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FUNDAÇÃO OSWALDO CRUZ
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Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

AVALIAÇÃO DAS CÉLULAS NATURAL KILLER (NK) DE INDIVÍDUOS INFECTADOS PELO ZIKA VÍRUS (ZIKAV)

GABRIEL ANDRADE NONATO QUEIROZ

Salvador – BA
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Tese de doutorado apresentada ao curso de Pós-graduação em Biotecnologia em Saúde e Medicina Investigativa para obtenção do grau de Doutor.

Orientadora: Profa. Dra. Maria Fernanda Rios Grassi
Coorientador: Prof. Dr. Vincent Vieillard

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“A dúvida é o preço da pureza”

(*Jean-Paul Sartre*)

QUEIROZ, Gabriel Andrade Nonato. **Avaliação das células Natural Killer (NK) de indivíduos infectados pelo Zika Vírus (ZIKAV)**. 2021. 82 f. Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) – Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2021.

RESUMO

O vírus da Zika (ZIKAV) foi identificado no Brasil em 2015 e resultou na maior epidemia já relatada por este vírus. A epidemia no Brasil foi associada a um aumento no número de bebês nascidos com microcefalia, sendo considerada uma emergência de saúde pública de importância internacional. A patogênese das complicações associadas ao ZIKAV complicações ainda é desconhecida e não estão claros os fatores ligados a estas complicações, como resposta imune, carga viral, fatores do hospedeiro, momento da infecção ou presença de comorbidades. As células NK são ativadas durante a infecção viral aguda e sua atividade contribui para o reconhecimento e resposta contra células infectadas. Desta forma, o presente trabalho buscou avaliar a resposta das células Natural Killer (NK) de indivíduos infectados pelo Vírus Zika por citometria de massa. Trata-se de um estudo de corte transversal, envolvendo pacientes atendidos em um pronto atendimento na cidade de Salvador. Foram avaliados os pacientes com quadro sugestivo de infecção viral aguda entre maio de 2015 e agosto de 2017. O diagnóstico de ZIKAV foi confirmado com resultado positivo ao rt-PCR no sangue ou urina ou saliva. Os pacientes também foram testados para infecção por Dengue ou Chikungunya (CHIKV) através do rt-PCR. Para avaliação da resposta imune, células mononucleares do sangue periférico (PBMC) foram obtidas e criopreservadas. Posteriormente, as células foram descongeladas e realizada a avaliação da resposta das células NK por citometria de massa (Cytof). Os resultados mostraram que as Células NK desses pacientes continha subconjuntos de células, ativadas que expressam níveis mais elevados de CD57, NKG2C e KIR3DL1 em comparação com os de doadores saudáveis. Além disso, as células NK KIR3DL1+ desses pacientes produziram altos níveis de IFN-γ e TNF-α, em resposta à estimulação in vitro. Adicionalmente, em pacientes infectados com ZIKV, foi observada uma superprodução de IFN-γ, que se correlacionou com o marcador de ativação de STAT-5. Desta forma, os resultados do presente estudo sugerem que as células NK contribuem para a geração de uma resposta imune anti-ZIKV eficaz e que pode afetar o desfecho da doença e/ou o desenvolvimento de sintomas persistentes.

P**alavras-chave**: ZIKV. CyTOF. Resposta immune.

QUEIROZ, Gabriel Andrade Nonato. **Evaluation of Natural Killer (NK) Cells of Individuals Infected by Zika Virus (ZIKAV)**. 2021. 82 f. Tese (Doutorado em Biotecnologia em Saúde e Medicina Investigativa) – Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2021.

ABSTRACT

The Zika virus (ZIKAV) was identified in Brazil in 2015 and resulted in the largest epidemic ever reported by this virus. The epidemic in Brazil was associated with an increase in the number of childs born with microcephaly, being considered a public health emergency of international importance. The pathogenesis of these complications is still unknown and the factors linked to these complications, such as immune response, viral load, host factors, time of infection or presence of comorbidities, are not clear. NK cells are activated during acute viral infection and their activity contributes to recognition and response against infected cells. Thus, the present work aimed to evaluate the response of Natural Killer (NK) cells from individuals infected by the Zika Virus by mass cytometry. This is a cross-sectional study, involving patients selected in an emergency in the city of Salvador. Patients with symptoms suggestive of acute viral infection between May 2015 and August 2017 were evaluated. The diagnosis of ZIKAV was confirmed with a positive result by rt-PCR in blood or urine or saliva. Patients were also tested for Dengue or Chikungunya infection (CHIKV) using rt-PCR. To assess the immune response, peripheral blood mononuclear cells (PBMC) were obtained and cryopreserved. Afterwards, the cells were thawed and the NK cell response was evaluated by mass cytometry (Cytof). The results showed that the NK cells from these patients contained subsets of activated cells that express higher levels of CD57, NKG2C and KIR3DL1 compared to those from healthy donors. In addition, KIR3DL1+ NK cells from these patients produced high levels of IFN-γ and TNF-α in response to in vitro stimulation. Additionally, in patients infected with ZIKV, an overproduction of IFN-γ was observed, which correlated with the STAT-5 activation marker. The results of the present study suggest that NK cells contribute to the generation of an efficacious adaptive anti-ZIKV immune response that could potentially affect the outcome of the disease and/or the development of persistent symptoms.

Keywords: ZIKV. CyTOF. Immune response.

SUMÁRIO

1 **INTRODUÇÃO**

O Zika vírus (ZIKAV) foi identificado pela primeira vez no Brasil em maio de 2015 (CAMPOS et al., 2015; ZANLUCA et al., 2015). Estima-se que o número de infectados tenha sido de 440.000 a 1.300.000 indivíduos (ECDC, 2015). Em novembro de 2015 o ministério da Saúde reconheceu uma associação entre microcefalia em recém-nascidos e o ZIKV. Até fevereiro de 2016, havia mais de 508 casos confirmados de microcefalia e outras alterações no sistema nervoso central (VENTURA et al., 2016; MLAKAR et al., 2016). Em consequência disso, a Organização Mundial da Saúde (OMS) decretou que a epidemia pelo ZIKV constituía uma emergência de saúde pública de Importância Internacional (ECDC, 2015) e é um problema de saúde pública no Brasil (FULLER et al., 2017). Além da microcefalia, existem evidências que apontam uma forte ligação entre a infecção pelo ZIKV e a ocorrência de complicações neurológicas como a síndrome de Guillan-Barré em adultos (CAO-LORMEAU et al., 2016).

