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REVIEW

Role of Monocytes in the Pathogenesis of Dengue

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Abstract
Diseases caused by dengue virus (DENV) are a major public health problem worldwide, considered one of the infections with more prevalence in tropical and subtropical zones of the world. Despite the intense research in the pathogenesis of DENV, this feature is not well understood. One of the main target cells for DENV infection is monocytes; these phagocytes can play a dual role, since they are essential to control viremia, but they also participate in the induction of tissue damage during DENV infection. Monocytes produce different pro-inflammatory cytokines and chemokines in response to infection, and also mediate endothelial damage. In peripheral blood, monocytes can be divided into three different subpopulations, namely classical, intermediate and non-classical, which differ in frequency, cytokine production, among others. Studies in the last years suggest that non-classical monocytes have higher affinity for microvasculature endothelium compared to other type of monocytes, which implies that they could be more involved in the increase of endothelial permeability observed during DENV infection. This review provides a general view of the role of monocytes and their subpopulations in DENV pathogenesis and its effect in viral replication. Finally, the potential contribution of these phagocytes in the alterations of endothelial permeability is discussed.

Keywords Dengue virus · Monocytes · Monocyte subpopulations · Endothelial permeability · Dengue virus pathogenesis

Introduction

Dengue disease is a viral infection caused by dengue (DENV) from which four distinct antigenically related serotypes (DENV-1, -2, -3, -4) have been described (Halstead 2007; Sun and Kochel 2013). The virus is transmitted through the bite of mosquitoes mainly of the species *Aedes aegypti*, a vector that is highly domesticated and prefers urban habitats (Whitehead et al. 2007). The World Health Organization (WHO) reports that DENV epidemics result in 50–100 million infections per year worldwide.

Dengue is a dynamic disease, with different clinical presentations and unpredictable clinical evolution. Infection with any of the four serotypes can lead to the development of disease in infected patients, and although almost in 60% of the cases the clinical outcomes are asymptomatic (Bhatt et al. 2013), the apparition of symptomatic dengue cases depends mainly of age and the experience of a second heterotypic dengue infection; this is a topic that has been extensively reviewed elsewhere by Dr. Halstead (Halstead 2008). In an attempt to improve the clinical follow-up and to decrease the morbidity and mortality of DENV-infected patients, in 2009 the WHO revised the guidelines for diagnosis and treatment of dengue, classifying the disease in two levels of severity: dengue (sub-classified in dengue with or without warning signs) and severe dengue (World Health Organization 2009). In 90% of the cases, the clinical outcome is dengue without warning signs, which is described as a mild self-limited flu-like infection, characterized by an abrupt onset of fever, accompanied by vomiting, abdominal pain, flushing, skin erythema, myalgia, arthralgia, headache and leukopenia. If patients develop any of the warning signs, such as mucosal

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bleed, liver enlargement and increase in hematocrit along with a rapid decrease in platelet count, the disease is classified as dengue with warning signs. If severe plasma leakage (evidenced by shock or fluid accumulation with respiratory distress), severe bleeding and/or severe organ involvement (such as hepatitis, encephalitis or myocarditis) is developed, the disease is classified as severe dengue. This clinical scenario affects the study of virological or immunological factors associated with severe dengue, as these types of patients will receive hospital-based management, therefore they could only be included in the studies on the late phase of the disease, whereas patients who do not develop severe outcomes would be enrolled in the early phase of the disease (before the fifth day of the onset of fever).

Of the 50 million cases of dengue reported annually, it is estimated that almost 500,000 correspond to severe dengue, of which 25,000 individuals die because of the infection with this virus. DENV is endemic in approximately 80 countries in the world and more than 2500 million people are at risk of getting infected with the virus (Simmons et al. 2012; Whitehorn et al. 2014). Although a large proportion of children is affected by uncomplicated dengue (Anderson et al. 2007), they are at higher risk of severe dengue, and particularly shock (Guzman et al. 2002), since they have lower ability to compensate the capillary leakage in comparison with adults. In addition, the neurologic symptoms that they usually develop (such as lethargy or irritability), can be easily confounded with other clinical entities (Méndez and González 2006). Indeed, the main cause of death in DENV-infected children is hypovolemic shock.

Different hypotheses have been proposed to explain the mechanism associated with the severe forms caused by DENV infection, among these, epidemiological and experimental evidence support the association between the severity of dengue illness and a previous dengue infection which confers the preexistence of enhancing antibodies (Halstead 2008). Also, the over-production of pro-inflammatory cytokines elicited by the activation of mononuclear phagocytes (monocytes, dendritic cells and macrophages), and cross-reactive B cells and T cells in response to DENV infection, seems to play a key role in the pathogenesis of infection, leading to an uncontrolled inflammatory immune response and the perturbation of vascular barrier integrity (Malavige and Ogg 2017; Pang et al. 2006; Srikiatkhachorn et al. 2017). Macrophages have been considered important targets of dengue infection, since DENV RNA and proteins have been detected in macrophage-type cells of several tissues including spleen, lymph nodes and liver (Aye et al. 2014; Balsitis et al. 2009; Jessie et al. 2004). In addition, it has been extensively described that monocytes are also a main target of DENV infection (Bhatt et al. 2013; Halstead et al. 1977; Simmons et al. 2012). In fact, in a human autopsy study, infected monocytes have been detected in lumen of blood vessels from lung and liver tissues (Jessie et al. 2004). Mononuclear phagocytes are important in the control of the infection and in the development of protective immune responses against this virus, since they are major producers of several cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, among others], antimicrobial peptides (lysozyme, cationic peptides) and chemokines (CCL5, CCL4, CCL3) (Whitehorn et al. 2014). Nevertheless, these phagocytes are FcR-bearing cells and seem to favor DENV replication, as well as tissue and organ damages, therefore considering them as key component of the immunopathogenesis of the disease. In fact, it has been reported that the viremia is the result of the massive replication of DENV in mononuclear phagocytes in draining lymph nodes; the newly produced viral particles then travel to the bloodstream where they infect mostly monocytes (Martina et al. 2009).

This review provides a general perspective of the role of monocytes and their subpopulations in DENV pathogenesis, as well as the effect of their response in viral replication. Finally, it discusses the contribution of these phagocytes in the alterations of endothelial permeability that is usually observed in severe dengue.

