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Dynamics of Immune Cell Recruitment during West Nile Encephalitis and Identification of a New CD19⁺B220⁻BST-2⁺ Leukocyte Population

Anne-Claire Bréhin, Juliette Mourières, Marie-Pascale Frenkiel, Gilles Dadaglio, Philippe Desprès, Monique Lafon, and Thérèse Couderc

West Nile virus (WNV) is an emerging neurotropic flavivirus. We investigated the dynamics of immune cell recruitment in peripheral tissues and in the CNS during WNV encephalitis in an immunocompetent mouse model. In the periphery, immune cell expansion can successfully limit viremia and lymphoid tissue infection. However, viral clearance in the periphery is too late to prevent viral invasion of the CNS. In the CNS, innate immune cells, including microglia/macrophages, NK cells, and plasmacytoid dendritic cells, greatly expand as the virus invades the brain, whereas B and T cells are recruited after viral invasion, and fail to control the spread of the virus. Thus, the onset of WNV encephalitis was correlated both with CNS viral infection and with a large local increase of innate immune cells. Interestingly, we identify a new immune cell type: CD19⁺B220⁻BST-2⁺, which we name G8-ICs. These cells appear during peripheral infection and enter the CNS. G8-ICs express high levels of MHC class II, stain for viral Ag, and are localized in the paracortical zone of lymph nodes, strongly suggesting they are previously unidentified APCs that appear in response to viral infection.

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the sequential events during lethal WNV encephalitis in immunocompetent hosts. Interestingly, characterization of leukocyte populations identified a new immune cell type: CD19⁺ B220⁺ BST-2⁺. The phenotypic characterization and location of these CD19⁺ B220⁺ BST-2⁺ cells in lymph nodes strongly suggest they could be a previously unidentified APC type.

**Materials and Methods**

**Virus and mice**

WNV strain Is-98-ST1 (GenBank accession no. AF_481864) (22) was produced on mosquito AP61 cell monolayers and purified as previously described (23). Virus titers, expressed as focus-forming units (FFU), were determined on AP61 cells by a focus immunodetection assay. The principles of good laboratory animal care were followed throughout all experimental processes. BALB/c mice (Janvier Laboratories) inoculated with either virus or diluent (mock infected) were anesthetized with isoflurane (Forene; Abbott Laboratories) at the indicated times after infection. Blood was collected and mice were perfused intracardiacally with PBS, then popliteal lymph nodes (LN), spleen, and the CNS (brain and spinal cord) were harvested.

**Leukocyte isolation**

Leukocytes were isolated from blood samples on Ficol density gradients. They were collected and resuspended in staining buffer (SB; pH 7.5) containing PBS with 1% inactivated FCS and 0.1% NaN₃ (w/v). To isolate leukocytes from organs, tissues were minced and homogenized in RPMI 1640 medium supplemented with 5% FCS. The suspension was digested at 37°C for 30 min with 1 mg/ml collagenase A (Life Technologies) and 10 μg/ml DNase I (Roche), then pressed through a nylon mesh and centrifuged at 1600 rpm for 5 min. For splenocyte and LN cell isolation, the cells were directly incubated in PBS supplemented with 5% FCS and 5 mM EDTA (PBS-EDTA) for 5 min, then pelleted and resuspended in SB. For CNS mononuclear cell isolation, each digested sample was applied onto a discontinuous 30/70% Percoll gradient and then cells were collected and washed. They were incubated in PBS-EDTA for 5 min and treated as described for lymphoid tissues.

**Fluorescent staining and flow cytometry analysis**

Before fluorescent labeling, cells in SB were counted. Nonspecific binding of Abs was blocked with anti-CD16/32 mAbs (2.4G2) in ice-cold SB. Cells were labeled in SB for 30 min on ice in the dark with the following fluorescently conjugated mAbs or biotinylated mAbs: anti-CD45 (2.104), anti-CD11b (M1/70), anti-CD19 (1D3), anti-CD3 (17A2), anti-B220 (RA3-6B2), anti-CD121c (HL-3), anti-CD122 (TM-β1), anti-Ly6G (RB6-8C5), anti-CD49b (DX5), anti-BSTS-2 (120G8 or PDCA-1), anti-CD19 (RA3-6B2), anti-CD11c (HL-3), anti-CD122 (TM-β1), anti-Ly6G (RB6-8C5), anti-CD49b (DX5), anti-BSTS-2 (120G8 or PDCA-1), and anti-CD3 (TM-β1). Biotinylated mAbs were detected with streptavidin-Pacific Blue.

