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In vivo Validation of Signaling Pathways Regulating Human Monocyte Chemotaxis

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Abstract

Identification of novel signal transduction pathways regulating monocyte chemotaxis can indicate unique targets for preventive therapies for treatment of chronic inflammatory diseases. To aid in this endeavor we report conditions for optimal transfection of primary human monocytes coupled with a new model system for assessing their chemotactic activity *in vivo*. This method can be used as a tool to identify the relevant signal transduction pathways regulating human monocyte chemotaxis to MCP-1 in the complex *in vivo* environment that were previously identified to regulate chemotaxis *in vitro*. MCP-1-dependent chemotaxis of monocytes is studied in an adoptive transfer model where human monocytes transfected with mutant cDNAs are transferred to mice followed by initiation of peritonitis. Harvesting peritoneal cells at 24 h diminishes the contribution of immunologic responses to the cross-species transfer. Validation of relevant regulatory molecules *in vivo* is critical for understanding the most relevant therapeutic targets for drug development.

Keywords

Monocytes; Transfection; adoptive transfer; chemotaxis; PKC β

1. Introduction

The nontransfectable nature of primary human monocytes has made it difficult to study the signal transduction pathways that are important for regulating monocyte function. Since transfection of primary monocytes with cDNA was not available until recently, we have instead used antisense oligonucleotides as specific inhibitors of mRNA and protein expression to explore monocyte signaling (Li and Cathcart, 1994; Li and Cathcart, 1997; Roy and Cathcart, 1998; Li et al., 1999; Bey and Cathcart, 2000; Carnevale and Cathcart, 2001; Bey and Cathcart, 2002; Zhao et al., 2002; Carnevale and Cathcart, 2003; Bey et al., 2004; Xu et al., 2004; Zhao et al., 2005; Bhattacharjee et al., 2006; Li et al., 2007). We report here the optimization of an efficient method for transfecting primary monocytes that has dramatically expanded the

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conventional approaches for studying potential signaling pathways involved in the pathogenic processes accompanying several inflammatory diseases including atherosclerosis.

Monocyte migration into the intima of an arterial wall is thought to be one of the regulatory steps in atherogenesis. Recruitment of monocytes from the peripheral blood is a multistep process in which locally produced chemokines are believed to play a crucial role. Monocyte chemoattractant protein 1 (MCP-1) is important in attracting monocytes to sites of inflammation and has been shown to be particularly important in atherosclerotic lesions (Wilcox et al., 1994; Boring et al., 1998; Chen and Dennis, 1998; Gu et al., 1998; Aiello et al., 1999; Ross, 1999). We have been studying the pathways that regulate monocyte chemotaxis to MCP-1 because of their pathologic significance and potential for offering new approaches for manipulating chronic inflammation.

Our studies to date have identified several unique signaling pathways that regulate monocyte chemotaxis *in vitro* (Carnevale and Cathcart, 2001; Carnevale and Cathcart, 2003) but it is important to validate the role of these pathways *in vivo* in relevant inflammatory responses. One kinase shown to regulate the *in vitro* chemotactic response of human monocytes to MCP-1 is PKC β (Carnevale and Cathcart, 2003). To confirm the relevant *in vivo* role for PKC β , we optimized transfection conditions in primary human monocytes and then transfected them with wild type and dominant negative PKC β cDNA. We then examined the *in vivo* relevance of PKC β for regulating monocyte chemotaxis using a novel model of inflammation incorporating adoptive transfer of human monocytes into mice. This model is based on the fact that migration of monocytes to the peritoneum in thioglycolate-induced peritonitis is dependent on MCP-1 (Lu et al., 1998). We therefore combined this adoptive transfer model and our optimal transfection protocols to evaluate the effect of a key regulatory molecule on MCP-1-induced chemotaxis of primary human monocytes *in vivo*.

