Journal of Immunological Methods 375 (2012) 196–206

Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Systematic validation of specific phenotypic markers for in vitro polarized human macrophages

C.A. Ambarus [a], S. Krausz [a], M. van Eijk [b], J. Hamann [c], T.R.D.J. Radstake [d], K.A. Reedquist [a,c], P.P. Tak [a], D.L.P. Baeten [a,*]

[a] Department of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, The Netherlands
[b] Department of Med ical Biochemistry, Academic Medical Center/University of Amsterdam, The Netherlands
[c] Department of Experimental Immunology, Academic Medical Center/University of Amsterdam, The Netherlands
[d] Department of Rheumatology, Radboud University Nijmegen Medical Center and Nijmegen Institute for Infection, Inflammation and Immunity, The Netherlands

ARTICLE INFO

Article history:
Received 11 July 2011
Received in revised form 17 October 2011
Accepted 18 October 2011
Available online 29 October 2011

Keywords:
Macrophage polarization
Cell surface molecules
Phenotypic markers
Flow cytometry
Inflammation

ABSTRACT

Background: Polarization of macrophages by specific micro-environmental conditions impacts upon their function following subsequent activation. This study aimed to systematically validate robust phenotypic markers for in vitro polarized human macrophages in order to facilitate the study of macrophage subsets in vivo.
Methods: Human peripheral blood monocytes were polarized in vitro with IFN-γ, IL-4, or IL-10. Similar experiments were performed with TNF, IL-13, dexamethasone, M-CSF and GM-CSF as polarizing stimuli. Phenotypic markers were assessed by flow cytometry and qPCR.
Results: IFN-γ polarized macrophages ($M\Phi_{IFN-\gamma}$) specifically enhanced membrane expression of CD80 and CD64, IL-4 polarized macrophages ($M\Phi_{IL-4}$) mainly upregulated CD200R and CD206, and downregulated CD14 levels, and IL-10 polarized macrophages ($M\Phi_{IL-10}$) selectively induced CD163, CD16, and CD32. The expression profiles of the most specific markers were confirmed by qPCR, dose–response experiments, and the use of alternative polarizing factors for each macrophage subset (TNF, IL-13, and dexamethasone, respectively). GM-CSF polarized macrophages ($M\Phi_{GM-CSF}$) upregulated CD80 but not CD64 expression, showing a partial phenotypic similarity with $M\Phi_{IFN-\gamma}$, and also upregulated the expression of the alternative activation marker CD206. M-CSF polarized macrophages ($M\Phi_{M-CSF}$) not only expressed increased levels of CD163 and CD16, resembling $M\Phi_{IL-10,}$ but also displayed high levels of CD64. The phenotype of $M\Phi_{M-CSF}$ could be further modulated by additional polarization with IFN-γ, IL-4, or IL-10, whereas $M\Phi_{GM-CSF}$ showed less phenotypic plasticity.
Conclusion: This study validated CD80 as the most robust phenotypic marker for human $M\Phi_{IFN-\gamma}$, whereas CD200R was upregulated and CD14 was specifically downregulated on $M\Phi_{IL-4}$. CD163 and CD16 were found to be specific markers for $M\Phi_{IL-10}$. The GM-CSF/M-CSF differentiation model showed only a partial phenotypic similarity with the IFN-γ/IL-4/IL-10 induced polarization.

1. Introduction

Macrophages play a key role in the innate immune system and drive tissue inflammation in a wide variety of immune-mediated inflammatory diseases. Originating from circulating monocytes, these cells differentiate upon entry into tissues where they can subsequently be activated by a wide array of microbial and self antigens. A large body of evidence

Abbreviations: APC, antigen presenting cell;ATM, adipose tissue macrophage;ERK, extracellular signal-regulated kinase;FIZZ-1, found in inflammatory zone-1;GAPDH, glyceraldehyde 3-phosphate dehydrogenase;JNK, Jun N-terminal kinase;MAPK, mitogen-activated protein kinase;PD-L2, programmed death ligand-2;TAM, tumor associated macrophage.

* Corresponding author at: Department of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 205662895.
E-mail address: d.l.baeten@amc.uva.nl (D.L.P. Baeten).

0022-1759/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2011.10.013

indicates that the macrophage response is not only determined by the type of activation but also heavily depends on the specific micro-environmental conditions in which cells were differentiated prior to their activation. The prototypical example is activation by TLR ligands such as LPS which, depending on macrophage priming by IFN-γ or immune complexes, leads to either pro- or anti-inflammatory cytokine production (Anderson and Mosser, 2002a,b; Edwards et al., 2006; Nathan, 1991).