Immunopathology of DENV infection

The study of the factors underlying the immunopathology of severe DENV infections has been hampered by the lack of a proper animal model of the disease, in which the tropism of the virus can be precisely observed. Nevertheless, based on in vitro, ex vivo and post-mortem studies, it is believed that the virus infects or interacts with several cell populations according to the scenario in which viral replication is taking place (Dalrymple and Mackow 2012; Martina et al. 2009) (Fig. 1): (1) cells of the immune system present where the mosquito bite has occurred (monocytes, dendritic cells and macrophages), (2) other cells in the regional lymph nodes and in peripheral blood such as monocytes, lymphocytes and dendritic cells, (3) liver cells, and (4) tissue macrophages (Aye et al. 2014; Balsitis et al. 2009; Dalrymple and Mackow 2012).

The first step in DENV infection is the inoculation of the virus into the dermis and epidermis of the host through the bite of DENV-infected *Aedes* mosquitoes. The first cells infected with the virus are Langerhans cells and other type of cells such as dendritic cells located in the initial site of infection (Navarro-Sánchez et al. 2005), mainly through interaction with various receptors described to date, including DC-SIGN receptor (dendritic cell-specific ICAM-grabbing non-integrin) (Cruz-Oliveira et al. 2015). DENV-infected dendritic cells migrate to regional lymph nodes as a process of their differentiation, favoring the spread and infection of

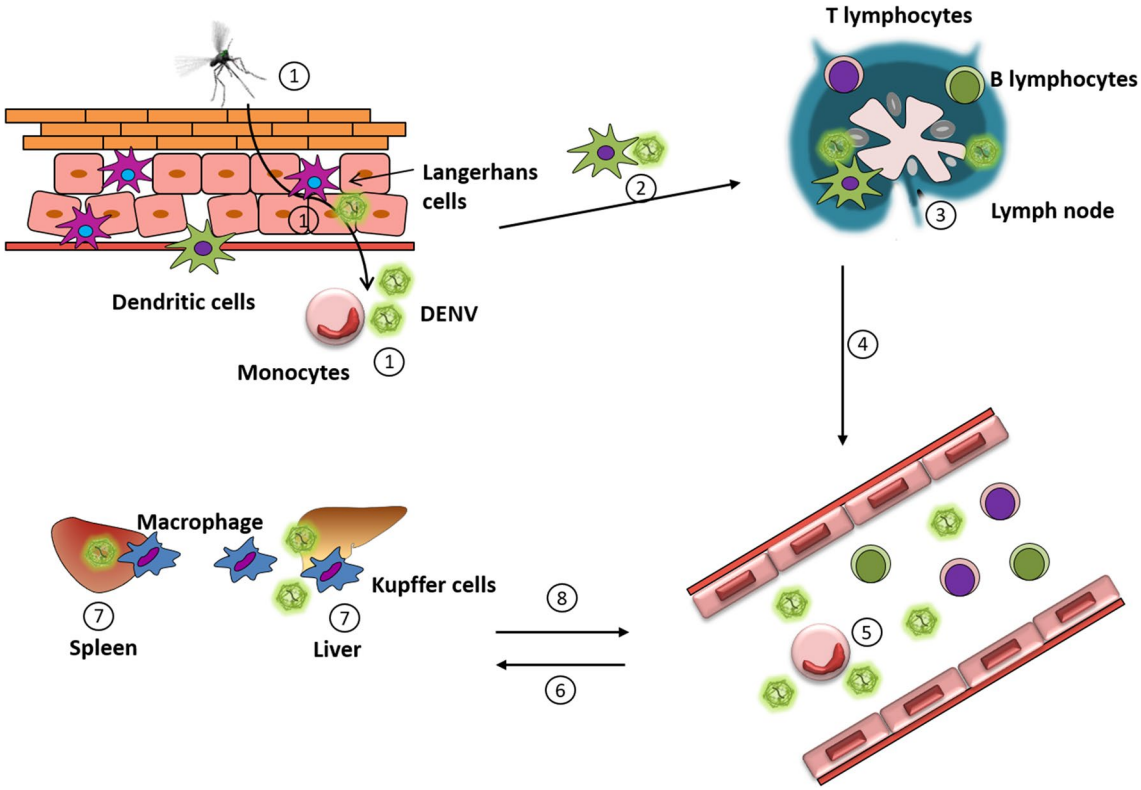

Fig. 1 Scenarios of monocyte infection during DENV natural infection. The virus is transmitted to humans by the bite of *Aedes* mosquitoes (1). Cells that are located intraepithelially and in blood vessels near the site of the bite, such as Langerhans cells, dendritic cells and monocytes, are the first targets of infection. Infected dendritic cells carry the virus to lymph nodes during its differentiation process (2), where the virus can undergo another replication cycle infecting new target cells (3). After this second round of replication the virus is released to the blood stream (4) where it can again be in close contact with monocytes that become infected (5). Due to viremia, DENV reaches other organs such as the liver and spleen (6) where other cells such as hepatocytes and macrophages can become infected (7). The viral particles produced in these last cells can return to blood stream (8) where they can infect more monocytes (8). The events depicted in this figure can be reviewed in detail elsewhere (Dalrymple and Mackow 2012; Jessie et al. 2004; Martina et al. 2009)

other cell populations such as monocytes and macrophages, enhancing viral replication and facilitating the dissemination of the virus throughout the host lymphatic system (Jessie et al. 2004). Subsequently, due to viral replication in the lymph nodes, the viral particles reach the peripheral blood, where mononuclear cells such as monocytes and myeloid dendritic cells become infected with the virus, thereby facilitating the way DENV can reach target cells of other tissues (spleen, liver, and bone marrow); in these sites, the virus can replicate in tissue macrophages but also in hepatocytes and megakaryocytes. Finally, due to new replication cycles that occur in these new target cells, the virus reaches again the peripheral blood spreading the infection even further (Clark et al. 2012; Martina et al. 2009).

It is still unclear why some people develop severe dengue, but it has been proposed that the immunological events elicited during DENV infection could be responsible for the development of severe manifestations. Among these events, the cross-reactivity of the humoral and cellular adaptive immune response against different DENV serotypes, but also the phenomenon known as cytokine storm (elevated levels of cytokines), have been associated with the compromise of endothelial integrity (Pang et al. 2006; Srikiatkhachorn et al. 2017). More recently, it has also been proposed that elements of the virus such as the NS1 protein could be inducing the severe manifestations (Beatty et al. 2015; Modhiran et al. 2015); some of these mechanisms are briefly explained as follows.

