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Review Article

The Relative Abundance of Monocyte Subsets Determines Susceptibility to Perinatal Hepatic Inflammation

Sarah Mohamedaly, Anas Alkhani, Amar Nijagal*

Department of Surgery and the Liver Center, University of California, San Francisco, CA 94143-0570, USA

ABSTRACT

The devastating consequences of perinatal liver inflammation contribute to a pressing need to develop therapeutics for the diseases that underly this condition. Biliary atresia (BA) is a perinatal inflammatory disease of the liver that results in obliterative cholangiopathy and rapidly progresses to liver failure, requiring transplantation. The ability to develop targeted therapies requires an understanding of the immune mechanisms that mitigate perinatal liver inflammation. This article reviews our recent findings demonstrating that in a murine model of perinatal hepatic inflammation, Ly6c^{Lo} non-classical monocytes express a pro-reparative transcriptomic profile and that the relative abundance of Ly6c^{Lo} monocytes promotes resolution of perinatal liver inflammation, rendering neonatal pups resistant to disease. We also examine the lineage relationship between monocyte subsets, reviewing data that suggests classical monocytes are a precursor for non-classical monocytes, and the alternative possibility that separate progenitors exist for each subset. Although a precursor-product relationship between classical and non-classical monocytes might exist in certain environments, we argue that they may also arise from separate progenitors, which is evident by sustained Ly6c^{Lo} non-classical monocyte expansion when Ly6c^{Hi} monocytes are absent. An improved understanding of monocyte subsets and their developmental trajectories during perinatal hepatic inflammation will provide insight into how therapies directed at controlling monocyte function may help alleviate the devastating consequences of diseases like BA.

Keywords: Biliary atresia (BA); Inflammation; Monocyte

INTRODUCTION

Perinatal hepatic inflammation can have devastating, life-threatening consequences. Biliary atresia (BA) is an example of a progressive inflammatory disease that occurs in the liver of neonates and infants. BA results in rapid obliteration of the biliary tree, leading to liver failure and cirrhosis. Accounting for approximately 75% of liver transplants performed in children under the age of 2 years, BA is the most common indication for pediatric liver transplant [1,2]. The primary treatment of BA relies on the Kasai portoenterostomy, a palliative procedure in most cases as 70% of children still develop progressive liver failure [2]. The etiology of BA is not well understood, but is thought to be attributed to multiple factors including genetic predisposition, immune dysregulation, and toxic and infectious causes [2]. The timing of injury is also not well understood. Compelling evidence supports the idea that the pathogenesis of BA occurs in utero [3]. The result of this perinatal insult triggers an inflammatory cascade in the liver. A better understanding of the mechanisms by which perinatal hepatic inflammation is initiated and resolved is an important step in identifying therapeutic targets that can halt the development of progressive liver injury and the need for liver transplantation in diseases like BA.

The uniform observation of periportal inflammation in patients with BA indicates that immune-mediated mechanisms are central to the pathogenesis and resolution of hepatic inflammation. Attempts to decrease periportal inflammation in patients with BA have included corticosteroids and intravenous immunoglobulin after portoenterostomy, but these treatments have not improved post-Kasai bile drainage or changed the overall survival of patients with their native liver [4,5]. The lack of clear efficacy using these treatments suggests that immunomodulatory agents that have broad effects may lack the specificity necessary to mitigate disease

Correspondence to: Amar Nijagal, Department of Pediatric Surgery, University of CA, San Francisco, CA 94143-0570, USA, Tel: 415-476-4086; E-mail: Amar.Nijagal@ucsf.edu

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progression.

IMMUNE SYSTEM MITIGATION OF PERINATAL LIVER INFLAMMATION

Current research supports the involvement of macrophages [6] and dendritic cells [7] in activating natural killer (NK) cells and neutrophils, respectively, to induce a proinflammatory response. Immune signals from NK cells [7], CD4+ T-helper cells [8-11], and gamma-delta T-cells [12] have also been implicated in the pathogenesis of perinatal inflammation in mice. Additionally, monocytes play an important role in mitigating hepatic inflammation and are among the initial wave of leukocytes recruited to hepatic parenchyma at the time of insult [13]. However, their role in perinatal hepatic inflammatory diseases like BA is poorly understood.

Our current understanding of monocyte function during inflammation is largely based on experiments in adult animals [14-17]. Previous data support the idea that classical monocytes (defined by cell surface expression of $Ly6c^{Hi}Ccr2^{Hi}Cx3cr1^{int}$) perform pro-inflammatory functions, whereas non-classical monocytes ($Ly6c^{Lo}Ccr2^{Lo}Cx3cr1^{Hi}$) aid in pro-reparative functions [13,17-20]. In the physiologic state, $Ly6c^{Lo}Ccr2^{Lo}Cx3cr1^{Hi}$ non-classical monocytes act as vascular scavengers, surveilling the endothelium and eliminating luminal micoparticles [21,22], whereas circulating $Ly6c^{Hi}Ccr2^{Hi}Cx3cr1^{int}$ classical monocytes are believed to be selectively recruited to inflamed tissues, where they participate in a pro-inflammatory response and give rise to monocyte-derived macrophages [23-25].