2. Materials and methods

2.1. Reagents

Human MCP-1 from Pharmingen was diluted to 50 μ g/ml with Dulbecco's PBS containing 1mg/ml BSA as a stock solution and was used at 50ng/ml to attract human monocytes. Anti-GFP antibody (monoclonal JL-8) was obtained from Clontech (Mountain View, CA). Antibody to PKC β II (anti-PKC β) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Human Dendritic Cell Nucleofector kit and Human Monocyte Nucleofector kit were both purchased from Amaxa Biosystems (Gaithersburg, MD). GFP-tagged PKC β wild type (GFP-PKC β -WT, pBK-CMV-GFP-PKC β II) and the dominant negative mutant (GFP-PKC β -DN, pBK-CMV-GFP-PKC β II K371R) were kindly provided by Yusuf Hannun (Medical University of South Carolina, Charleston, SC). PKH26 red fluorescent cell linker kits were obtained from Sigma-Aldrich Co (St. Louis, MO). Four to eight week old BALB/CJ female mice were from The Jackson lab, Bar Harbor, Maine. Isoflurane USP was from Abbott Laboratories, North Chicago, IL. Heparin was obtained from Amersham Life Sciences, Arlington Heights, NJ.

2.2. Isolation of Human Monocytes

Human peripheral blood monocytes (PBM) were isolated either by separation of mononuclear cells followed by adherence to BCS-coated flasks as described earlier (Cathcart et al., 1989) or by Ficoll-Hypaque sedimentation followed by countercurrent centrifugal elutriation (Wahl et al., 1984a; Wahl et al., 1984b). PBM purified by these two methods were consistently >95% CD14⁺. These studies complied with all relevant federal guidelines and institutional policies regarding the use of human subjects. All data shown in this manuscript are from studies with elutriated monocytes.

2.3. Immunoblotting

PBM were either nucleofected with pmaxGFP or GFP-PKC β -WT or GFP-PKC β -DN using the method providing optimal transfection efficiency. Whole cell extracts were prepared by previously published protocols (Rosen et al., 1996; Roy et al., 2002). Lysates were resolved by 12% SDS-PAGE, transferred to a PVDF membrane, blocked with 5% BSA in PBS with 0.1% Tween 20 and subjected to immunoblotting with monoclonal anti-GFP antibody (diluted 1:1000 in 3% BSA in PBS with 0.1% Tween 20) overnight. The hybridization signal was detected using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). The immunoblots were then stripped and reprobed with PKC β antibody according to our previously published protocol.

2.4. Transfection of primary human monocytes

Monocytes (5×10^6 in 2 ml 10% BCS/DMEM) were kept in polypropylene tubes in a 37°C incubator with 10% CO₂ for 2 h. The tubes were then centrifuged at 1200 rpm for 10 min. The supernatant was completely aspirated off and the cell pellet was resuspended in 100 μ l (25°C) of either in Human Dendritic Cell Nucleofector™ Solution (DC solution) or in Human Monocyte Nucleofector Solution (MN solution) (Amaxa, Gaithersburg, MD). Either pmaxGFP (provided by Amaxa as a positive control) or highly purified (endonuclease free) plasmid DNA (2 μ g) was mixed with this 100 μ l cell suspension and transferred into an Amaxa-certified cuvette. The cuvette was inserted in the Amaxa nucleofector apparatus and the nucleofection was performed either by using the program U-02 (in DC solution) or Y-01 (in MN solution). After nucleofection the monocytes were carefully transferred to 6-well plates containing either 2 ml of Opti-MEM I medium (OPT) or Human Monocyte Nucleofector medium (MNM). Monocytes were then incubated for another 4 to 48 h depending on the experiment.

2.5. In vitro chemotaxis assay

Migration of monocytes to MCP-1 was quantified using a microchamber chemotaxis assay as described in our previous study (Carnevale and Cathcart, 2001). After chemotaxis for 90 min, cells were fixed in 4% paraformaldehyde (30 min) and mounted in DAPI-containing Vectashield. GFP positive cells were counted in five high-powered fields using a FITC filter whereas untransfected cells were counted using the DAPI filter on a Leica upright microscope (model DMR).

2.6. Fluorescent labeling of monocytes with PKH26

Freshly isolated primary human monocytes were washed with serum-free DMEM and labeled with the fluorescent dye PKH26 per kit instructions.