Role of cytokines in DENV pathogenesis

One of the best described immunological events occurring in DENV infection that has been classically observed in DENV-infected patients is the exacerbated cytokine production, explained in part by the uncontrolled activation of different innate immune cell populations such as dendritic cells, NK cells, macrophages, monocytes and T cells (Beltrán and López-Vergès 2014; Chen and Wang 2002; Pang et al. 2006). A great variety of studies performed in different countries including Vietnam (Nguyen et al. 2005), India

(Chakravarti and Kumaria 2006), Singapore (Kumar et al. 2012), Brazil (Bozza et al. 2008), Cuba (Pérez et al. 2004), Colombia (Restrepo et al. 2008), and among others, have described high levels of cytokines and chemokines in the serum of patients infected with DENV; these include IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, transforming growth factor β, interferon (IFN)-γ, macrophage inflammatory protein (MIP)-1β, and monocyte chemoattractant protein (MCP)-1, highlighting the pivotal role of cytokine production in DENV pathogenesis. In some studies, a positive correlation has been observed between the severity of the disease (severe dengue and dengue with alarm signs) and the serum levels of IL-6 and TNF-α (Arias et al. 2014) and MIP-1β (Bozza et al. 2008). Also, some experimental studies suggest that this phenomenon is related to an increase in endothelial permeability due to damage of the endothelial cell-to-cell junctions. Despite the high impact of this response in the endothelial tissue, it is considered that such response is essential for the control of viral replication and spread during the first days of infection, but that at latter stages it may be contributing significantly to the functional alterations in endothelial cells observed in severe cases, as it will be discussed further.

Antibody-dependent enhancement in DENV pathogenesis

It has also been observed that during a second infection there is a higher risk of developing severe dengue. This is in part due to the phenomenon described as antibody-dependent enhancement (ADE), in which it was demonstrated that antibodies generated in a first infection by a given virus serotype are non-neutralizing against the other serotypes. When a secondary infection occurs with a different serotype (heterotypic infection), these non-neutralizing antibodies bind to the infectious one and mediate its entry and infection of FcγR-bearing cells, such as monocytes and macrophages, resulting in an increase in the proportion of infected cells (Halstead and O'Rourke 1977; Halstead et al. 2010), as well as an increased level of some cytokines such as TNF-α, IL-6, IL-1β, IL-10, and among others (Puerta-Guardo et al. 2013; Tsai et al. 2014). This phenomenon has been validated in primary DENV infections of infants with sub-neutralizing antibodies acquired of dengue-immune mothers, which was associated with development of severe disease (Chau et al. 2009; Halstead 2008; Simmons et al. 2007). In addition, it has been reproduced in mouse models of ADE (Martínez Gómez et al. 2016; Ng et al. 2014).

The infection with DENV-4 by way of the ADE mechanism leads to a significant increase of viral replication and viral load in an in vivo model of Rhesus macaques that has been additionally related to the severity of infection (Goncalvez et al. 2007; Halstead 1979). This phenomenon has also been observed using in vitro models, with both serum from DENV-infected patients and with monoclonal antibodies specific for E protein epitopes (mAB IgG 1A5) used at sub-neutralizing concentrations, which induces an increase in the number of plaque forming units of approximately 10- to 10,000-fold in the K562 cell line (Goncalvez et al. 2007). In AG129 mice, where a lethal ADE-triggered infection is achieved, the role of FcγR mediating this process has been demonstrated, since the use of a modified antibody that lacks the Fc region reverted the lethal effect of the non-modified antibody (Balsitis et al. 2010).

Furthermore, ADE infection has also been observed with immature DENV particles, which lacks a proper mature form of M protein in the membrane. Maturation of prM to the mature form M takes place in the Golgi apparatus due to the action of the cellular protease furin that induces conformational changes in the structural E protein (Mukhopadhyay et al. 2005). Given the high number of immature particles produced during the DENV replication cycle, the presence of prM antibodies with a sub-neutralizing potential could be mediating the binding and entry of immature DENV particles to FcγR-bearing cells, making them fully infectious particles; this hypothesis has been proved so far in K562 cells through interaction with FcγR-II (Rodenhuis-Zybert et al. 2010), in U937 promonocytes (Dejnirattisai et al. 2010) and in P388D1 mouse macrophages (da Silva Voorham et al. 2012). These evidences highlight the role of antibodies in the process of facilitating the entry and infection of DENV in target cells. As it was mentioned before, ADE is the main mechanism that explains the epidemiological observations of severe disease development after a second heterotypic dengue infection, or by a primary dengue infection in babies born of dengue-immune mothers. Furthermore, ADE-facilitating antibodies can play an indirect role in the increase of endothelial permeability, inducing the production of high levels of inflammatory cytokine as described in some studies (Nguyen et al. 2004; Puerta-Guardo et al. 2013).

Cross-reactivity with self-antigens

Another important aspect of DENV pathogenesis is the production of anti-NS1 and anti-E antibodies that cross-react with self-antigens (Wan et al. 2013). High levels of IgM antibodies have been observed in DENV-infected patients that react with platelets of healthy individuals and could induce elimination of such platelets, which can also explain the thrombocytopenia usually observed in DENV-infected patients (Lin et al. 2001). The presence of these antibodies has been associated with the severity of the disease in patients that have secondary infections (Saito et al. 2004). Other studies have also demonstrated the presence of autoantibodies, showing that these types of antibodies in DENV-infected patients serum react against mouse (Falconar 1997)

and human endothelial cells (Lin et al. 2003). Antibodies of the IgG and IgM isotypes that can be found in DENV-infected patients recognize antigens in human umbilical vein endothelial cells (HUVECs), and a greater proportion of such antibodies is found in serum from patients with severe dengue (Lin et al. 2003). The binding of these antibodies with both platelets and endothelial cells leads to apoptosis of these cells and to the activation of macrophages as evaluated through nitric oxide (NO) production (Wan et al. 2013). For these reasons, it is believed that autoantibodies can considerably contribute to the severity of the disease.

Cross-reactivity of T cell responses

Besides the role of antibodies in the development of severe dengue, other adaptive immune components, such as T cells, have also been implicated in the pathogenesis. Without ignoring the role of the polyfunctional dengue-specific T cell response in the protection against DENV infection (Weiskopf et al. 2013), it has been noted in some patients with secondary dengue infections, that when memory $CD8^+$ T lymphocyte clones specific of previous infecting serotypes are activated, they have a low affinity for the new infecting serotype. These lymphocytes lead to a weak cytotoxic response against infected cells, but instead produce high levels of pro-inflammatory cytokines including TNF-α and IL-6 (Mongkolsapaya et al. 2003), which could be contributing to the high production of cytokines observed in severe dengue infections.