We investigated the role of classical and non-classical monocyte subsets in the pathogenesis and resolution of perinatal liver inflammation using a mouse model of perinatal hepatic inflammation. Rhesus rotavirus (RRV) infection in neonatal BALBc mice results in progressive hepatic inflammation that resembles the histologic findings of human BA [26]. Our findings demonstrate that (i) $Ly6c^{Lo}$ non-classical monocytes express a pro-reparative transcriptomic signature during the perinatal period, (ii) the abundance of $Ly6c^{Lo}$ non-classical monocytes inversely correlates with susceptibility to RRV-mediated perinatal liver injury, and (iii) experimental manipulation of $Ly6c^{Lo}$ non-classical monocytes can render neonatal pups resistant to perinatal liver injury [27].

The transcriptomic profile of Ly6cLo non-classical monocytes in the perinatal liver had higher expression of anti-inflammatory genes such as Il4ra and Tgfb1 than did Ly6cHi classical monocytes. Furthermore, we observed a physiologic abundance of $Ly6c^{Lo}$ non-classical monocytes compared to $Ly6c^{Hi}$ classical monocytes in the late-gestation liver. Specifically, $Ly6c^{Lo}$ non-classical monocytes were the predominant monocyte population in the late-gestation liver, outnumbering classical monocytes by 2.5-fold, before equalizing to the known 1:1 relationship that exists in circulation after birth [20].

Given the known anti-inflammatory profile of $Ly6c^{Lo}$ non-classical monocytes, we questioned whether this relative abundance of $Ly6c^{Lo}$ non-classical monocytes confers resistance to perinatal liver inflammation. Using fetal surgery techniques that we previously described [28,29], we infected late-gestation fetuses with RRV and confirmed inflammatory changes in the fetal liver. We observed that 75% of live-born mice infected with RRV in utero lived beyond 21 days, which was significantly higher than the proportion surviving after neonatal injection (21%). Furthermore, unlike mice injected postnatally, the mice infected in utero did not demonstrate neonatal growth restriction or weight loss. These findings suggested that the observed physiologic differences in monocyte subsets may account for the resistance to disease observed in late-gestation fetal mice.

We then hypothesized that experimental expansion of $Ly6c^{Lo}$ non-classical monocytes in the neonatal liver would spare postnatal mice from hepatic inflammation. Indeed, after depleting both $Ly6c^{Hi}$ classical monocytes and neutrophil populations in a postnatal pup we found a significant increase in $Ly6c^{Lo}$ non-classical monocytes and a high $Ly6c^{Lo}$:$Ly6c^{Hi}$ ratio similar to that of the late-gestation fetus. We found that this environment of $Ly6c^{Lo}$ non-classical monocyte expansion conferred protection against RRV-mediated perinatal inflammation. Our findings were confirmed histologically, demonstrating less severe inflammatory changes in the liver parenchyma with near resolution of inflammation in most pups. This result suggested that resistance to perinatal inflammation was either due to diminished levels of pro-inflammatory classical monocytes and neutrophils, or due to the abundance of non-classical monocytes. To distinguish these possibilities, we inhibited $Ly6c^{Lo}$ non-classical monocytes in the setting of $Ly6c^{Hi}$ classical monocyte and neutrophil depletion and found that susceptibility to RRV-mediated inflammation was restored. These findings demonstrate that the abundance of $Ly6c^{Lo}$ non-classical monocytes is indeed associated with resolution of RRV-mediated hepatic inflammation [27]. Collectively, the results of these experiments indicate that non-classical monocytes are crucial for resolution of perinatal hepatic inflammation and suggest that these cells should serve as targets for therapies designed to mitigate the effects of perinatal liver inflammation.

LINEAGE RELATIONSHIP BETWEEN $LY6C^{Hi}$ CLASSICAL MONOCYTES AND $LY6C^{Lo}$ NON-CLASSICAL MONOCYTES

Our data also informs the lineage relationship between non-classical monocytes and classical monocytes. The expansion of $Ly6c^{Lo}$ non-classical monocytes that occurs in the absence of $Ly6c^{Hi}$ classical monocytes suggests that non-classical monocytes may not originate from classical monocytes during RRV-mediated perinatal hepatic inflammation.

There are currently two hypotheses that address the development of classical and non-classical monocyte subsets. The most accepted hypothesis supports the idea that classical monocytes serve as a precursor for non-classical monocytes [17,24,30-32]. An alternative hypothesis that has been proposed is that separate progenitors exist for each subset [23,30]. Studies exploring these hypotheses have focused on bone marrow monocytes in the adult mouse. To the best of our knowledge, studies have not been replicated in the neonatal mouse or specifically in the setting of perinatal hepatic inflammation.

The first hypothesis, originally described by Sunderkötter et al., demonstrated that non-classical monocytes are generated from classical monocytes in peripheral circulation [24]. In the original experiments, the authors ablated all monocyte populations by intravenous administration of clodronate-loaded liposomes and then used fluorescence-activated cell sorter analysis to monitor re-emergence of the monocyte subsets in peripheral blood [24]. $Ly6c^{Hi}$ classical monocytes re-emerged on day 2 after ablation, whereas $Ly6c^{Lo}$ non-classical monocytes re-emerged on days 3 and 5 [24]. These findings suggest that $Ly6c^{Hi}$ classical monocytes serve as a

precursor for Ly6c^{Lo} non-classical monocytes.