2.7. Adoptive transfer of monocytes and initiation of peritonitis

Recipient mice (8–10 months, 2–3 per group,) were lightly anesthetized with avertin. Thioglycolate (1 ml of 4% in physiological saline) was injected into the peritoneal cavity to initiate peritoneal inflammation. Tail veins were dilated with limonene and cleaned with 95% ethanol. Primary human monocytes were injected in the tail vein (1.5 million in 0.2 ml of saline per animal). The injected monocytes were equal to approximately 11–14% of total peripheral blood mononuclear cells of similar weight animals. After 24 h, mice were sacrificed and peritoneal cells were harvested, washed three times in saline, resuspended in PBS (1 ml) and counted. Cells were fixed with paraformaldehyde (0.4% on ice for 45 min), washed and used to prepare ten cytopspins for each sample. Five cytopspins were stained with HEMA per the manufacturer's instruction and were used to determine the number of monocytes in the peritoneal lavage based on their morphological characteristics. The remaining five cytopspins were mounted in the DAPI-containing Vectashield and used to count PKH26 positive or GFP

expressing cells on a Leica upright microscope (model DMR) using a Texas red or FITC filter, respectively.

2.8. Statistical analysis

The significance of observations was calculated using unpaired student t-tests.

3. Results

3.1. Nucleofection of primary human monocytes with pmaxGFP

We performed several comparative experiments to optimize nucleofection of primary human monocytes using modifications of the Human Dendritic Cell and Human Monocyte nucleofector kits from Amaxa. We tested their transfection efficiency as well as cytotoxicity. For each nucleofection protocol, we performed two controls to assess the effect of nucleofection on cell viability (total DNA). For the untransfected control, we resuspended the cells in the nucleofector solution but did not subject them to nucleofection. As a control for transfected cells we resuspended the cells in nucleofector solution without DNA and subjected them to nucleofection. As a positive control we transfected monocytes with the pmaxGFP vector which encodes green fluorescence protein (GFP) and was provided in the kits. GFP-expressing cells were analyzed by fluorescence microscopy to monitor transfection efficiency. Monocytes were resuspended and transfected in either Dendritic Cell Nucleofector (DC) or Monocyte Nucleofector (MN) solutions provided in the DC and MN kits respectively. After transfection monocytes were incubated in either Monocyte Nucleofector Medium (MNM, from the Monocyte Kit) or Optimem (OPT).

Primary monocytes isolated by elutriation always showed better transfection efficiency as compared to monocytes isolated by the adherence method. Maximum transfection efficiency was achieved when monocytes were resuspended and transfected in DC solution and kept in MNM (Fig. 1A). Using this method we found ~75% and ~68% nucleofection efficiency in elutriated primary human monocytes after 24 h and 48 h of pmaxGFP expression (Fig. 1A), whereas using the same method we were only able to achieve ~50% (50.6 ± 2.3)% and ~44% (44.7 ± 3.1)% nucleofection efficiency in adherence-isolated monocytes after 24 h and 48 h of GFP expression respectively (data not shown). Our results showed that our optimized method, employing transfection in DC solution followed by incubation in MNM, significantly increased transfection efficiency in elutriated monocytes as compared to monocytes isolated by adherence ($p < 0.0005$ for 24 h and < 0.001 for 48 h). This method is also significantly better than the other methods with respect to GFP transfection efficiency (Fig. 1A). We did not prolong the time of pmaxGFP expression beyond 48 h since monocyte viability began to decline.

3.2. Cytotoxicity of primary monocytes after nucleofection with pmaxGFP

Nucleofection affects cell survival; cell viability is an important issue after successful transfection. Although monocyte survival in each of these four transfection protocols was affected by nucleofection, the extent of cell death was greater when we used the combinations of MN solution/OPT or DC solution/OPT. Using the DC solution/MNM combination, we found that the cell viability was ~90% and ~80% respectively after 24 h and 48 h of GFP transfection. As an indicator of cytotoxicity we measured total DNA concentrations of GFP-transfected monocytes (positive control) as well as the negative controls. Our results showed a decrease of ~10% and ~23% in total DNA concentration, respectively, after 24 and 48 h of GFP transfection using the same protocol which manifested maximum transfection efficiency (Fig. 1B). Our optimized method was also significantly better than the other methods (MN solution/OPT, DC solution/OPT and MN solution/MNM) with respect to cell viability. The total DNA concentration, a reflection of cell number, measured after 24 h of GFP transfection