DENV NS1 contribution

Finally, in addition to the contribution of NS1 in dengue pathogenesis through autoimmune events (Amorim et al. 2014), there is several evidence that support its direct role in endothelial alteration. It has been observed that the direct contact of DENV NS1 with endothelial cells leads to increase vascular permeability via Toll-like receptor 4 (TLR4) (Beatty et al. 2015; Modhiran et al. 2015) or endothelial glycocalyx layer disruption (Puerta-Guardo et al. 2016). In addition, NS1 protein can activate immune cells, such as monocytes/macrophages, via TLR4 (Beatty et al. 2015; Modhiran et al. 2015) or TLR2/TLR6 activation (Chen et al. 2015), favoring cytokine production of IL-6, TNF-α and IL-8, which in turn could also contribute significantly to the overall increase in endothelial permeability. Although there is some controversial data about the association of DENV NS1 levels with severe disease (Adikari et al. 2016; Duong et al. 2011; Libraty et al. 2002), it is possible that NS1 mechanisms exposed so far could also explain severe dengue manifestations during both primary and secondary infection.

Monocytes and their role in DENV infection

Human monocytes can be divided into three different subpopulations according to the expression of the membrane receptor for lipopolysaccharide CD14, and the isoform FcγRIII-A of the low affinity receptor for the Fc region of IgG antibodies (CD16) (Wong et al. 2012b; Ziegler-Heitbrock 2000). Taking into account the expression of these receptors, monocytes can be divided into $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate), and $CD14^+CD16^{++}$ (non-classical) monocytes (Wong et al. 2012b; Ziegler-Heitbrock 2000). In healthy individuals, $CD14^{++}CD16^-$ (classical) monocyte subpopulations have more frequency (80–90%) compared to $CD14^+CD16^+$ (non-classical) (10–15%) and $CD14^{++}CD16^+$ (intermediate) (1–5%) monocytes. $CD14^{++}CD16^-$ and $CD14^{++}CD16^+$ monocytes are characterized by the expression of high levels of the chemokine receptors CCR2 and CCR5 and low levels of CX3CR1. On the other hand, non-classical monocytes have a high expression of CX3CR1 but low expression of CCR2 and CCR5 (Ancuta et al. 2003; Geissmann et al. 2003; Wong et al. 2011). Other important characteristics and alterations described in these monocyte subpopulations can be further reviewed in Saha and Geissmann (2011) and Ziegler-Heitbrock and Hofer (2013).

In several studies, which included in vitro approaches, but also during acute dengue disease, monocytes were shown to be one of the main target cells for DENV infection and replication among peripheral blood mononuclear cells (PBMC) (Chen et al. 1999; Durbin et al. 2008; Halstead et al. 1977; Halstead and O'Rourke 1977; Kou et al. 2008; Miller et al. 2008; Neves-Souza et al. 2005). Infection of PBMCs from healthy individuals with DENV-2 using a multiplicity of infection of five resulted in 4% $CD14^+$ cells positive for E antigen, as determined by flow cytometry, whereas only 0.02% and 0.4% of $CD3^+$ and $CD19^+$ cells, respectively, were positive for the same antigen (Kou et al. 2008). In DENV-infected patients in the febrile period, monocytes ($CD14^+$ cells) constitute 49% of cells positive for the viral antigens E and NS1 among all peripheral blood cells, whereas lymphocytes only represent 2% of infected cells (Neves-Souza et al. 2005). The difference in the frequency of infected monocytes between the ex vivo (more than 40%) and in vitro (not more than 10%) results may be partly explained by the fact that in the context of natural infection monocytes follow different scenarios where they can interact with the virus, such as at the initial site of infection and in the bloodstream during viremia (Fig. 1). However, it is important to point out that DENV infection enhancement in monocytes could attributed to their FcγR expression

and the presence of non-neutralizing antibodies. In fact, DENV is growth better in cultures of monocytes obtained from dengue-immune individuals (Halstead et al. 1976). Significant increase in monocyte infection can be achieved using antibodies through ADE-triggered infection, enhancing monocyte infection up to 20–30% (Sun et al. 2011). It is possible that this phenomenon is related to the quick increase of serum antibodies that has been observed in DENV-infected patients (Balakrishnan et al. 2011; Garcia-Bates et al. 2013), which could be promoting the infection of monocytes via FcR, even in primary infections, and in higher proportions in secondary infections. Furthermore, this hypothesis is more plausible due to the fact that human $CD14^{++}CD16^{+}$ monocytes infected in vitro with DENV-2 promote rapid differentiation of B lymphocytes into plasmablasts, compared to non-infected monocytes (Kwissa et al. 2014). The frequency of infected monocyte could also depend on the characteristics of DENV strains (Diamond et al. 2000), which could also explain the differences observed using in vivo and in vitro approaches. In the next sections, we attempt to better explain the effect of DENV infection in monocytes, as well as the potential role of these cells in the immunopathogenesis of dengue disease.

DENV-induced monocyte activation

Monocytes express a high variety of pattern recognition receptors (PRRs) including TLRs, RIG-I-like receptors, NOD-like receptors, DNA sensors, scavenger receptors, and also other receptors related to the activation of the inflammasome (Lech et al. 2010; Schneberger et al. 2011). These receptors are believed to allow monocytes to recognize different pathogen-associated molecular patterns characteristic of DENV (Nasirudeen et al. 2011; Qin et al. 2011) (Fig. 2). For instance, it has been observed that mouse macrophages deficient in TLR3 expression are more susceptible to in vitro infection by DENV-1, leading to higher levels of NS3 protein evaluated by immunoblot, compared to wild-type mouse macrophages (Nasirudeen et al. 2011). Additionally, monocyte activation can be observed during dengue infection through FcR-dependent pathway (Sun et al. 2011).