**ELISA**

LN, spleen, and CNS homogenates were analyzed by ELISA for the production of IFN-α. Supernatants were collected from nonstimulated and virus- or R848-stimulated plasmacytoid dendritic cell (pDC) cultures after 24 and 48 h and analyzed IFN-α content. IFN-α was assayed by coating anti-IFN-α mAb (clone RMMA-1; PBL Biomedical) at 2 μg · ml⁻¹ in a Na₂HPO₄ buffer (pH 6.5) overnight at 4°C. After washing with 0.1% Tween 20 (Sigma-Aldrich) in PBS, samples were incubated overnight at 4°C. Rabbit polyclonal anti-IFN-α Abs (PBL Biomedical) were diluted to 0.05 μg · ml⁻¹ in washing buffer supplemented with 10% FCS, and incubated for 24 h at room temperature. HRP-conjugated donkey anti-rabbit IgG Abs (Southern Biotechnology Associates) were diluted 1/4000 in washing buffer supplemented with 10% FCS and incubated for 1 h at room temperature. Two hundred microliters of o-phenylenediamine was then added and the reaction was stopped after 15–20 min by adding 50 μl of H₂SO₄, and the OD was measured at 450 nm. rIFN-α was used to standardize the ELISA.
FIGURE 2. Cells isolated from LN, blood, spleen, and CNS of individual mice on days 4 and 6 after infection with either diluent (Mock) or WNV were analyzed by flow cytometry. CNS analysis (A–D). Microglia (CD11b<sup>+</sup>CD45.2<sup>+</sup>) and leukocytes (CD11b<sup>+</sup>CD45.2<sup>-</sup>) were gated from total cells from the CNS (A). Microglia and leukocytes were gated as shown in A. Histograms represent the mean values of total cell number from at least three independent experiments. Error bars, SEM. Asterisks indicate a significant difference between cells isolated from mock-infected and WNV-infected mice (***, *p < 0.001) (B). Microglia (CD11b<sup>+</sup> as gated in A) from mock (filled histogram) or WNV-infected mice on day 4 (thin line) or day 6 (thick line) were analyzed for CD45.2 expression to distinguish resting (CD45.2<sup>+</sup>) from activated (CD45.2<sup>-</sup>) microglia (gated in M1) (C). Percentage of activated microglia was determined (gated in M1) and plotted against viral titer in the CNS for individual mice (D). Representation of leukocyte populations (B, T, NKT and NK cells, neutrophils (Neutro), macrophages (Mac), cDCs, and pDCs) (gated as shown) in LN from mock- or WNV-infected mice on days 4 and 6 (E). Histograms represent the mean total cell numbers for each leukocyte population (as gated in E) from at least three independent experiments (F). For the CNS, cells were gated from among leukocytes, excluding CD11b<sup>+</sup> cells. Error bars, SEM. Asterisks indicate a significant difference between cells isolated from mock-infected and WNV-infected mice (*, *p < 0.01; **, *p < 0.001; nd, not determined). Data from an individual mouse from one representative experiment of two, including three to four mice per group, are depicted in A, C, and E.
We followed the kinetics of the viral load in the CNS of infected animals (Fig. 1B). On day 3, there was noninfectious virus in the brain, but on day 4, the viral load was $10^2$–$10^5$ FFU/g of brain tissue. By day 6, all mice suffered clinical signs of encephalitis, associated with an increased WNV infection of the CNS ($10^5$ FFU/g). We also determined the viral load in the blood, LN, and spleen at the time when infectious virus enters the CNS (day 4) and at the onset of encephalitis (day 6; Fig. 1C). On day 4, the viral load in the LN and spleen was similar to that in the brain, but it was lower in serum ($<10^2$ FFU/ml). By day 6, the viral burden had declined in all of these peripheral tissues, whereas it increased in the CNS.

Thus, following s.c. WNV infection, viremia occurs and WNV replicates both in lymphoid tissues and the CNS; however, the infection appears to decrease quickly in the periphery, while it expands in the CNS until the onset of encephalitis.