Subsequent studies utilizing similar methods to Sunderkötter et al. also observed this precursor-product relationship between the two monocyte subsets [30,33,34]. Using pulse chase technology, Yona et al. supported this linear development relationship [31]. The authors mapped BrdU (5-bromo-2'-deoxyuridine) incorporation in monocyte subsets, showing that Ly6c^{Hi} classical monocytes were rapidly present in the peripheral blood, whereas Ly6c^{Lo} non-classical monocytes were observed in the peripheral blood after 3 days despite incorporation of BrdU in the bone marrow at 6 hours [31]. The appearance of classical monocytes first, followed by non-classical monocytes, support the idea that Ly6c^{Hi} monocytes give rise to Ly6c^{Lo} monocytes.

Del Sacco et al. investigated the relationship between monocyte subsets in the liver using spinning-disk fluorescent confocal intravital microscopy (SD-IVM) [17]. Monocytes subsets were identified by the cell surface markers using Ccr2 and Cx3cr1, with classical monocytes being Ccr2^{Hi} Cx3cr1^{Lo} and non-classical monocytes defined as Ccr2^{Lo} Cx3cr1^{Hi}. At baseline, ~5% of patrolling lymphocytes in the liver vasculature were Ccr2^{Hi} classical monocytes and no Cx3cr1^{Hi} non-classical monocytes were detected. Upon injury, Ccr2^{Hi} classical monocytes began to accumulate around the injured area between 8 and 72 hours later, whereas Cx3cr1^{Hi} non-classical monocytes became prevalent between 48 and 72 hours with identical localization to the Ccr2^{Hi} cells [17]. There was also a transition from Ccr2^{Hi} Cx3cr1^{Lo} to Ccr2^{Lo} Cx3cr1^{Hi} within the tissue microenvironment surrounding the focal site of injury that was dependent on a local milieu of cytokines and independent of the recruitment of additional cells to the injury site [17]. This phenotypic transition was correlated with tissue repair and thus suggestive of a local, cytokine-driven in situ reprogramming of pro-inflammatory classical monocytes into pro-reparative non-classical monocytes during liver injury.

Arguably, the most compelling evidence supporting the idea that Ly6cHi classical monocytes may give rise to Ly6c^{Lo} non-classical monocytes was demonstrated by adoptive transfer methods. Varol et al. demonstrated that in the absence of inflammation, transferred bone marrow Ly6c^{Hi} classical monocytes home back to the bone marrow of recipient mice in which myeloid dendritic cell progenitors (MDPs) are depleted and participate in Ly6cLo non-classical monocyte differentiation [35]. The authors excluded MDPs on the basis that they are Ly6c negative; however, recent evidence supports the presence of common monocyte progenitors (cMoPs) that are Ly6c positive, confounding the results of this study [36]. The recent discovery of common monocyte progenitors (cMoPs) certainly raises the question of whether other precursors exist in the lineage relationship between MDPs and final monocyte subsets that have yet to be discovered.

Challenging this precursor-product relationship between monocytes subsets, Nahrendorf et al. used adoptive transfer methods to demonstrate sequential recruitment of different monocyte subsets to the myocardium after a myocardial ischemic injury in a biphasic manner [37]. Findings demonstrated that Ly6c^{Hi} classical monocytes were present at the site of injury during the first 3 days (phase I) and Ly6c^{Lo} non-classical monocytes increased between days 4-7 (phase II) [37-39]. The adoptive transfer methods in CCR2 knockout mice demonstrated the following (i) efficient accumulation of adoptively transferred Ly6c^{Lo} monocytes in infarcts in phase II; (b) the absence of Ly6c^{Hi} monocytes in phase I, but presence of Ly6c^{Lo} monocytes in phase II, in infarcts of CCR2$^{-/-}$ mice; and (c) presence of Ly6c^{hi} monocytes in phase I, but absence of Ly6c^{Lo} monocytes in phase II, in infarcts of CX3CR1$^{-/-}$ mice [37]. The expansion of Ly6c^{Lo} non-classical monocytes when Ly6c^{Hi} classical monocytes are absent argue against in situ conversion of Ly6c^{Hi} classical monocytes to Ly6c^{Lo} non-classical monocytes during tissue repair.

CONCLUSION

While our findings demonstrate the importance of Ly6c^{Lo} non-classical monocytes in resolution of perinatal liver inflammation and raise the possibility of a separate progenitor relationship between the monocyte subsets, the evidence supporting an alternate hypothesis of a precursor-product relationship demonstrates the need for continued investigation and further understanding of this developmental relationship. This is especially relevant in the setting of perinatal liver inflammation, given its morbid consequences. Deeper understanding of the role of monocyte subsets and particularly their developmental lineage during inflammation can provide insight for future targeted therapy that could halt inflammation and alleviate the burden of devastating inflammatory conditions, such as BA.

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