The activated phenotype of monocytes during dengue infection has been reported in different studies. $CD14^{+}$ monocytes from febrile DENV-infected patients express ex vivo higher levels of intercellular adhesion molecule (ICAM)-1, TLR2 and TLR4, and lower levels of HLA-DR, compared to monocytes from healthy controls; further, in vitro infection of these cells with DENV-2 leads to a significant increase in the expression of TLR2 and TLR4 (Azeredo et al. 2010). Also, monocytes from DENV-infected patients with severe dengue infection exhibit a higher expression of CD32, CD86 and CD11c molecules compared to patients with mild forms of the disease, being the main cells positive for the prM and NS3 antigens among PBMCs (Durbin et al. 2008). In addition, in an in vitro model, infection enhancement of monocytes through ADE mechanism results in an increase of CD86 and CD40 expression as well as TNF-α production (Sun et al. 2011). Another important evidence supporting the observations concerning monocyte activation upon DENV infection, is an up-regulation of the inducible NO synthase enzyme, observed in DENV-infected patients and also in in vitro DENV-1-infected monocytes from healthy individuals (Neves-Souza et al. 2005). Additionally, it has been observed that dengue NS1 protein can also activate mouse macrophages and PBMC, especially monocytes, via TLR4 receptor, inducing the expression of TNF-α, IL-6 and IL-1β (Modhiran et al. 2015). Considering that NS1 antigen levels can persist also beyond day 5 of illness (Devignot et al. 2010), this could also represent a mechanism of monocyte activation even when viral replication has been controlled. Finally, in ADE infection of both monocyte type cell line (THP-1 cells) and human peripheral blood monocytes, Fcγ-receptor signaling also favors the high production of IL-10 (Chareonsirisuthigul et al. 2007; Tsai et al. 2014; Ubol et al. 2010) through a mechanism known now as "intrinsic ADE" (Halstead et al. 2010); this mechanism involves the down-regulation of different pro-inflammatory mediators, included type-I IFN, thus favoring an increase in viral production.

These findings suggest that DENV infection can activate monocytes, possibly explaining the high production of pro-inflammatory and immunomodulatory molecules associated with vascular leakage disorders observed in patients with severe dengue infections, as it will be described further in the next section.

Production of cytokines

In several in vitro studies, monocytes have been used as a model to describe the production of cytokines in response to DENV infection. In vitro, DENV-2-infected human monocytes obtained from healthy individuals produce high amounts of TNF-α (Espina et al. 2003), IL-10 (Torrentes-Carvalho et al. 2009) and IL-8 (Bosch et al. 2002), compared to unstimulated monocytes. In this case, TNF-α production was dependent on viral replication, since there was a diminished production of this cytokine when the infection was performed with heat-inactivated viruses (Espina et al. 2003). Monocytes infected with DENV-2 for 48 h secrete high levels of several cytokines and chemokines such as MCP-1, interferon γ-induced protein (IP)-10, IL-6, IL-8, IL-10 and IL-1β, similar to that observed with stimulation using the TLR7/TLR8 agonist R848 (Kwissa et al. 2014). In addition, it has been described that monocyte-derived

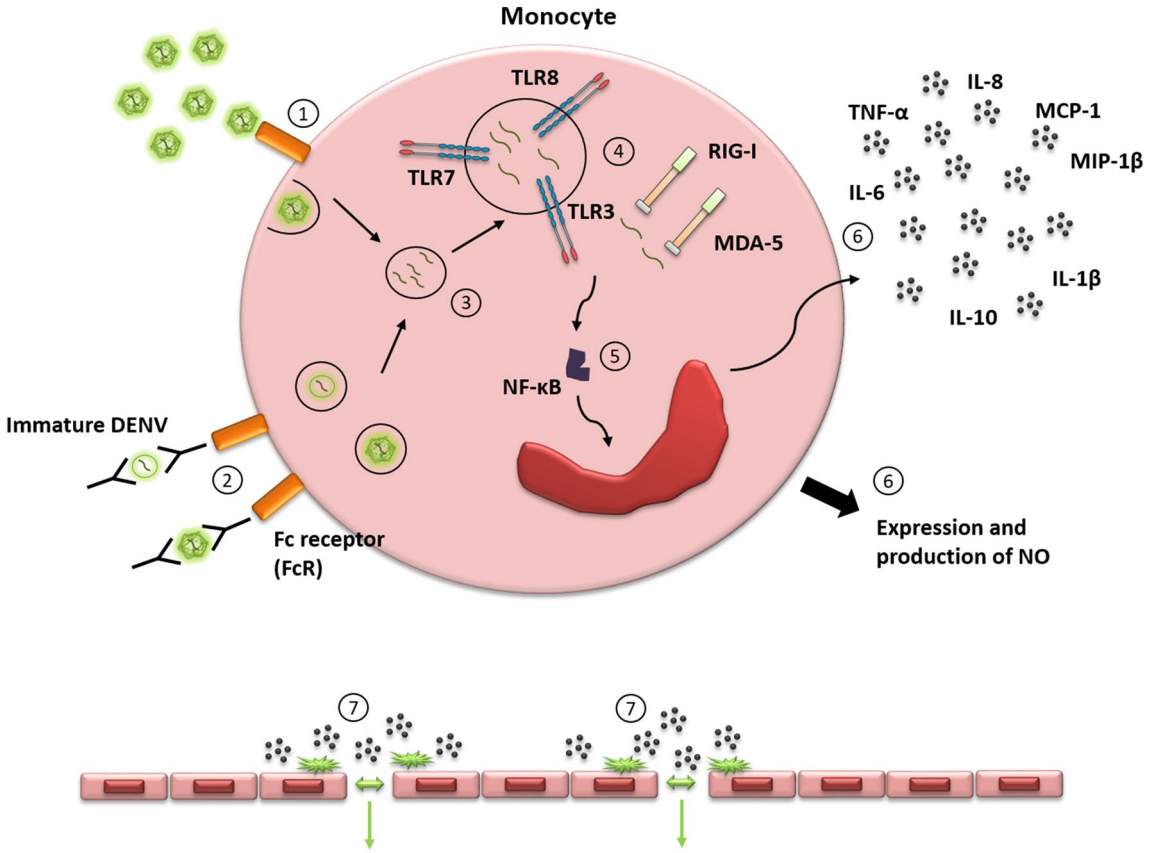

Fig. 2 Role of monocytes in the endothelial alterations. Monocytes are one of the main targets for DENV infection. Viral entry into the cell occurs mainly through mannose receptors (1) (Reyes-del Valle et al. 2014). The virus can also infect monocytes using Fc receptors (FcRs) by the antibody-dependent enhancement (ADE) mechanism (2) (Sun et al. 2011). The viral particle is endocytosed and after the change of pH within the endosome, viral RNA is released from the capsid into the cytoplasm where viral proteins are transcribed (3) (Urcuqui-Inchima et al. 2010). Products of viral replication such as single-stranded and double-stranded RNA are recognized by TLR3, TLR7, TLR8, RIG-I and MDA5 (4) (Nasirudeen et al. 2011; Qin et al. 2011), but also NS1 is recognized by TLR4, leading to activation of the transcription factor NF-κB (5). In this way, the expression and production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, among others, is increased (6) (Espina et al. 2003; Kwissa et al. 2014). It has also been reported that monocyte activation can increase the expression and production of nitric oxide (NO) (Neves-Souza et al. 2005). Finally, some pro-inflammatory mediators such as TNF-α, IL-1β and NS1 can directly increase the endothelial permeability (7) (Burke-Gaffney and Keenan 1993; Dewi et al. 2004; Modhiran et al. 2015)

macrophages produce high levels of IL-1β, IL-8, IL-12, TNF-α and MIP-1β in response to in vitro DENV-2 infection for 44 h, compared to uninfected cells (Chen and Wang 2002). Finally, high concentrations of NS1 can activate monocytes and trigger the in vitro production of IL-10 at 24 h of culture (Adikari et al. 2016), and also induce the up-regulation of *TNF-α* and *IL-6* expression in mouse macrophages and human monocytes after 3 h of stimulation (Modhiran et al. 2015).