Até o momento a resposta imune no curso da infecção pelo ZIKV foi pouco avaliada e ainda existem importantes lacunas nesta área de conhecimento. A patogênese destas complicações ainda não está esclarecida e não estão claros quais fatores estariam ligados aos desfechos clínicos, a exemplo da carga viral, fatores do hospedeiro, momento da infecção ou presença de outras comorbidades. O papel da resposta imune na proteção e/ou na associação dessas complicações necessita ser investigado. As células Natural Killer (NK) têm um papel fundamental na resposta imune celular em infecções virais. Os estudos com estas células na infecção pelo ZIKV ainda são restritos e insuficientes em revelar resultados que contribuam com o conhecimento acerca do papel destas células na infecção.

No presente trabalho, foi realizada uma caracterização do fenótipo e função das células Natural Killer de indivíduos infectados pelo Zika Vírus.

A execução do presente estudo pôde contribuir para o entendimento da resposta das células NK no contexto da infecção pelo ZIKAV, além de contribuir no entendimento da fisiopatologia da infecção e de identificação de possíveis assinaturas imunológicas que possam auxiliar na compreensão do papel da resposta imune inata nesta infecção.

2 **REVISÃO DE LITERATURA 14**

2.1 VÍRUS ZIKA

O vírus Zika (ZIKAV) é um arbovírus, envelopado, esférico, com cerca de 50nm de diâmetro. Pertencente à família *Flaviviridae* e ao gênero *Flavivírus*, este vírus apresenta uma fita de RNA positivo, com cerca de 10.794 bases e possui três proteínas estruturais (envelope, membrana e capsídeo) e sete não estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) (Figura1) (LINDENBACH et al., 2003; CLARKE et al., 2015; PAUL; BARTENSCHLAGER., 2015; DOUGHTY et al. 2017).

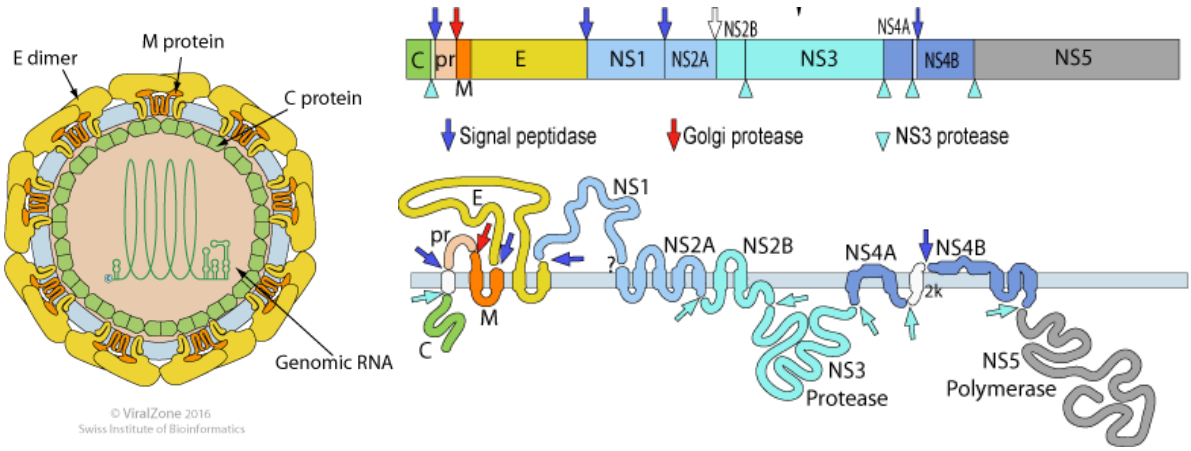

Figura 1 – Estrutura, proteínas estruturais e não estruturais do ZIKV
Fonte: (adaptado VIRAL ZONE, 2021)

2.2 HISTÓRICO E EPIDEMIOLOGIA DO ZIKAV

O ZIKAV foi isolado pela primeira vez em 1947, de um macaco *Rhesus* proveniente da floresta Zika, localizada em Entebbe, na Uganda. Em seguida, o vírus foi isolado de mosquitos *Aedes africanus*, no mesmo local. (DICK, 1952). Posteriormente, em 1954, o vírus foi isolado em humanos na Nigéria (MCNAMARA et al., 1954). O ZIKAV permaneceu endêmico restritamente a alguns países da África equatorial e da Ásia, até que em 2007, foi relatada uma grande epidemia do vírus nas ilhas de Yap, que compõe a Federação dos Estados da Micronésia. As estimativas da soro prevalência do ZIKAV nestas ilhas demonstraram que aproximadamente 70% da população havia sido infectada (DUFFY et al., 2009). Entre os anos de 2007 a 2013, foram relatados poucos casos da infecção em indivíduos que viajavam para África e Ásia (FOY et al., 2011; KWONG et al., 2013).

No ano de 2013 foi relatada uma epidemia de ZIKAV na Polinésia Francesa, que apresentou mais de 300 indivíduos com infecção confirmada e com aproximadamente 29.000 pessoas (10% da população) com sintomas sugestivos do ZIKAV. Em seguida, o vírus foi identificado em outras regiões do pacífico, como nas Ilhas Cook, Nova Caledônia e Ilha de Páscoa e nas Américas (IOOS et al., 2014; MUSSO et al., 2014; ROTH et al., 2014; ZHANG et al., 2017)

Nas Américas, o vírus foi identificado pela primeira vez no Brasil por pesquisadores da Universidade Federal da Bahia (UFBA), em 2015 (CAMPOS et al., 2015; ZANLUCA et al., 2015). Nos meses seguintes, a infecção se espalhou pelos diversos Estados do Brasil, caracterizando-se como a maior epidemia do ZIKAV já relatada, estimada em cerca de 440.000 a 1.300.000 indivíduos infectados (ECDC, 2015; GRASSI et al., 2016).

Existem algumas hipóteses para estimar como o ZIKAV tenha chegado ao Brasil. Acredita-se que o vírus possa ter sido introduzido durante a Jornada Mundial da Juventude, em 2013, devido à grande circulação de jovens da África e Ásia no país (EPELBOIN et al., 2017). No mesmo período, em 2013, ocorreu a Copa das Confederações, que pode ter introduzido o vírus no Brasil (FARIA et al., 2016). Acredita-se ainda que o vírus pode ter sido introduzido na Copa do Mundo em 2014, através de visitantes vindos da Polinésia Francesa (SALVADOR; FUJITA, 2016). Outras hipóteses sugerem que a chegada do vírus no Brasil tenha sido advinda do Campeonato Mundial de Canoagem em 2014 (MUSSO, 2015).