using the optimized method was significantly higher than MN solution/OPT and DC solution/OPT combinations (Fig. 1B). DNA concentration measured after 48 h of GFP transfection using the optimized method was also significantly higher than MN solution/MNM combination (Fig. 1B). After 48 h of transfection with GFP, MN solution/OPT and DC solution/OPT methods showed significantly lower DNA levels as compared to the other methods (data not shown). Since cell viability was greater with our optimized method and since we found maximal transfection efficiency with this method, we selected this method for future studies. Due to the greater transfection efficiency and lower cytotoxicity at 24 h of GFP transfection, we chose to transfect the monocytes for 24 h prior to measuring chemotaxis or prior to injection into mice.

3.3. Time-dependent expression of GFP after nucleofection

To assess the expression of GFP in monocytes we performed a time-dependent nucleofection experiment. Primary human monocytes were transfected with pmaxGFP using the optimal method (DC solution/MNM combination) and GFP protein expression was measured over time. GFP expression was detectable after 4–8 h, showed a considerable level of expression after 16 h of GFP transfection and after 24 h the expression of GFP reached its maximum level (Fig. 2A, upper panel). The immunoblot was further assessed to ensure equal loading by reprobing with a constitutively expressed protein, PKC β (Fig. 2A, lower panel). After 24 h of GFP transfection, monocytes were also monitored by fluorescence microscopy to assess the expression level of GFP. The maximal number of fluorescent cells was detected after 24 h of GFP expression (Fig. 2B and data not shown). The representative figure shows 65% GFP-positive cells after nucleofection (Fig. 2B).

3.4. Expression of GFP-PKC β -WT and the GFP-PKC β -DN mutant in monocytes to determine the optimal method for transfection

Previous studies from our lab identified PKC β as a critical regulator of human monocyte chemotaxis to MCP-1 *in vitro* using pharmacologic inhibitors and specific antisense oligonucleotides (Carnevale and Cathcart, 2003). To functionally validate our method of transfection, we investigated the role of PKC β in monocyte migration by overexpressing GFP-PKC β -WT as well as the dominant negative mutant, GFP-PKC β -DN plasmid DNA. We measured the expression of PKC β *in vitro* in different groups by using both GFP as well as PKC β antibodies in Westerns. After 24 h of nucleofection although both GFP and GFP-tagged PKC β were detected in the immunoblot, the GFP protein expression level was much higher compared to the GFP tagged PKC β molecule. The same antibody detected no GFP or GFP-tagged PKC β in the negative controls (Fig. 2C, upper panel). To show that the over expressed GFP-PKC β is different from the constitutively expressed endogenous PKC β ; we further stripped and reprobed the same blot with PKC β antibody. Our results showed the presence of over expressed GFP-PKC β only in those groups where the nucleofection was performed with the GFP-PKC β -WT and -DN plasmids, whereas endogenous PKC β was present in all the groups (Fig. 2C, lower panel). Using the optimal method we found ~64% and ~58% transfection with PKC β -WT and -DN mutant respectively compared to ~75% nucleofection efficiency of pmaxGFP expression after 24 h of transfection in elutriated primary human monocytes.

3.5. GFP-PKC β -DN expressing monocytes display reduced chemotaxis to MCP-1 *in vitro*

Monocytes expressing GFP-PKC β -DN showed 92% reduction as compared to either pmaxGFP or GFP-PKC β -WT expressing monocytes (Fig. 3). Interestingly, a significant reduction was also observed in the basal migration of GFP-PKC β -DN expressing monocytes in the absence of MCP-1. These data support our previous observations, using pharmacologic inhibitors and antisense oligonucleotides, that identified an important regulatory role of PKC β in monocyte

chemotaxis to MCP-1 *in vitro* (Carnevale and Cathcart, 2003). They also highlight the success of our transfection procedure and feasibility of using GFP-expressing monocytes for *in vivo* studies. Our results also indicate a general role of PKC β in monocyte migration without a chemotactic stimulus.