Monocyte cell death

DENV-induced monocyte activation can also lead to programmed cell death. At what extend this cell death is triggered by monocytes as a regulatory process or instead is triggered by the virus for beneficial purposes, should be further studied. However, it has been observed that DENV infection in monocytes can induce an apoptotic-type of cell death related with an increase in TNF-α production (Espina et al. 2003; Torrentes-Carvalho et al. 2009). Further, in a model of ADE-DENV infection in human monocytic cell line U937, apoptosis induction was observed in response to the four serotypes; a mechanism dependent on the activation of caspases 7, 8 and 9 (Klomporn et al. 2011). On the other hand, DENV can also trigger another type of programmed cell death associated with an activation of caspase-1 and release of IL-1β in monocytes, known as pyroptosis (Tan and Chu 2013).

Further studies are needed to explore the molecular pathways of programmed cell death in monocytes induced by DENV infection, and its role in pathogenesis. We hypothesize that monocyte death may be explaining the decline of

non-classical monocytes observed during DENV infection, as well as the greater release of proinflammatory components, damage-associated molecular patterns, and viral particles that could contribute with the inflammatory response.

Monocyte subpopulation alterations in DENV infection

Despite the evidence regarding monocyte infection by DENV, little is known about monocyte subpopulation behavior in DENV infection and its role in the development of severe dengue. It has been reported that non-classical monocytes are a very important source of TNF-α. Furthermore, these same types of monocytes have a greater affinity for microvasculature compared to classical monocytes, which instead have more affinity for macrovasculature (Collison et al. 2015). An increase in the percentage of the $CD14^{++}CD16^{+}$ monocyte subpopulation has been reported in DENV infected patients with mild and severe forms of the disease, along with a decrease in the percentage of $CD14^{+}CD16^{++}$ subpopulation compared to healthy individuals (Kwissa et al. 2014). Further, CD16 molecule is up-regulated in monocytes from infected patients compared to healthy controls, as well as in those monocytes infected in vitro with DENV-2 (Azeredo et al. 2010). Currently, these findings observed in DENV infected patients have been corroborated by us in on-going studies (Fig. 3).

It has also been reported that both $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{++}$ monocyte subpopulations are equally susceptible to DENV-2 infection in vitro, and that they both produce similar levels of anti-viral factors such as IFN-α, CXCL10 and TNF-related apoptosis-inducing ligand. However, $CD14^{+}CD16^{++}$ monocyte subpopulations produced higher levels of TNF-α, IL-1β, IL-6, CCL2 and CCL4 in response to infection (Wong et al. 2012a). It was suggested that possibly the non-classical monocyte subpopulation mediates a protective role through its recruitment to infected sites. Nevertheless, since TNF-α and IL-1β are related to an increase in endothelial permeability (Burke-Gaffney and Keenan 1993; Dewi et al. 2004), this subpopulation could be mediating an endothelial alteration in patients with severe DENV infection. Finally, co-culture assays between intermediate monocytes infected with DENV-2 and autologous B lymphocytes have shown that this interaction induces a significant increase in the differentiation of B cells to IgM producer plasmablasts, which was dependent of B cell activating factor, a proliferation-inducing ligand (APRIL), and IL-10 (Kwissa et al. 2014). These results suggest that DENV infection modulates the number of monocytes in blood, leading to an increase of the intermediate subpopulation, which seems to be recruited to draining lymph nodes where they modulate the response and differentiation of B cells. This interaction could lead to a quick increase in the production of low affinity antibodies against the virus that can be

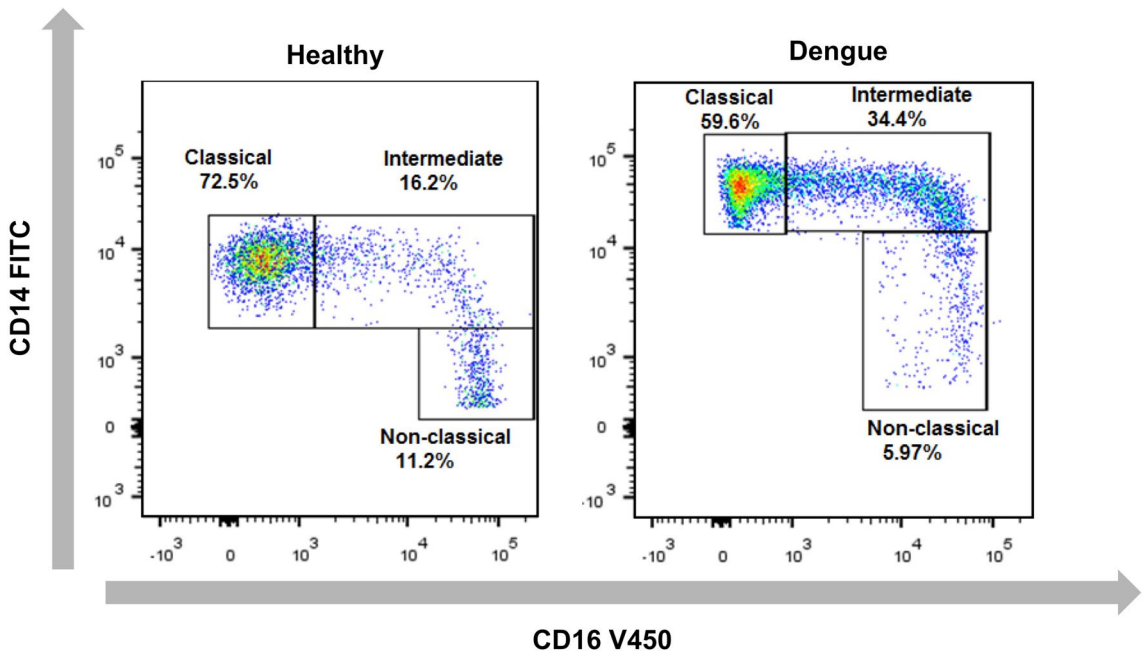

Fig. 3 Increase in the frequency of $CD14^{++}CD16^{+}$ and decrease of $CD14^{+}CD16^{++}$ subpopulations of monocytes in patients infected with DENV. Representative dot-plot figure of classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocyte subpopulations based on the expression of CD14 and CD16 in $HLA\text{-}DR^{+}$ cells using flow cytometry. In the left panel, a healthy control is presented, and in the right panel a patient infected with DENV is shown

associated with an increase in DENV infection and replication through other mechanisms such as ADE, which could be important not only during secondary but also in primary infections.