Em fevereiro de 2016, casos autóctones da infecção pelo ZIKV já haviam sido relatados em 26 países (Barbados, Bolívia, Brasil, Colômbia, Costa Rica, Curaçao, República Dominicana, Equador, El Salvador, Guiana Francesa, Guadalupe, Guatemala, Guiana, Haiti, Honduras, Jamaica, Martinica, México, Nicarágua, Panamá, Paraguai, Porto Rico, Saint Martin, Suriname, Ilhas Virgens e Venezuela) (WHO, 2016). Assim, a Organização Mundial da Saúde (OMS) passou a considerar a epidemia pelo ZIKAV uma emergência de saúde pública de importância internacional (ECDC, 2015).

De acordo com o Boletim Epidemiológico do Ministério da Saúde, no Brasil, foram registrados, como casos prováveis, 37.011 casos de ZIKAV em 2015, 215.327 em 2016, 17.593 em 2017, 7.544 em 2018 e 10.768 em 2019. Já em 2020, só no primeiro semestre, foram notificados 905.912 casos prováveis (taxa de incidência de 431,1 casos por 100 mil habitantes). A figura 1 demonstra a propagação do ZIKAV da Floresta Zika em Uganda até as Américas (Figura 2).

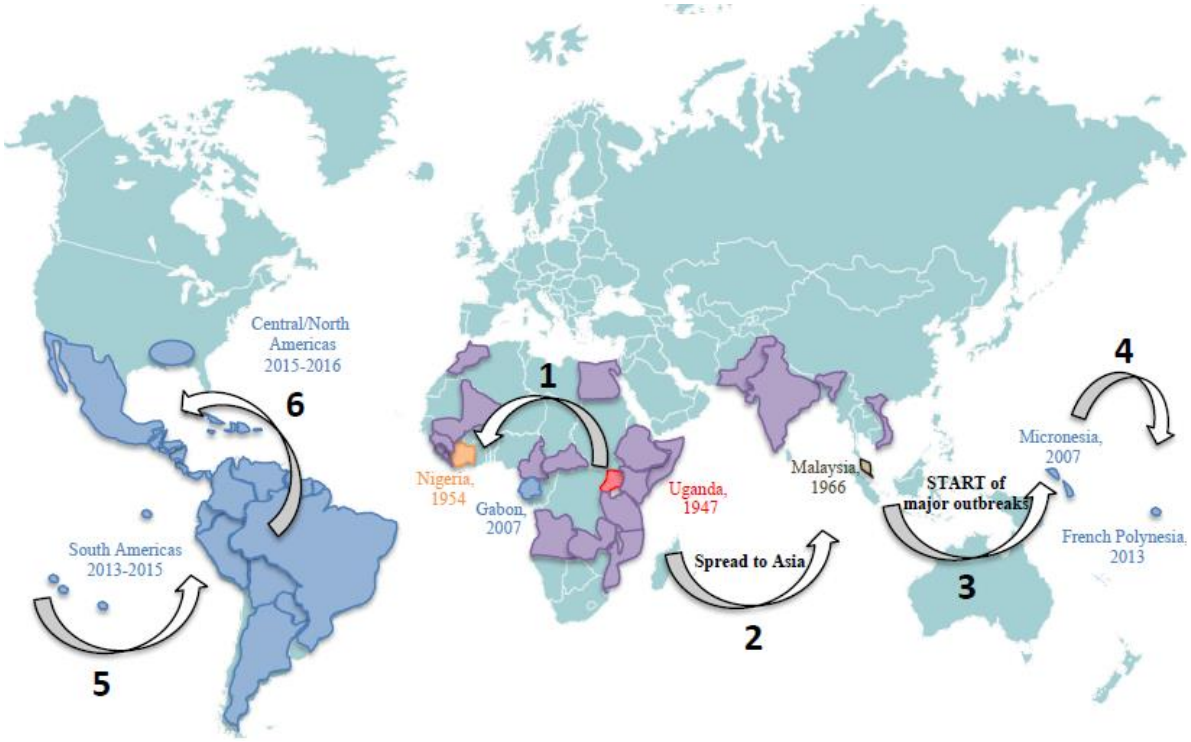

Figura 2 - Propagação do vírus Zika da Floresta Zika em Uganda até as Américas
Fonte: (MAUCOURANT; QUEIROZ et al., 2019).

2.3 MODOS DE TRANSMISSÃO DO ZIKV

O ZIKAV pode ser transmitido pela via vetorial, pela via sexual, pela via vertical, além de ser transmitido através de transplante de órgãos e doação de sangue (Figura 3). Na transmissão vetorial, as espécies de mosquitos do gênero *Aedes*, principalmente a espécie *Aedes aegypti* atuam como principal vetor. Além deste, existe relato de outras espécies transmissoras, como *Aedes albopictus*, *Aedes dalzieli*, *Aedes furcifer*, *Aedes hensilii*, *Aedes hirsutus*, *Aedes luteocephalus*, *Aedes metálico*, *Aedes taylori*, *Aedes unilineatus*, *Aedes vittatus*, dentre outros. Apesar das diversas espécies possuírem papel na transmissão do ZIKAV, devido sua grande adaptação ao ambiente urbano, acredita-se que o os surtos de 2015 ocorreram principalmente pela transmissão através do *Aedes aegypti* (LANCIOTTI et al., 2008; HAYES, 2009; KRAEMER et al., 2015; AYRES, 2016; SMARTT et al., 2017). Acredita-se também que em uma única picada o *Aedes aegypti* seja capaz de transmitir o Dengue vírus (DENV), o Zika vírus (ZIKV) e o Chikungunya vírus (CHIKV)(RUCKERT et al., 2017).

Além da transmissão vetorial, estudos mostram a infecção pelo ZIKAV associada a transmissão por via sexual (MUSSO et al., 2015a), devido à presença e persistência do vírus no sêmen e em fluidos vaginais (BARZON et al., 2016; MURRAY et al., 2017), a transmissão vertical por via transplacentária e a transmissão parenteral também já foram relatadas

(BESNARD et al., 2014; MUSO et al., 2014b; ADIBI et al., 2016). Em relação a localização, o ZIKV já foi observado na saliva humana (MUSSO et al., 2015b), no sangue (MUSSO et al., 2015a), além do sêmen e urina (GASKELL et al., 2017).