Dynamics of immune cell recruitment in the course of lethal WNV encephalitis

We analyzed the distribution of leukocyte populations in lymphoid organs (LN and spleen), blood, and the CNS in WNV-infected mice before (day 4) and at the onset of encephalitis (day 6). After cardiac perfusion, cells were isolated from blood on a Percoll gradient and from LN, spleen, and the CNS after enzymatic digestion. In addition, cells from CNS samples were isolated by Percoll gradient. Note that cells isolated from CNS samples obtained from perfused mice in which leptomeninges were not removed allow the detection of cells present both in meninges and in nervous parenchyma. The cells in the various samples were then quantified and phenotyped by flow cytometry.

In the CNS, cells can be classified according to their expression of the myeloid marker CD11b and of the hematopoietic marker CD45.2. CD11b$^+$CD45.2$^{low}$ cells represent resting microglia, the resident hematopoietic cells of the CNS, whereas CD11b$^+$CD45.2$^{high}$ cells are leukocytes (Fig. 2A). In mock-infected mice, microglia was the main cell type, whereas leukocytes were rare (Fig. 2B). Microglia was the most expanded population during WNV infection, increasing up to 10-fold at day 6 (Fig. 2B). Although expanding, microglia up-regulated CD45.2 expression, a marker that increases with microglia activation (Fig. 2C). These cells, CD11b$^+$CD45.2$^{high}$, cannot be phenotypically distinguished from peripheral macrophages (24, 25); thus, their origin in the CNS may be activated microglia as well as infiltrating peripheral macrophages (gated in M1, Fig. 2C). To determine the extent to which microglia/macrophages became activated, we examined the acquisition of activation markers, CD86 and MHC class II (MHC II), and the expression of both CD11c, the dendritic cell marker, and of BST-2, first described to be specific for pDCs (26). BST-2 was expressed by microglia on day 4 and expressed more strongly on day 6, concomitantly with CD86, MCH II, and CD11c expression (Fig. 2C). BST-2 can be considered to be an earlier marker of maturation of microglia, because it was expressed before the up-regulation of CD86 and MHC II.

The whole microglia/macrophage population was activated on day 6 (Fig. 2B) and activation of microglia was fully correlated with WNV invasion of the CNS (Fig. 2D). At the same
time, despite the number of leukocytes entering the CNS being small relative to the microglia/macrophage population, leukocyte recruitment into the CNS also increased as the infection progresses (Fig. 2B).

We phenotyped B, T, NKT, and NK cells, neutrophils, macrophages, conventional DC (cDCs), and pDCs (Fig. 2E for LN) and followed the kinetics of recruitment of leukocyte populations at the periphery and in the CNS (Fig. 2F). In the spleen, B and T cells were the only leukocyte populations showing changes in numbers during WNV infection, with a decline by days 4 and 6, respectively. In contrast, they increased substantially in LN by day 4 despite not trafficking through the blood, suggesting they proliferated in situ. Both cell types emerged in the CNS only on day 4 despite not trafficking through the blood, suggesting that they proliferated in situ. B cell counts increased in LN and the CNS by day 4, while T cell counts increased in LN and the CNS by days 4 and 6; however, their numbers remained stable in the blood. At the same time points, NK cell counts also increased in LN with substantial trafficking in the blood and these cells invaded the CNS. On day 6, they made up 25% of all leukocytes in the blood (21.7 x 10^6 cells) and 30% in the CNS (1.47 x 10^6 cells). Neutrophils reached the CNS as early as day 4 and showed a large expansion in the blood by day 6, albeit to a lesser extent than NK cells. Similarly, macrophages became more numerous in the blood. They may have entered the CNS, increasing the number of microglia/macrophages. cDCs accumulated in LN on and after day 4, despite no trafficking through the blood, suggesting they enter the LN via afferent lymph vessels. In the CNS, microglia can differentiate into dendritic-like cells (27), as confirmed by the acquisition of CD11c and cannot be phenotypically distinguished from peripheral cDCs. Remarkably, pDC counts remained constant in the periphery, but became more abundant in the CNS as early as day 4.

These observations show that LN and blood were the sites of leukocyte expansion in the periphery and that the CNS displayed high innate immune cell expansion during WNV encephalitis.