In addition, we have observed in unpublished data that $CD14^{+}CD16^{++}$ monocytes are the main subpopulation expressing the activation marker CD86 and show more frequency of TNF^{+} cells in dengue patients compared to other monocyte subsets. Taking this evidence in consideration, and because $CD14^{+}CD16^{++}$ monocytes have more affinity for the microvasculature endothelium, this subpopulation may play a critical role mediating the endothelial dysfunction observed in severe cases of DENV infection.

Role of monocytes in endothelial dysfunction observed in DENV infections

As mentioned throughout the review, one of the pivotal evidences in DENV infections is an increase in endothelium permeability, observed through the loss of body fluids throughout the body that leads to hemoconcentration, pleural effusion, hematuria, and other types of hemorrhages (Martina et al. 2009). This phenomenon is mainly mediated by an endothelial dysfunction and reorganization of junctions rather than a direct cytotoxic effect of the virus in endothelial cells. Some evidences that might explain the increase of endothelium permeability induced by DENV have been described. As a possible effect caused directly by the virus, it was observed in a model that infection of EA.hy926 cells (HUVEC fused with lung carcinoma cells, line A549) with DENV-2 disrupts the organization of the actin cytoskeleton and also down-regulates the expression of VE-cadherin (component of adheren junctions) and ZO-1 (component of tight junctions) along with an alteration of the distribution of these molecules (Kanlaya et al. 2009). Further, as an effect caused indirectly by the virus, it has been described that plasma from DENV-infected patients can disrupt the expression of VE-cadherin and ZO-1 in HUVEC cell cultures; this effect was due to the presence of pro-inflammatory components, since the blocking of IP-10, MCP-1, IL-9, among others, reversed the effect (Appanna et al. 2012).

Although during DENV infection monocyte binding to endothelial cells that could provoke functional damage (observed as an increase in permeability) has not been demonstrated, inflammatory mediators produced by monocytes, such as TNF-α, are likely to play an important role. In fact, using an in vitro model of endothelial permeability, it was observed that supernatants from DENV-infected THP-1 cells induce the up-regulation of ICAM-1, VCAM-1 and E-selectin along with increase in endothelial permeability in HMVEC, that depended on the action of TNF-α and IL-8 (Kelley et al. 2012). Likewise, IL-8 produced by DENV-2-infected primary monocytes can increase endothelial permeability (Bosch et al. 2002). Finally, MCP-1, which is highly produced by monocytes infected in vitro with DENV, seems to be important in increasing endothelial permeability since its neutralization with antibodies reverted partially the effect caused by monocyte supernatants in HUVEC (Lee et al. 2006). These evidences suggest that once they are infected with DENV, pro-inflammatory mediators produced by monocytes can play an important role in the increase of endothelial permeability (Fig. 2). Some soluble factors produced by monocytes upon DENV infection can be more important than other in terms of increasing endothelial permeability, regarding the extent of the alteration that they can induce in endothelial cells. Such is the case of TNF-α, IL-1β and IFN-γ that have been correlated with an increase in endothelial permeability (Burke-Gaffney and Keenan 1993; Dewi et al. 2004).

Finally, other important scenario in which monocytes play an essential role in pathogenesis is in ADE infection mechanism, described above. In addition to increasing the entry and replication of the virus, ADE-triggered infection also increases the expression and production of cytokines, thereby increasing the endothelial permeability induced by some inflammatory factors. Indeed, supernatants from U937 cells infected through the ADE mechanism induce an increased permeability of Madin–Darby canine kidney cells, along with a redistribution and degradation of proteins involved in cell-to-cell binding (Puerta-Guardo et al. 2013). These same authors found that neutralization of TNF-α, IL-6 or IL-12 using specific antibodies restores the function of these cells, suggesting an important role of these cytokines in this process (Puerta-Guardo et al. 2013).

Conclusions and perspectives

As already mentioned in this review, monocytes express a wide array of PRR which allow them to trigger a proper and effective innate immune response upon DENV infection. However, these phagocytes are also important components of the immunopathology of severe dengue, contributing to viral replication, nitric oxide production, and high levels of cytokine production, factors that can have a direct effect on endothelial cells, causing an increased endothelial permeability. However, other mononuclear phagocytes such as macrophages could be also important in pathogenesis, since they share a wide range of molecules and functions with monocytes, and their responses can also contribute to severe dengue. Therefore, monocytes and other mononuclear phagocytes could be promising therapeutic targets for DENV-infected patients.

Despite the findings described in the literature so far, the role of monocyte subpopulations in the development of