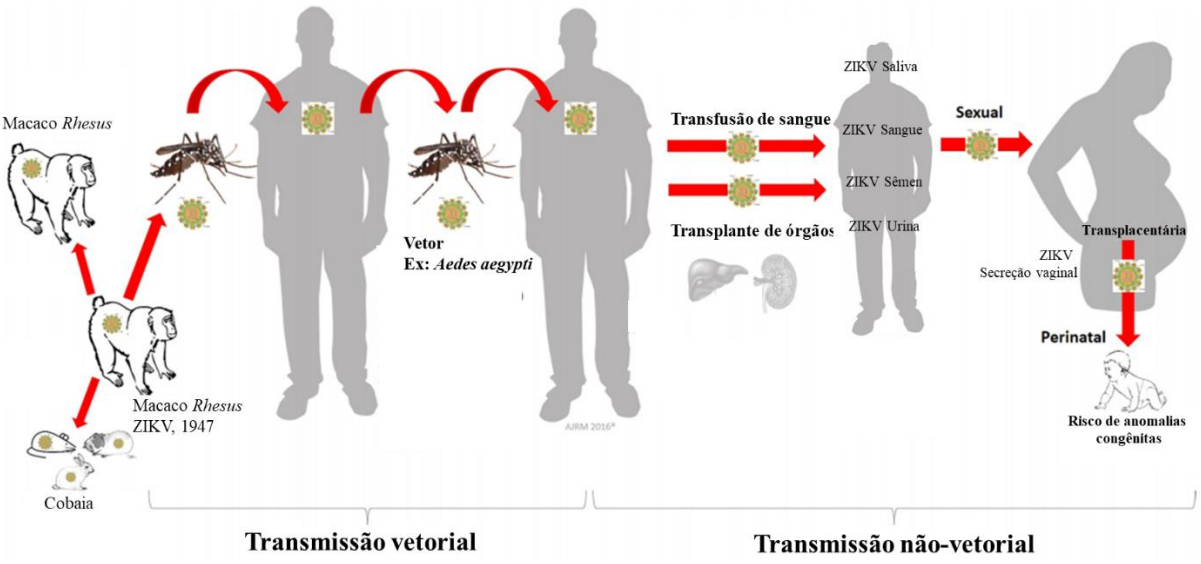

Figura 3 - Vias de transmissão do ZIKV
Fonte: (adaptado RODRIGUEZ-MORALES et al., 2016).

2.4 MANIFESTAÇÕES CLÍNICAS

Historicamente, o quadro clínico inicial da infecção pelo ZIKV foi principalmente associado a uma doença febril leve, autolimitada e de curta duração (GOULD; SOLOMON, 2008). Estima-se que 80% dos casos de infecções pelo ZIKV sejam assintomáticos e a maior parte dos casos sintomáticos não se associam com complicações graves. O período de incubação dura cerca de 10 dias e os sinais e sintomas são semelhantes a infecção pelo DENV, mas sem graves associações hemorrágicas. Geralmente os sinais mais frequentes na infecção pelo ZIKV são a febre baixa, erupção maculopapular pruriginosa e mialgia. Mas também são relatados cefaleia, conjuntivite, artralgia, dores de garganta e vômito (DUFFY et al., 2009; HAYES, 2009). Geralmente estes sintomas permanecem por três a sete dias, mas a mialgia e artralgia pode persistir por mais dias em alguns pacientes (TAPPE et al., 2014). Além destas manifestações, a infecção pelo ZIKV tem sido associada ao contexto de outros desfechos, como a síndrome de Guillain-Barré e a Síndrome congênita do ZIKV (CAO-LORMEAU et al., 2016).

2.4.1 **Síndrome de Guillain-Barré**

Evidências também mostraram a associação da infecção pelo ZIKAV ao desenvolvimento da síndrome de Guillain-Barré (SGB), uma neuropatia autoimune, pouco frequente caracterizado por fraqueza muscular progressiva dos membros e paraparesia dos membros superiores e inferiores, parestesias nas extremidades, mialgia, insuficiência respiratória, dentre outros (OEHLER et al., 2013; HUGH et al., 2016; PINHEIRO et al., 2016, WILLISON et al., 2016).

A incidência mundial da SGB é de aproximadamente 1 caso por 100.000 pessoas e em casos raros, pode levar à morte (IOOS et al., 2014). Em 2015, a OMS relatou 1708 casos de SGB no Brasil, embora isso deva ser interpretado com cautela, pois vários casos não foram testados para infecção por ZIKAV (WHO, 2016). As razões para o aumento na incidência de SGB no Brasil, mas também em El Salvador e Suriname são desconhecidas, particularmente porque potencialmente outros patógenos virais, como DENV ou CHIKV podem estar envolvidos. É importante ressaltar que a SGB na infecção pelo ZIKAV foi associada a uma maior morbidade durante a fase aguda, em comparação com o SGB desencadeado por outras etiologias (NAVECA et al., 2018).

2.4.2 **Síndrome congênita do ZIKAV**

Após a epidemia do ZIKAV que ocorreu no Brasil em 2015, um progressivo número de casos de crianças nascidas com microcefalia chamou a atenção das autoridades, que levou a uma série de investigações para verificar a transmissão transplacentária do vírus. Em 2016 a hipótese foi confirmada, quando o ZIKAV foi detectado o fluido amniótico de duas gestantes de fetos confirmados com microcefalia (CALVET et al., 2016; NUNES et al., 2016).

A infecção pelo ZIKV durante a gravidez pode levar a chamada Síndrome Congênita do ZIKV e a outra gama de alterações congênitas. Diversas infecções podem levar a síndromes congênitas, entretanto, Moore e colaboradores (2017), relatam que na infecção pelo ZIKV, a síndrome leva a alterações na morfologia craniana, anomalias cerebrais, anomalias oculares e contraturas congênitas. Além de restrições no crescimento intrauterino e baixo peso ao nascer. Estes autores listam cinco características principais que são observadas na síndrome congênita do ZIKV e raramente são observadas em outras síndromes congênitas, são elas: microcefalia severa; diminuição do tecido cerebral com um padrão específico de dano cerebral, incluindo calcificações subcorticais; danos na região anterior do olho, incluindo cicatrizes maculares e

mancha pigmentar da retina; contraturas congênitas e Hipertonia que restringe o movimento do corpo logo após o nascimento (MLAKAR et al., 2016; MOORE et al., 2017).