**Tissue distribution of pDCs paralleled the production of IFN-α**

We observed that pDCs were not significantly increased in the periphery in the course of WNV infection, but could be detected in the CNS as early as day 4 and their number significantly increased as the infection progressed into the CNS (Fig. 2F, bottom panels). pDCs produce substantial amounts of IFN-α (28); therefore, we
investigated whether WNV-stimulated pDCs produced IFN-α. IFN-α secreted into the supernatants of cultures of pDCs harvested from spleen of mice was assayed following in vitro incubation with WNV for 24 and 48 h. R848 was used as a positive control of pDC activation. Nonstimulated pDCs did not produce IFN-α, whereas pDCs cultivated in the presence of WNV secreted large amounts of IFN-α (5 ng; Fig. 3A). Five-fold more than that IFN-α produced by R848-stimulated pDCs. Moreover, 25% of the pDCs were found to be Ag positive after 48 h. Thus, WNV triggered IFN-α production by pDCs in vitro. We then determined the production of IFN-α by the different tissues of WNV-infected mice. The cytokine was undetectable in LN and spleen (Fig. 3B). This finding differs from that of another study showing an increase of IFN-α mRNA by day 3 in LN (29); the disagreement could be due to experimental differences, notably the method of detection of the expression of IFN-α (i.e., the detection of mRNA by RT-PCR and immunological detection of the protein). In the CNS, IFN-α was first detected by day 4 at the time when the pDC count rises and was greatly increased by day 6 (Fig. 3A). Thus, the detection of IFN-α at the periphery and in the CNS paralleled the distribution of pDCs.

**Discussion**

Using an immunocompetent mouse model infected s.c. with WNV, we analyzed the dynamics of immune cell recruitment in the periphery and in the CNS during the course of WNV encephalitis.

In the periphery, lymphoid tissues and blood were transiently infected. LN and the blood were the sites of leukocyte expansion and trafficking, and all immune cell populations analyzed, except pDCs, increased either in LN or blood. The most expanded populations were T and B cells and cDCs in LN and innate immune cells including NK cells, neutrophils, and macrophages in the blood. Surprisingly, these innate immune cell populations, except NK cells expanded only by day 6, a time by which peripheral infection had declined. These data suggest that the immune cell populations developed in LN; i.e., T and B cells and cDCs, as well as circulating NK cells can successfully limit viremia and lymphoid tissue infection. B cells are central to containing viremia, and T cells have an important function in clearing infection from tissues (16, 18). Spleen leukocyte populations displayed no change, except a slight decrease of T and B cells. Other studies on T cell number in spleen show that T cells were stable (17). Experimental differences, such as the virus and the mouse strains used and the route of injection, could explain these discrepancies. In contrast to the previous study, here we injected mice s.c. and used a neuroinvasive strain phylogenetically close to WNV strains responsible for the American outbreak.

However, viral clearance in the periphery is too late (day 6) to prevent viral invasion of the CNS (day 4). Following entry into the CNS, viral infection increases until the onset of encephalitis. The difference in progress of infection in the periphery and in the CNS might be a consequence of the particular immune status of the CNS. The healthy CNS is an immunologically quiescent organ, presumably due to the blood-brain barrier and the relative lack of intraparenchymal leukocytes and MHC II expression. However,
our data show substantial expansion of innate immune cells, including microglia/macrophages, NK cells, neutrophils, and pDCs and the production of IFN-α, as soon as virus invaded the CNS (day 4). Concomitant with the peak of infection (day 6), they expanded dramatically, while B and T cells appeared in the CNS. Similar observations have been reported for mouse hepatitis virus encephalitis (30). Thus, WNV replication increased in the CNS despite early and massive expansion of innate immune cells, suggesting that they were unable to control viral infection efficiently. T cells have a crucial role in clearing infection from the CNS (13, 14, 16, 17, 21, 31). It is likely that they were recruited too late after viral invasion to control viral infection. However, another study with a mouse-adapted strain of WNV suggests that T cells can have immunopathological effects, depending on the viral dose injected (17). Although we used a lower dose of another strain of WNV than that used in this previous study, we cannot exclude the possibility that the entry of T cells into the CNS was deleterious in our model.

We found that the onset of WNV encephalitis was correlated both with CNS viral infection and with a large local increase of innate immune cells. These cells seem to be unable to control viral infection and, furthermore, may contribute to infection and/or damage of the CNS. Activated microglia/macrophages that comprise the largest component of CNS infiltrates during WNV encephalitis can initiate an inflammatory cell response. Inflammation is part of a physiology process for repairing damage and recruiting cells of the adaptive immune response. However, when it is not controlled and extended, inflammation loses its repairing function and can be the cause of damage (32). Indeed, the region of highest neurodegeneration correlates with the region of highest concentration of activated microglial cells present in many CNS diseases, including Alzheimer’s and AIDS dementia (33), suggesting that macrophage-lineage cells can be deleterious during CNS disease. Depletion of microglia could allow the role of the most prominent cells in the CNS to be elucidated during WNV encephalitis. Neutrophils might also be deleterious during WNV encephalitis. Indeed, it has been shown that depletion of neutrophils resulted in prolonged survival in mice infected with Murray Valley encephalitis, another neurotropic flavivirus (34). We show here substantial recruitment of NK cells into the CNS during WNV encephalitis, but their function is unclear because it has been previously shown that depletion of NK cells did not affect the course of WNV encephalitis (31).