IFN-γ was originally described to polarize macrophages towards classically activated cells (M1) which secrete high amounts of TNF, IL-12, IL-1β and low amounts of IL-10 upon subsequent activation and play an important role in fighting intracellular pathogens (Mantovani et al., 2005; Mosser, 2003; Nathan, 1991; O'Shea and Murray, 2008). In contrast, IL-4 induces alternatively activated macrophages (M2), which are characterized by low pro-inflammatory cytokine and high IL-10 production, and are involved in tissue repair, and anti-parasitic and allergic reactions (Gordon, 2003; Gordon and Taylor, 2005; Stein et al., 1992). This polarization model has been further refined as factors such as IL-10, glucocorticoids, TGF-β, and immune complexes were also described to lead to M2 profiles (Anderson and Mosser, 2002a; Bogdan et al., 1991; Goerdt and Orfanos, 1999; Mantovani et al., 2004; Martinez et al., 2008, 2009; Schebesch et al., 1997). Besides the mentioned differences in cytokine production, the concept of polarization has been confirmed by clear differences in chemokine production, NO metabolism, phagocytosis (Gordon and Taylor, 2005; Mantovani et al., 2004; Martinez et al., 2008, 2009; Mosser and Edwards, 2008) and transcriptional profiles (Ghassabeh et al., 2006; Lang et al., 2002; Martinez et al., 2006). Macrophage polarization is also accompanied by specific changes in cell morphology and phenotype (Gordon and Taylor, 2005; Mantovani et al., 2004; Martinez et al., 2008, 2009; Mosser and Edwards, 2008). Already described phenotypical markers are the mannose receptor CD206 and the scavenger receptor CD163, expression of which is enhanced by IL-4 (Chroneos and Shepherd, 1995; Stein et al., 1992) and IL-10, respectively (Högger et al., 1998).

The use of subset-specific phenotypic markers may open a new avenue for in vitro functional studies as well as a more accurate characterization of the macrophage infiltration in a variety of immune-mediated inflammatory diseases. However, recent studies have highlighted the complexity and limitations of this conceptual model. As a classical example, the tumor associated macrophage (TAM) shares pro- and anti-inflammatory properties and was therefore described as a separate subset (Chen et al., 2005; Duluc et al., 2007; Mantovani et al., 2002). Another example is the adipose tissue macrophage (ATM), which includes M1, M2, and a mixed M1/M2 subset, where both phenotype and function depend on the local microenvironment and recruited monocyte subset (Dalmas et al., 2011; Shaul et al., 2010; Wentworth et al., 2010; Zeyda et al., 2007). These observations raise the crucial question of the exact relationship between phenotype and function in macrophage biology. On the other hand, however, the fact that these macrophage types cannot be easily classified according to the polarization model may be due to the relative lack of well-validated and specific phenotypic markers. Firstly, many phenotypic markers, such as the mouse M2 markers FIZZ-1 and YM-1, have been identified in animal models, but are not expressed on human macrophages (Mantovani et al., 2004). Secondly, for many molecules it still needs to be established whether their differential expression at mRNA level truly translates into robust differences in protein expression. Thirdly, other factors have been proposed to steer polarization besides IFN-γ, IL-4, or IL-10. Polarization toward M1 versus M2 was, for example, also described to be induced by in vitro exposure to GM-CSF or M-CSF, respectively (Fleetwood et al., 2007, 2009; Sierra-Filardi et al., 2010; Verreck et al., 2004, 2006). It remains largely unknown whether $M\Phi_{GM\text{-}CSF}$/$M\Phi_{IFN\text{-}\gamma}$ and $M\Phi_{M\text{-}CSF}$/$M\Phi_{IL\text{-}4}$/$M\Phi_{IL\text{-}10}$ are phenotypically similar or rather represent distinct cell subsets. Finally, macrophages do not necessarily undergo genuine lineage commitment as polarization can be reversed both in vitro and in vivo (Biswas et al., 2008; Gratchev et al., 2006; Khallou-Laschet et al., 2010; Porcheray et al., 2005; Stout et al., 2005). Therefore, the present study was designed to systematically validate surface markers for the three main polarized macrophage subsets, $M\Phi_{IFN\text{-}\gamma}$, $M\Phi_{IL\text{-}4}$ and $M\Phi_{IL\text{-}10}$, in humans and to confirm their specificity in different in vitro conditions.

2. Materials and methods

2.1. Monocyte isolation from peripheral blood and in vitro polarization

Monocytes from peripheral blood of healthy volunteers were isolated by gradient centrifugation with Lymphoprep (Axis-Shield PoPAS, Oslo, Norway) and, subsequently, Percoll gradient separation (GE Healthcare, Uppsala, Sweden). Monocytes were cultured at a concentration of 0.5×10^6/ml in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany) in 6 well culture plates (Corning Incorporated, New York, NY, USA) (Van Eijk et al., 2005). Unless indicated otherwise, cells were polarized with human recombinant IFN-γ (50 ng/ml; R&D Systems, Abingdon, UK), IL-4 (40 ng/ml; Miltenyi Biotec, Bergisch Gladbach, Germany), or IL-10 (50 ng/ml; R&D Systems) for 4 days. In confirmatory experiments, the cells were polarized with TNF (50 ng/ml, Biosource, Breda, The Netherlands), IL-13 (20 ng/ml, Peprotech, London, UK), dexamethasone (5 nM, Sigma Aldrich, Zwijndrecht, The Netherlands), M-CSF (50 ng/ml, R&D Systems), or GM-CSF (50 ng/ml, R&D Systems). Finally, in specific experiments, monocytes were first differentiated with GM-CSF or M-CSF for 4 days and subsequently polarized with IFN-γ, IL-4 or IL-10 for another 3 days. Macrophages from different donors were polarized in independent experiments.

2.2. Flow cytometry

Monocyte-derived macrophages were recovered by scraping of the plate. Surface marker expression was analyzed by flow cytometry on day 4 (BD FACS Calibur Flow Cytometer, Erembodegem, Belgium). Purity was assessed by staining with anti-CD14 (clone 61D3, eBioscience, San Diego, CA) and was around 90%. Fluorochrome-labeled