2.5 DIAGNÓSTICO LABORATORIAL

A infecção pelo ZIKV pode levar a alterações laboratoriais como leucopenia transitória e, em alguns casos, trombocitopenia. Além destes achados, pode-se observar elevação nas enzima séricas aspartato-aminotransferase/transaminase glutâmico-oxalacética (AST/TGP) e/ou alanina aminotransferase/transaminase glutâmico-pirúvica (ALT/TGP). Apesar disso, estes achados são inespecíficos e insuficientes para o diagnóstico diferencial do ZIKV (TAPPE et al., 2014).

O diagnóstico laboratorial da infecção pelo ZIKV é baseado na detecção do RNA viral por RT-PCR. Usualmente, o RNA viral no soro dos pacientes infectados é detectado somente até o quinto dia da infecção. Já na urina, é possível do RNA viral duas a três semanas da infecção (CAMPOS et al., 2015; GOURINAT et al., 2015; CHARREL et al., 2016; PESSÔA et al., 2016).

Embora a IgM específica seja detectada por ELISA, poucos laboratórios têm essa capacidade. O diagnóstico laboratorial é limitado devido à baixa viremia e a reatividade cruzada de anticorpos ZIKAV com outros Flavivirus (como DENV e vírus da febre amarela) e requer normalmente confirmação por ensaios de neutralização em placa (CHARREL et al., 2016).

2.6 A INFECÇÃO E PATOGÊNESE

A patogênese das complicações associadas a infecção pelo ZIKAV ainda é desconhecida. Além disso, não estão claros quais fatores estariam ligados aos desfechos clínicos, a exemplo da carga viral, fatores do hospedeiro, momento da infecção ou presença de outras comorbidades.

Sabe-se que o tropismo do ZIKAV por regiões específicas pode ter um importante papel nos desfechos clínicos e na transmissão do vírus. Estima-se que a infecção no mosquito transmissor se dá através da ingestão de sangue de indivíduos infectados durante a infecção aguda. Cerca de cinco a dez dias após o repasto, o vírus já pode ser detectado na saliva do vetor (WONG et al., 2013). A partir daí, durante novos repastos, o mosquito transmite o vírus para os seres humanos. Acredita-se que fibroblastos e queratinócitos da pele do indivíduo são permissivos à infecção pelo ZIKAV. Estas células possuem um importante papel na

disseminação inicial do vírus, mas os mediadores envolvidos neste processo, ainda não estão completamente elucidados. Estima-se que a etapa inicial do ciclo é a ligação do vírus ao(s) fator(es) de entrada na célula hospedeira, incluindo DC-SIGN, AXL e TYRO3 que demonstraram ser importantes para mediar a infecção pelo ZIKAV (HAMEL et al., 2015; NOWAKOWSKI et al., 2016).

Após a entrada, o ZIKAV se replica em macrófagos teciduais e células dendríticas que levam o vírus para os nódulos linfáticos e outros tecidos linfoides. São, assim, recrutados números aumentados de macrófagos que amplificam ainda mais a replicação viral (NGONO & SHRESTA, 2018) e assim, disseminação e tropismo do vírus para diversos tecidos e locais do organismo, como o sistema nervoso central (SNC), cérebro (progenitores neurais, neurônios maduros e astrócitos), olhos (células ganglionares, neurônios bipolares, nervo óptico, córnea, câmara anterior do humor aquoso), útero e vagina (epitélio vaginal, fibroblastos uterinos), placenta (células de Hofbauer, trofoblastos, células endoteliais), testículos (células de Leyding, células de Sertoli, espermatogônias), fluidos corporais (lágrimas, saliva, sêmen, muco cervical, urina) (CALVET et al., 2016; MLAKAR et al., 2016; MINER; DIAMOND 2017). Ainda assim, os mecanismos nos quais o vírus chega a estes locais ainda não estão totalmente esclarecidos.

2.7 RESPOSTA IMUNE

A resposta imune ao ZIKAV foi pouco avaliada até o momento, mas sabe-se que a resposta imune inata é uma das primeiras linhas de defesa do hospedeiro. Muitos receptores estão envolvidos na resposta imune inata contra vírus, como Toll-like receptores (TLR) e receptores induzidos pelo ácido retinoico I (RIG-I), que detectam diferentes padrões moleculares associados a patógenos e desencadeiam respostas antivirais produzindo interferons tipo I (IFNs), em particular IFN-β e múltiplos subtipos de IFN-α, que montam uma defesa inata rápida e potente contra vários vírus (MEYLAN et al, 2006; SADLER et al., 2008). A produção de IFNs tipo I é iniciada através do reconhecimento destes padrões moleculares associados ao patógeno (PAMPs), gerados durante a infecção viral (HONDA; TANIGUCHI, 2006). Apesar disso, a importância dos IFNs tipo I e III na imunidade inata antiviral do hospedeiro é destacada pela diversidade de estratégias virais para evadir essas respostas (VERSTEEG; GARCIA-SASTRE, 2010). A capacidade do ZIKAV de neutralizar (ou interferir com) a produção de IFNs tipos I e II poderia contribuir para sua capacidade de atravessar a placenta durante a gravidez e causar doença neuronal no feto em desenvolvimento (LUM et al., 2018)

Os IFNs tipo I e tipo III não são as únicas citocinas que são direcionadas (ou cuja produção é modulada) pelo ZIKAV. Um estudo longitudinal de uma coorte de pacientes infectados pelo ZIKAV em Cingapura revelou altos níveis de citocinas e quimiocinas inflamatórias, incluindo GM-CSF, IFN-γ, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-9, IL-17, IL-22, CXCL10, CCL2 e CCL5 (LUM et al., 2018) que foram identificados apenas na fase aguda da infecção viral, tornando-os marcadores úteis para infecção aguda pelo ZIKAV. Entretanto, a superexpressão de três mediadores pró-inflamatórios dominantes, IFN-γ, CXCL10 e CCL5, parece ser suficiente para gerar uma resposta anti-ZIKAV efetiva e, consequentemente, uma doença leve. O IFN-γ é importante neste contexto, pois coordena a transição da resposta imune inata à adaptativa, apoiando a ativação de macrófagos e o recrutamento de outras células imunológicas, como os linfócitos TH1, para o local da infecção (SCHNEIDER et al., 2014).