3.6. In Vivo model of human monocyte chemotaxis

Despite identification of many signaling molecules that regulate monocyte migration to MCP-1 *in vitro*, our understanding of their *in vivo* significance is limited. *In vitro* assays of chemotaxis are conducted under highly controlled experimental conditions using purified monocytes, whereas in contrast *in vivo* chemotaxis takes place in a heterogeneous and complex environment. Thus it is important to verify that *in vitro* observations are also relevant *in vivo*. Our success in transfecting primary human monocytes prompted us to develop a unique human to mouse adoptive transfer model to investigate MCP-1-dependent chemotaxis *in vivo*. We have validated this model using human monocytes transfected with dominant negative PKC β .

This model system (Fig. 4) is based on the following premises: 1) Monocytes in MCP-1 knockout mice display a profound defect in migration to the peritoneum in response to thioglycolate (Lu et al., 1998). This observation suggests that migration of monocytes in thioglycolate-induced peritonitis is an MCP-1-dependent process that can be exploited as an *in vivo* assay for monocyte chemotaxis to MCP-1. 2) By virtue of being fluorescently labeled, adoptively transferred human monocytes can easily be distinguished from the endogenous unlabeled pool of leukocytes of the recipient animals. 3) Deficiency of a signaling molecule in adoptively transferred monocytes that is required for monocyte chemotaxis to MCP-1 would cause migration of significantly fewer human monocytes to the peritoneum. 4) Since adoptively transferred human monocytes constitute only a small fraction of total mononuclear cells (11–14%), their presence should not significantly affect migration of either endogenous total leukocytes or total monocytes to the peritoneal cavity and 5) Human monocyte migration to the peritoneum is linear over time (Henderson et al., 2003), therefore monocyte migration to the peritoneum was monitored after only 24 h to minimize immunologic interference by the recipient animal.

To test the feasibility of this model, *in vivo* migration of PKH26 labeled primary human monocytes was evaluated in thioglycolate-induced peritonitis. As expected, there was increased migration of total monocytes/macrophages and total leukocytes to the peritoneum in response to thioglycolate (Fig. 5A). Thioglycolate injection also induced significant migration of adoptively transferred monocytes (PKH26 positive) to the peritoneum. These observations demonstrate that adoptively transferred primary human monocytes can respond similarly to endogenous mouse monocytes.

3.7. Validation of the peritonitis model using the GFP-tagged dominant negative mutant of PKC β

We exploited the fluorescence of GFP or GFP-PKC β (WT or DN) expressing monocytes to discriminate and quantify thioglycolate-induced migration of adoptively transferred human monocytes from endogenous mouse cells (Fig. 5B). For this purpose, primary human monocytes expressing GFP, GFP-PKC β -WT or GFP-PKC β -DN were adoptively transferred to the recipient mice and peritonitis was induced. After 24 h cells were harvested from the peritoneum and scored for GFP-positive cells (representing adoptively transferred human monocytes), total monocyte/macrophages and total leukocytes. In response to thioglycolate, 4-fold more GFP or GFP-PKC β -WT expressing monocytes migrated to the peritoneum (Fig. 5B, left panel). In contrast, significantly fewer (54%) monocytes expressing GFP-PKC β -DN responded to the thioglycolate stimulus as compared to either GFP or GFP-PKC β -WT expressing monocytes. Thioglycolate also induced ~4-fold increased migration of total

monocytes/macrophages to the peritoneal cavity and this was not altered by injection of the human monocytes (Fig. 5B, middle panel). A similar pattern was observed in migration of total leukocytes where total leukocyte recruitment was unaltered by injection of human monocytes (Fig. 5B, right panel).

4. Discussion

It is well known that cell lines, particularly human monocytic cell lines, do not always utilize similar signaling pathways as their primary monocyte counterparts (Brinckmann et al., 1996; Xu et al., 2003). It has therefore become necessary to investigate these pathways in freshly isolated primary monocytes even though experimental approaches have historically been somewhat more limited than those available for cell line studies. Using a unique combination of electroporation and cell-type specific solutions, primary cells can also be efficiently transfected by the Amaxa nucleofector technology. Combining available reagents and nucleofector settings, we optimized a transfection protocol for primary human monocytes. This particular method was shown to be significantly better than other methods tested in terms of both transfection efficiency and cell viability. We therefore used this protocol to explore the role of certain signal transduction pathways in regulating human monocyte chemotaxis to MCP-1.

