producing pDCs (1E) normalized to the mean percent pDC IFN- α and the mean percent pDC TNF- α production at baseline which was set to 100% (baseline = 0h time point indicating immediate processing after venipuncture) following 20hrs of stimulation with CL097. The different times (0h, 6h, 12h and 24h) specify the time from blood draw to processing.

Figure 1F and 1H represent the Median Fluorescent Intensity (MFI) of IFN- α (1F) and the MFI of TNF- α (1H) after gating on all CD123+ pDCs following 20hrs of stimulation with CL097. P values represent the results of the Repeated Measures ANOVAs.

Figure 1G and 1I show the mean percent of the MFI of pDC IFN- α (1G) and pDC TNF- α (1I) production following 20hrs of stimulation with CL097 of all four donors in relation to the respective mean MFI at baseline which was set to 100%.

Figure 2. delayed processing of blood leads to loss of functional capacity of mDCs

Figure 2A shows representative Flow plots of CD11c+ mDCs producing TNF- α (upper panel) or CD11c+ mDCs producing IL-12 (lower panel) following 20hrs of stimulation with the TLR7/8 ligand CL097. Eight tubes of blood were drawn from each of four donors and two tubes of blood per donor were processed at different time points following venipuncture. The times specified on top of the upper panel indicate the time of venipuncture to processing. Figure 2B and 2D show the percent of TNF- α (2B) or IL-12 (2D) producing mDCs following 20hrs of stimulation with CL097. The different shades of the columns indicate the time from blood draw to processing (0h= baseline, 6h, 12h and 24h). The indicated p values represent the results of Repeated Measures Analysis of Variances (ANOVAs).

Figure 2C and 2E show the mean percent TNF- α producing mDCs (2C) and the mean percent-IL-12 producing mDCs (2E) normalized to the mean percent mDC TNF- α and the mean percent mDC IL-12 production at baseline which was set to 100% (baseline = 0h time point indicating immediate processing after venipuncture) following 20hrs of stimulation with CL097. The different times (0h, 6h, 12h and 24h) specify the time from blood draw to processing.

Figure 2F and 2H represent the Median Fluorescent Intensity (MFI) of TNF- α (2F) and the MFI of IL-12 (2H) after gating on all CD11c+ mDCs following 20hrs of stimulation with CL097. P values represent the results of the Repeated Measures ANOVAs.

Figure 2G and 2I show the mean percent of the MFI of mDC TNF- α (2G) and mDC IL-12 (2I) production following 20hrs of stimulation with CL097 of all four donors in relation to the respective mean MFI at baseline which was set to 100%.

Figure 3. delayed processing of blood leads to loss of functional capacity of monocytes

Figure 3A shows representative flow plots of one donor demonstrating TNF- α production of monocytes after 20hrs of incubation with CL097. Indicated times (0h, 6h, 12h and 24h) specify the time of blood draw to sample processing. Figure 3B shows the percent monocytes producing TNF- α of all monocytes of four different donors of one blood draw after 20h of stimulation with CL097 with different times to sample processing as specified by the shades of the columns. Figure 3C points out the percent monocytes producing TNF- α of all monocytes of indicated time points as compared to immediate processing (baseline, 0h= 100%). Figure 3D shows the

MFI of monocyte TNF- α production (gated on all CD14⁺ monocytes) of one blood draw of four different donors with four different times to processing (0h, 6h 12h and 24h after blood draw as specified by shade of the column) following 20hrs of incubation with CL097. Figure 3E illustrates the mean percent monocyte TNF- α MFI of several time points of whole blood rest since venipuncture as compared to immediate processing (0h time point= 100%). P values specify the results of Repeated Measurements ANOVA tests.