A imunopatologia dos distúrbios associados a síndrome congênita do ZIKAV ainda não estão bem descritos. Apesar disso, um mecanismo potencial para a microcefalia observada, é a capacidade do ZIKAV de infectar preferencialmente as células progenitoras neurais humanas e desencadear sua apoptose (CALVET et al., 2016). A capacidade da microglia de interagir com os tecidos infectados pelo ZIKAV também pode contribuir para uma maior disseminação do vírus no cérebro em desenvolvimento (VAN DEN POL et al., 2017). A ativação da microglia leva à produção de citocinas pró-inflamatórias como TNF-α, IL-1β, IL-6, IL-12 e moléculas citotóxicas, como o óxido nítrico, que agravam os danos inflamatórios (DIOP et al., 2018). Além disso, Naveca e colaboradores (2018) revelam também uma superexpressão de CXCL10, uma quimiocina ligada ao dano neuronal, durante a infecção aguda por ZIKAV (NAVECA et al., 2018). Além disso, uma extensa análise que avaliou 69 citocinas revelou que as quimiocinas CXCL10, CCL8 e CCL2 foram especificamente associadas à infecção sintomática por ZIKAV durante a gravidez e mais importante, que níveis elevados de CCL2 e sua correlação inversa com os níveis CD163, TNF-α e CCL22 estavam aparentemente associados ao nascimento anormal induzido pelo ZIKAV (FOO et al., 2018). Juntas, essas observações sugerem que o excesso de produção de algumas citocinas e quimiocinas induzidas por determinadas células em mulheres grávidas com ZIKAV poderia contribuir para os danos neuronais que afetam o desenvolvimento do cérebro fetal e o desenvolvimento da microcefalia.

Dados também sugerem que a resposta imune à infecção por ZIKAV pode estar parcialmente implicada na sintomatologia da síndrome de Guillain-Barré, embora a imunopatologia dos distúrbios associados Síndrome de Guillain-Barré no ZIKAV não esteja esclarecida. Sabe-se que as citocinas pró-inflamatórias desempenham vários papéis na patogênese da SGB, como TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-18 e IL-23, mas também

mediadores inflamatórios, como TGF-β, IL-4 e IL-10, bem como IL-27, que exerce efeitos pró-inflamatórios e anti-inflamatórios, (NYATI; PRASAD, 2014; WANG et al., 2017). Parece que IL-23 e IL-27, dois membros da família IL-12, estão mais particularmente associados à recuperação do GBS (PENG et al., 2018), enquanto o CXCL10 foi implicado na patogênese do GBS (CHIANG; UBOGU et al., 2013). Assim, hipotetizou-se que altos níveis de CXCL10 em pacientes com ZIKAV podem contribuir para danos neuronais que afetam o desenvolvimento do cérebro fetal e possivelmente atingir nervos periféricos na síndrome de Guillain-Barré, bem como (NAVECA et al., 2018).

2.7.1 **Células NK**

As células NK, foram identificadas pela primeira vez em 1975, fazem parte das células mononucleares do sangue periférico (CMSP) e participam da resposta imune inata (KIESSLING et al., 1975; JOSEPH e LEWIS, 2009). Estas células atuam especialmente na resposta contra vírus, sem necessitar de sensibilização prévia. Exercem também um papel fundamental na imunovigilância contra células cancerígenas (ZAMAI et al., 1998; CERWENKA; LANIER, 2001; BAR et al., 2014).

A expressão de moléculas CD16 e CD56, bem como a ausência da molécula CD3 na superfície das células NK, representam marcadores fenotípicos importantes para estas células. As células NK também não expressam o Receptor de Células T (TCR). Contudo, há uma população celular que expressa o TCR e o CD3, além das moléculas CD16 e CD56, são as células NKT (COOPER et al., 2001; CALIGIURI, 2008). Estas células atuam por meio da produção de citocinas e de mecanismos citotóxicos, eliminando células tumorais e células infectadas por patógenos intracelulares (JANEWAY et al., 2006). Além destes marcadores, o CD57 é um importante marcador de células senescentes com capacidade proliferativa reduzida e propriedades funcionais alteradas (KARED et al., 2016).

As células NK são um elemento-chave do sistema imunológico inato e representam uma defesa de primeira linha contra uma variedade de infecções virais. Eles desempenham tanto papéis antivirais quanto regulatórios através da liberação de fatores solúveis e operam através de um balanço de sinais inibitórios e ativadores que lhes permitem detectar e lisar células alvo infectadas por vírus. (O'CONNOR et al., 2005; LIANG et al., 2008; CHEENT; KHAKOO, 2009; CHAPLIN, 2010; GASSER ; RAULET, 2006; SUN; LANIER, 2011; VIVIER et al., 2011). Quando há um predomínio de sinais ativadores, a célula exibirá os seus mecanismos citotóxicos. Por outro lado, se houver mais sinais de inibição a célula não realizará a lise da célula alvo. Além disso, acredita-se que se os sinais de ativação e inibição forem equivalentes,

os sinais inibitórios prevalecerão. A atividade das células NK é regulada por um equilíbrio dos sinais relacionados a estes receptores. Estes receptores também possuem um papel essencial na produção de citocinas e na regulação da atividade lítica. (FARAG et al., 2002; ALMEIDA-OLIVEIRA; DIAMOND, 2008).

Os receptores de ativação e inibição, presentes na superfície das células NK e o seu papel no reconhecimento de células infectadas por vírus está relacionado ao equilíbrio na expressão desses receptores e da ligação com seus respectivos ligantes na membrana da célula alvo. Desta forma, a interação destes receptores pode determinar a atividade efetiva das células NK sobre a célula alvo. (LIMA et al., 2001; KELLEY et al., 2005; CHEENT; KHAKOO, 2009; IGLESIAS et al., 2009). Os receptores são classificados de acordo com a estrutura, tipo de ligante ou função. Com base na estrutura, esses receptores são divididos em quatro famílias principais: Receptores de Citotoxicidade Natural (NCR, *Natural Cytotoxicity Receptors*)*;* Receptores Killer Semelhantes às Imunoglobulinas (KIR, *Killer cell immunoglobulin like receptors*); Receptores Killer tipo Lectina (KLR, *Killer Cell Lectin-Like Receptors*) ou Receptores da família semelhante a lectina do tipo-C e Receptores Leucocitários semelhantes às imunoglobulinas (LILR, *Leucocyte Ig-Like Receptors*). Para as famílias KIR, KLR e LIRL, em geral, os ligantes são moléculas de MHC, mas para a família dos NCR, os ligantes ainda são pouco conhecidos (LIMA et al., 2001).