There are several advantages of this transfection protocol as compared to other non-viral transfection methods:

- a. As nucleofected DNA can directly enter the nucleus, high transfection efficiencies can be obtained. In primary human monocytes, transfection efficiencies of over 70% can be achieved.
- b. This method is not dependent on cell division for the transfer of DNA into the nucleus. Expression of the transfected gene can be analyzed within a short period of time. In primary monocytes, expression of GFP can be detected after 4 h of transfection and maximizes after 24 h.
- c. This is a simple and quick method to perform. In human monocytes we can transfer a gene of interest within an hour.

This method has substantially expanded the scope of our studies related to defining requisite signal transduction pathways in primary human monocytes. Earlier we were restricted to the use of pharmacological inhibitors and/or antisense oligonucleotides to manipulate signal transduction candidate activity or expression. We can now successfully nucleofect primary human monocytes with dominant-negative, constitutively active or kinase deficient mutants of a specific gene and then identify the effect of that gene in a specific signaling pathway and downstream biological response.

To examine the feasibility of applying this transfection protocol to understanding the signaling pathways regulating monocyte chemotaxis, we applied our transfection protocol to test the effect of PKC β on monocyte chemotaxis to MCP-1 both *in vitro* and *in vivo*. In a previous publication we demonstrated that PKC β (a cPKC isoform) regulated the MCP-1-stimulated chemotaxis of human monocytes *in vitro* (Carnevale and Cathcart, 2003). To evaluate the role for PKC β in regulating monocyte chemotaxis *in vivo* we first transfected human monocytes with GFP-PKC β -WT and the kinase deficient mutant (GFP-PKC β -DN) and confirmed the role of PKC β in regulating the monocyte chemotaxis to MCP-1 *in vitro*. These data along with the PKC β protein expression data after transfection demonstrated the success of our nucleofection protocol and poised us to proceed with initiating *in vivo* studies with these constructs.

The peritonitis model presented in this study is a new approach to validate *in vivo* roles of signaling molecules that participate in MCP-1-mediated chemotaxis of primary human monocytes. We harvested the peritoneal cells at 24 h to minimize any immune attack on the human monocytes and although, we have used GFP as a reporter molecule to distinguish endogenous cells from adoptively transferred monocytes, labeling cells with PKH26 works equally well. An alternative approach is to use mouse mononuclear cells for adoptive transfer as reported in our latest study (Mishra, R.S., K. Carnevale and M.K. Cathcart, J. Exp Med. *in press*, 2008). Though mouse mononuclear cells may at first glance appear to be a better choice due to the intraspecies nature of the cells, it is difficult to obtain sufficient quantities of mouse monocytes to conduct meaningful studies and the bulk of our preexisting data have been obtained in primary human monocytes and would need to be entirely reexamined for mouse cells. Additionally, we do not have a current protocol for transfecting mouse monocytes or mononuclear cells thus transfection approaches are not feasible at this time *in vivo*.

In summary, we report two new approaches to study the regulation of signal transduction pathways in monocyte chemotaxis. The optimal transfection protocol allows the identification of critical signaling components *in vitro*. We also present a novel model system for utilizing transfected primary human monocytes to assess the relevance of signal transduction pathways in monocyte chemotaxis to MCP-1 *in vivo*. These approaches can be widely applied to identify relevant new targets for regulating monocyte chemotaxis and controlling inflammation.

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Abbreviations used

MCP-1, Monocyte chemoattractant protein 1; OPT, Opti-MEM I medium; MNM, Human monocyte nucleofector medium; BCS, Bovine calf serum.