pDC have been reported to be absent from the CNS (35) and, in contrast to cDCs which have been described to invade the inflamed CNS, there have been few studies of the presence of pDCs in the CNS in pathological conditions. pDCs have been found in the cerebrospinal fluid in patients suffering from multiple sclerosis or meningitis (36), and pDCs were recruited to the brain parenchyma by Flt3L treatment (37). Our study describes for the first time the presence of pDC in the CNS in the course of infection. We did not observe any expansions of peripheral populations of pDCs suggesting that infiltration of blood-derived pDCs is unlikely; our observations strongly support a local source of pDCs. Our techniques for CNS sample preparation did not separate nervous parenchyma from meninges or choroid plexuses; therefore, the initial localization of the pDCs cannot be determined. This requires additional studies, although, as proposed by Curtin et al. (37), they may originate from meninges or choroid plexus and also from resident brain microglia.

The main function of the pDCs appears to be the production of large amounts of IFN type I in response to viral or bacterial infection. pDCs in vitro secreted IFN-α in response to WNV stimulation, strongly suggesting that pDCs recruited in the CNS of WNV-infected mice are a source of IFN-α in the brain: IFN-α was indeed produced in the CNS during infection and its abundance increased as the infection progressed and migratory cells invaded. However, IFN-α was only able to inhibit viral production when mice or cells, including neurons, were treated before but not after WNV infection (38–39). Thus, the function of the pDCs in WNV encephalitis remains to be clarified.

In this study, we report isolation of a new CD19⁺ BST-2⁺ hematopoietic population, G8-ICs, that is not detected in mock-infected animals and appears in the course of WNV encephalitis. Interestingly, this cell population has never been described before. The origin of G8-ICs cannot be determined from the expression pattern of lineage markers, and it remains to be determined whether G8-ICs are recruited or expanded in situ. BST-2 is known to be up-regulated by several cell types following IFN-α production during viral infection (40), suggesting its expression by G8-ICs could reflect an activated state. However, during WNV infection, we did not detect any IFN-α in the periphery and B cells remained negative for this marker, while G8-ICs expressed low levels, suggesting that BST-2 expression by G8-ICs could be a lineage marker more than an activation marker. Nevertheless, G8-ICs express high levels of MHC II and up-regulate CD86, showing that they are activated cells. In addition, a B1 progenitor displaying a CD19⁺ BST-2⁺ phenotype has been described by Montecino-Rodriguez et al. (41). However, this progenitor is located in the bone marrow of naive mice and is not yet differentiated, whereas G8-ICs are activated cells, present in the secondary lymphoid organs, and migrate to the CNS following infection, excluding the possibility that G8-ICs are B cell progenitors.

The role of G8-ICs remains to be defined, and it is still unknown whether they have a beneficial or a deleterious effect on infection. However, these cells appeared in the periphery when the infection was controlled. They up-regulate CD86 and MHC II molecules on day 4 postinfection and are Ag positive for viral protein. Furthermore, they are localized in the T cell area of the LN where T cells and G8-ICs are in close interaction. These various observations suggest that G8-ICs may be a new APC presenting WNV Ag to T cells leading to Ag-specific T cell responses against infection. The fact that these cells appeared in periphery during the clearance of the virus strongly suggests that they may be involved in the protective immunity against the virus. However, in the CNS, T cells cannot control infection and, as previously suggested (17), they may have a pathological effect. Indeed, G8-ICs may be deleterious in the CNS, by exacerbating immune responses. Furthermore, they may also influence innate immunity by their capacity to produce cytokines and chemokines. Actually, the direct role of these cells needs to be elucidated by determining their pattern of cytokine production, their capacity to stimulate T cells, and their influence on viral immunity. The determination of their origin would also be informative and help identify their immune functions. It would be also valuable to determine whether G8-IC-like cells are present in humans during infections and identify their role, if any, in the course of the disease.

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**Disclosures**

The authors have no financial conflict of interest.
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