Os Receptores de Citotoxicidade Natural (NCR, *Natural Cytotoxicity Receptors*) são compostos apenas por receptores de ativação. Dentre eles, o NKp30 (CD336 ou NCR2), NKp46 (CD335 ou NCR1) e o NKp44 (CD337 ou NCR3). A nomenclatura atribuída a cada NCR está ligada ao seu peso molecular. Acredita-se que o NKp44 seja expresso apenas em células NK ativadas, enquanto o NKp46 e o NKp30 são expressos constitutivamente nas células NK circulantes. Outros autores relatam que estes receptores não estão restritos a células NK, e foram detectados em graus variáveis na maior parte dos tipos de células hematopoiéticas (MCMAHON; RAULET, 2001; FOSTER et al., 2003; ALMEIDA-OLIVEIRA; DIAMOND, 2008a-b.

Os Receptores Killer Semelhantes às Imunoglobulinas (KIR, *Killer cell immunoglobulin like receptors*), apresentam receptores com função de ativação e de inibição. Os nomes atribuídos a cada KIR é baseado na estrutura da proteína. Letra D, relacionada a quantidade de domínios extracelulares semelhantes a imunoglobulinas, letra L (*large),* quando a cadeia citoplasmática é longa, ou S (*short)* quando curta, e por fim, um número, de acordo ao número de genes no cromossomo (MORETTA; MORETTA, 2004; MARANGON et al., 2008).

Os Receptores Killer tipo Lectina (KLR, *Killer Cell Lectin-Like Receptors*) ou Receptores da família semelhante a lectina do tipo-C, estão presentes em células NK e compõem receptores de ativação e de inibição. Compõem este grupo os receptores de inibição NKG2A (CD159 ou CD94) e o NKGK2B, e os receptores de ativação NKG2D (CD314) e NKR-P1A (CD161). (LEE et al., 1998; PAROLINI et al., 2000; RAULET, 2003; LI., et al 2001; KIM et al., 2005; CHEENT e KHAKOO, 2009; ORR; LANIER, 2010).

A exaustão celular é caracterizada pela perda progressiva das funções celulares e culmina com a apoptose das células exaustas. Geralmente está associada a uma estimulação antigênica persistente. Durante o processo de exaustão, ocorre gradativamente aumento na quantidade e na diversidade dos marcadores de exaustão. Dentre esses marcadores estão o PD-1 (Morte Programada), TIM-3 (Domínios de mucina e imunoglobulina de célula T), CTLA-4 (Antígeno 4 associado ao linfócito T citotóxico), ICOS (Coestimulador induzível de células T) e LAG-3 (Gene 3 de ativação linfocitária) (GOLDEN-MASON et al., 2009; WHERRY et al., 2007; WHERRY et al., 2011)

A célula NK também é o principal tipo de célula envolvida na citotoxicidade mediada por células dependente de anticorpos (ADCC). A expressão do receptor FcγRIII (CD16) liga-se a uma porção Fc de um anticorpo na membrana de uma célula infectada, em seguida a célula NK pode liberar IFN-γ, perforina e granzimas para realizar a lise das células alvo (CLYNES et al., 2000; JANEWAY et al., 2006).

As células NK possuem mecanismos de citotoxicidade, através da liberação de grânulos citotóxicos e citocinas que são essenciais para o controle de células infectadas (HENKART, 1985; CARVALHO et al., 2001; JANEWAY et al., 2006). A indução de apoptose na célula alvo consiste no principal mecanismo de controle das infecções. O IFN-γ, dentre outras funções, atua como um mediador químico que promove um aumento da expressão de MHC-I e auxilia o processamento antigênico, favorecendo uma resposta destas células contra as células alvo (CARVALHO et al., 2001; ABBAS et al., 2008).

Os grânulos citotóxicos contêm moléculas proteicas de perforina, granzimas, granulisina, serglicina e catepsinas que são fundamentais no processo de citotoxicidade celular. Durante o processo de degranulação, glicoproteínas presentes na membrana de vacúolos que contêm estes grânulos, como a molécula CD107a (LAMP-1), ficam retidas transitoriamente na membrana da célula citotóxica, sendo este um importante marcador de degranulação recente (PETERS et al., 1991; KRENSKY, 2000; TRAPANI, 2001; AKTAS et al., 2009; DE SAINT BASILE et al., 2010).

Quando a célula citotóxica é estimulada e libera o conteúdo dos vacúolos granulares de perforina, se ligam à membrana da célula alvo e formam poros, de 5 a 20 nm na bicamada lipídica (SAUER et al., 1991; KAGI et al., 1994; LOWIN et al., 1994; PIPKIN & LIEBERMAN, 2007). Estes poros alteram a integridade da membrana da célula alvo e promovem um desequilíbrio hidroeletrolítico, que associado a entrada de outras moléculas pro-apoptóticas presentes podem levar estas à morte (BARRY; BLEACKLEY, 2002; PIPKIN; LIEBERMAN, 2007).