Figure 1. Transfection efficiency and viability of nucleofected human monocytes using different methods

Primary human monocytes (5×10^6 /group), isolated by elutriation, were untransfected or nucleofected using either DC solution or MN solution in the presence or absence of $2 \mu\text{g}$ of pmax GFP as described in Materials and methods. After nucleofection the cells were kept in either OPT or MNM and the nucleofected cells were incubated up to 48 h. Comparison of % transfection efficiency of primary monocytes (24 and 48 h post nucleofection) using the four different transfection protocols are shown in Figure 1A. As an indicator of cell viability, DNA concentration (Qiagen kit, Valencia, CA) was determined in post-nucleofected (24 and 48 h after GFP transfection) primary human monocytes as well as monocytes suspended with DC or MN solution and transferred to either OPT or MNM with and without nucleofection. DNA concentrations of different groups are presented in Figure 1B. Data are from three independent experiments and shown as the mean $\pm$ SD. Significant differences were determined by comparing each group to the DC solution/MNM group (black bar, * $p < 0.01$, ** $p \leq 0.02$ and *** $p < 0.05$).

Figure 2. Expression of GFP constructs in primary human monocytes

Elutriated primary human monocytes (5×10^6 /group) were untransfected or transfected using DC solution either in absence of any plasmid DNA or in presence of pmaxGFP (Fig. 2A and Fig. 2C), GFP-PKCβ-WT (Fig. 2C) or GFP-PKCβ-DN (Fig. 2C) mutant plasmid (2μg) as described in Materials and methods. After nucleofection cells were kept in MNM either for different time intervals as indicated (Fig. 2A) or for 24 h (Fig. 2C) and then whole cell extracts were prepared. Equal amounts of lysate proteins were resolved by SDS-PAGE for immunoblotting with monoclonal GFP antibody to check the expression of both GFP and GFP-tagged PKCβ. The same blot was then stripped and further reprobed with anti-PKCβ to assess

equal loading. After nucleofection with pmaxGFP vector (2 μ g), primary human monocytes were kept in MNM for 24 h and observed under brightfield and fluorescence (Fig. 2B).

Figure 3. Dominant negative PKC β expressing primary human monocytes display reduced chemotaxis to MCP-1 *in vitro*

Primary human monocytes (5×10^6) were transfected with GFP (black bar), GFP-PKC β -WT (hatched bar) or GFP-PKC β -DN (cross-hatched bar). These cells (1×10^5 cells/well) were allowed to migrate towards MCP-1 (5.75 nM) using a microchamber chemotaxis assay (Carnevale and Cathcart, 2003). Cells were fixed, mounted in DAPI-containing Vectashield and GFP-expressing monocytes were quantified using an upright fluorescence microscope. Results are expressed as the mean number of migrated cells in five high-powered fields (5HPF) $\pm$ SD.

Figure 4. Schematic representation of the peritonitis model

Primary human monocytes were either labeled with PKH26 or transfected with GFP-tagged DNA. Monocytes were injected into the tail vein of recipient mice and peritonitis was induced by thioglycolate injection. Peritoneal cells harvested after 24 h of peritonitis were scored for primary human monocytes (PKH26-labeled or GFP positive cells), total monocyte/macrophages and the total leukocytes.

Figure 5. Validation of human monocyte chemotaxis *in vivo*

(A) Adoptively transferred primary human monocytes respond to MCP-1/thioglycolate *in vivo*. As predicted above, thioglycolate increased the migration of adoptively transferred human monocytes (left panel), total monocytes/ macrophages (middle panel) and total leukocytes (right panel). Data represent basal migration of cells (in the absence of thioglycolate) and thioglycolate-induced migration of cells are the mean $\pm$ data range; (n=2) and mean $\pm$ SD; (n=3) respectively.

(B) GFP-PKCβ-DN expressing monocytes display reduced migration to MCP-1/thioglycolate *in vivo*. Primary monocytes expressing GFP (black bar), GFP-PKCβ-WT (hatched bar) or GFP-PKCβ-DN (cross-hatched bar) were adoptively transferred to recipient mice. Peritonitis was induced and GFP-monocytes, total monocyte/macrophages and total leukocytes were enumerated after 24 h. The open bar represents basal migration of cells in the absence of thioglycolate stimulus. Data are the mean $\pm$ SEM; n=3.