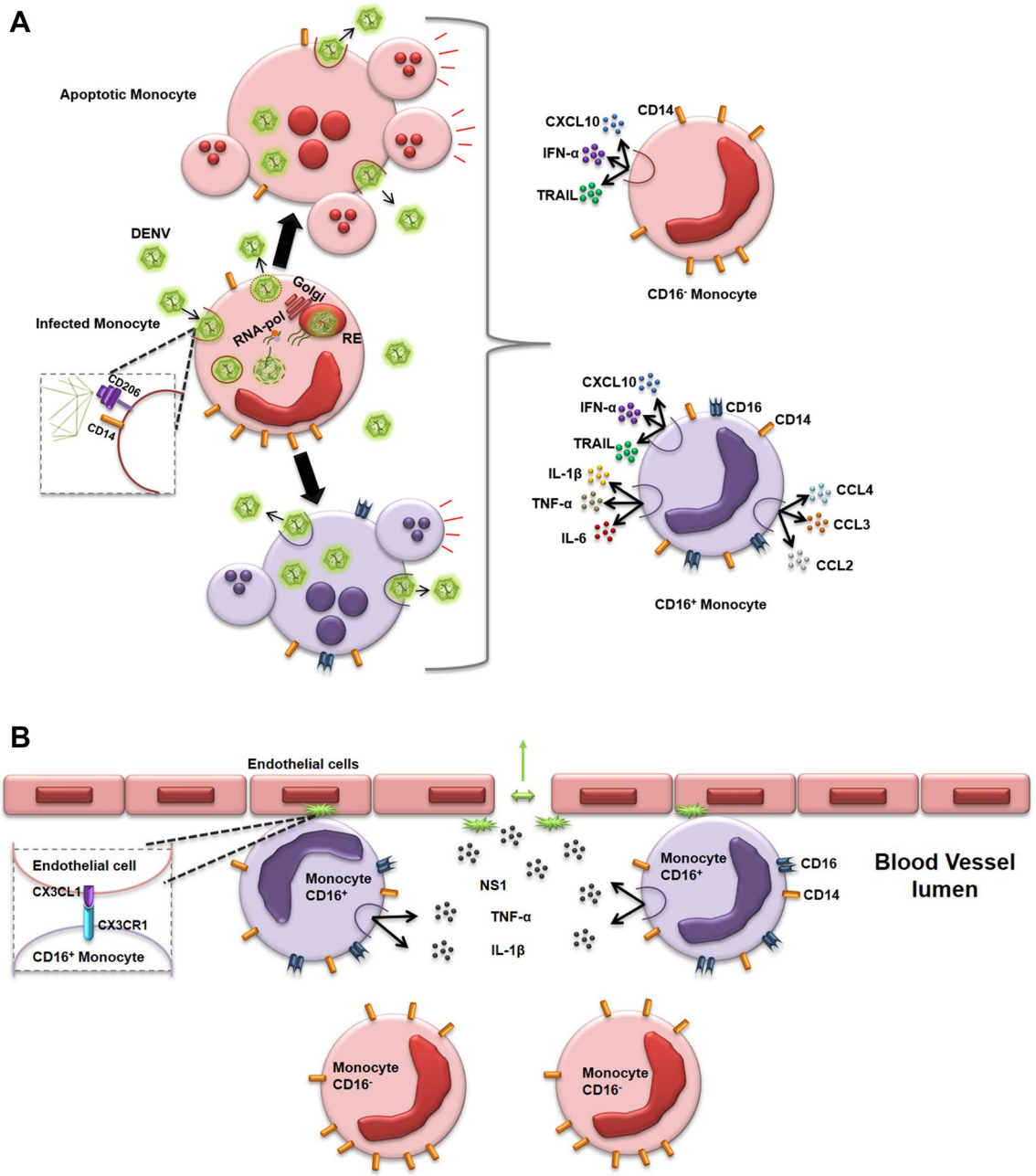

Fig. 4 Hypothetical model of the potential effect of $CD14^{+}CD16^{++}$ monocyte subpopulations on the endothelium during DENV infection. **a** Monocytes can be activated rapidly by DENV infection, possibly through the recognition of viral particles, NS1 and by apoptotic bodies of DENV-infected cells, since DENV induces cell death of different cell subpopulation including monocytes. In response to these stimuli, $CD14^{++}CD16^{-}$ monocytes can produce high quantities of CXCL10, TNF-related apoptosis-inducing ligand (TRAIL) and IFN-α, whereas $CD14^{+}CD16^{++}$ monocytes can produce these same cytokines but also high quantities of IL-1β, TNF-α, IL-6, CCL4, CCL3 and CCL2. **b** In basal conditions, $CD14^{+}CD16^{++}$ monocytes have a patrolling function over the endothelium, mainly through the interaction of CX3CL1 on microvasculature endothelial cells with CX3CR1 on monocytes. However, we propose that when these monocytes are activated by NS1 and by other viral components, they can be retained in the endothelial surface, which could contribute to endothelial alterations through direct secretion of pro-inflammatory mediators, such as TNF-α, IL-1β and NS1. Although it is not shown in this graph, other factors such as autoantibodies can contribute directly to endothelial permeability

severe manifestations of the disease remains poorly understood. Specifically, we propose that $CD14^{+}CD16^{++}$ monocytes can be one of the main targets in the development of an alternative and novel treatment for dengue patients. $CD14^{+}CD16^{++}$ monocytes can bind robustly to the endothelial cells via CX3CL1 under continuous blood flow (Ancuta

et al. 2003; Collison et al. 2015). Using an adoptive transfer model of cells in *Rag2*-deficient mice, it was observed that $CD14^{+}CD16^{++}$ monocytes patrol and bind to microvasculature endothelium, respond to viral antigens through the activation of TLR7 and TLR8 and mediate endothelial damage (Cros et al. 2010). For these reasons, and taking into account that this subset is one of the most important sources of inflammatory cytokines such as TNF-α in response to different stimuli including dengue infection (Wong et al. 2012a, b), it is possible that $CD14^{+}CD16^{++}$ monocytes can target endothelial cells in DENV infection.

Since we and other authors have demonstrated that $CD14^{+}CD16^{++}$ monocyte subpopulation is decreased in peripheral blood from DENV-infected patients (Fig. 3), it is plausible to infer that this decrease is due to a sequestration of these cells, after activation, in the endothelium of peripheral small blood vessels leading to a lesser frequency of circulating $CD14^{+}CD16^{++}$ monocytes (Fig. 4). It is possible that here lies the key to the increased endothelial permeability, since $CD14^{+}CD16^{++}$ monocytes attached to peripheral blood vessels could be releasing inflammatory mediators that contribute to an increased permeability. Another possibility is that these monocytes migrate into different tissues, as have been proposed already for other inflammatory diseases (Yoshimoto et al. 2007). Therefore, further studies are needed to confirm these hypotheses and to attribute a precise role to $CD14^{+}CD16^{++}$ monocytes in the endothelial alterations observed in DENV infections. Also, further studies to explore the biology and role of monocyte subpopulations in DENV pathogenesis are required.

Finally, it is important to mention that alterations in frequency and number of non-classical and intermediate subsets in other diseases with a clear endothelial dysfunction have been described, such as atherosclerosis (Mikołajczyk et al. 2016), sepsis (Chousterman et al. 2016; Skrzeczyńska et al. 2002) and lupus (Burbano et al. 2018). Hence, the contribution of monocyte subsets in endothelial homeostasis and/or damage can have an effect in different inflammatory conditions. Thus, it is also important to further evaluate the role of activation and regulation of monocyte subsets in the maintenance of endothelial integrity in the context of DENV pathogenesis.

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