Até o momento, poucos dados sobre o envolvimento de células NK na infecção por ZIKAV foram relatados na literatura (GLASNER et al., 2017; LUM et al., 2018; MAUCOURANT et al., 2019). Mas dados sugeriram que as células infectadas com ZIKAV realizaram uma suprarregulação do MHC de classe I, mediada pelo feedback positivo de IFN-β e consequente inibição da atividade das células NK. Isso se deve ao fato de que o receptor KIR2DL2 e Receptores LILRB1 podem exibir ligação aumentada com HLA-A (KIR2DL2, LILRB1), HLA-B (LILRB1) e HLA-C (LILRB1), facilitando assim o escape imunológico de ZIKV (GLASNER, 2017). Entretanto, outro estudo relatou que a infecção por ZIKAV induz a expressão de muitos genes estimulados por IFN-γ (CHAUDHARY et al., 2017). Além disso, outros grupos mostraram que o pré-tratamento de fibroblastos com IFN-γ restringe a replicação do ZIKAV (HAMEL et al., 2015), possivelmente através do início de um mecanismo de feedback inibitório. Lum e colaboradores (2018b), realizaram uma Imunofenotipagem de células do sangue periférico de pacientes infectados com ZIKV e observaram que o vírus foi capaz de contrabalançar a atividade de monócitos e/ou células NK devido a superexpressão de CXCL9, CXCL10, CXCL11 e CCL5 durante a fase aguda da infecção. Além disso, níveis de IL-23, IL-27 e IL-12, também aumentaram, bem como a análise do transcriptoma revelou que IL-18, TNF - α, CD107 e IFN-γ potencializaram a atividade das células NK. O estudo realizado por Schanoski e colaboradores (2019), avaliou a granzima A (GzmA), que é um importante marcador de degranulação e morte celular. Os resultados mostraram um grande aumento de GzmA em indivíduos durante a infecção na fase aguda de ZIKV. Tal resultado pode pressupor uma grande participação das células NK como células de defesa da infecção. Em um outro estudo, que avaliou casos fatais de Microcefalia induzida por ZIKV, os autores observaram que células NK contribuíram diretamente para o dano celular. Células NK marcadas com CD57 induziram a produção de IFN-γ no parênquima neural e no quadro inflamatório perivascular. Essas células também exibiram a expressão de caspase 3, TNF-α, IFN-α e IFN-β (AZEVEDO et al., 2018).

Nos trabalhos com modelos animas, um estudo que avaliou macacos *Rhesus* como modelo experimental, as células NK foram associadas à uma resposta antiviral eficiente, através da secreção de granzimas e perforinas que eliminam células infectadas por vírus, principalmente durante o pico da viremia (SILVEIRA et al., 2017). Além disso, um estudo preliminar também em macacos *Rhesus* mostrou que a via de inoculação está diretamente relacionada à resposta das células NK. Neste contexto, a infecção por ZIKAV por inoculação intravaginal nestes animais diminuiu a expressão da proteína 1 de morte celular programada (PD-1) em Células NK (WOOLLARD et al., 2018).

Assim, o entendimento do papel entre os receptores das células NK, bem como os grânulos citotóxicos e citocinas envolvidas na infecção e o seu desfecho no desenvolvimento da imunidade e patogênese do ZIKAV, ainda necessita de mais investigação. Desta forma, embora muitos estudos estejam sendo desenvolvidos em indivíduos infectados pelo ZIKAV, a patogênese desta doença e papel das células NK na infecção ainda não são bem conhecidos. Estudos com estas avaliações podem ajudar a compreender a influência da resposta de células NK nestes pacientes.

3 **OBJETIVOS**

3.1 GERAL

Avaliar a resposta das células Natural Killer (NK) de indivíduos infectados pelo Vírus Zika.

3.2 ESPECÍFICOS

- Revisar a literatura destacando o papel das citocinas e quimiocinas na infecção pelo ZIKAV e suas ligações potenciais à patogênese;
- Avaliar o fenótipo e a função das células NK por citometria de massa (CyTOF);
- Realizar ensaio funcional de infecção de Mo-DCs com ZIKV, K562 e cocultura com células NK;
- Avaliar a degranulação de células NK e produção intracelular de citocinas por citometria de fluxo;
- Realizar Análise de PCR em tempo real de IL-12 p35 e IL-12 p40.

4 **CAPÍTULO 1 - ARTIGO 1**

Para entender o papel das citocinas e quimiocinas na infecção pelo ZIKAV e suas ligações potenciais à patogênese foi realizada uma revisão da literatura intitulada “Zika virus in the eye of the cytokine storm”, que foi publicada no Periódico Eur. Cytokine Netw. Vol. 30, n° 3, Setembro de 2019 74-81, apresentado no capítulo 1 desta tese.

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REVIEW

Zika virus in the eye of the cytokine storm

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ABSTRACT. Zika virus (ZIKV) is an emerging arbovirus that causes a mosquito-borne disease. Although infection with ZIKV generally leads to mild disease, its recent emergence in the Americas has been associated with an increase in the development of the Guillain-Barré syndrome in adults, as well as with neurological complications, in particular congenital microcephaly, in new-borns. Over the five past years, through the combined efforts of the scientific community, comprehensive remarkable progress aimed at deciphering the clinical, virological, physiopathological, and immunological features of ZIKV infection. This review highlights some of the most recent advances in our understanding of the role of cytokines and chemokines in ZIKV infection, and discusses potential links to pathogenesis.

Key words: Zika virus, Flavivirus, Cytokines, Chemokines, Microcephaly, Guillain-Barré syndrome

INTRODUCTION

Zika virus (ZIKV) is an emerging arbovirus of the *flavivirus* genus discovered in 1947 near Entebbe, Uganda, where it circulates in the forests between nonhuman primates and sylvatic mosquitoes [1]. Until 2007, ZIKV has silently circulated in many parts of Africa and Asia, with less than 20 documented human infections that represented only cases of spillover transmission from the sylvatic cycle, in which humans became infected as an accidental host [2]. The first large outbreak of human infection by ZIKV occurs in 2007 in Micronesia [3], followed by the one in French Polynesia in late 2013 [2], that subsequently spread across the Pacific to the Americas in a short timeframe [4] (*figure 1*). Nowadays, ZIKV is making headlines around the world, and the World Health Organization (WHO) has declared a public health emergency of international concern for this virus [5].

For more than sixty years, the early clinical picture of natural human ZIKV infection has mainly been associated with a self-limiting, mild febrile illness of short duration. However, like many other flaviruses, including yellow fever (YFV), dengue (DENV), and West Nile (WNV) virus [6], ZIKV has turned out to be a significant human pathogen. During the outbreak in Micronesia in 2007, ZIKV disease started to be associated with rash, high fever, arthralgia, and conjunctivitis, whereas during later outbreaks, several cases of Guillain-Barré syndrome were observed in French Polynesia, as well as meningoencephalitis in the Pacific Islands, and myelitis in Guadeloupe [7-9]. Most strikingly, the ZIKV epidemic in Brazil in 2015 has brought to light a temporal relation between fetal microcephaly and ZIKV infection of the childbearing mothers during the first trimester of pregnancy [10-12], prompting several national agencies to issue advisories to pregnant women and those considering pregnancy. Additionally, several case reports on sexual transmission of ZIKV [13], due to its persistence in semen [14] and vaginal secretions [15], have been published. Thus, unlike other flaviviruses, ZIKV is now characterized by its capacity of transplacental and sexual transmission, causing life-threatening neurological complications, that has highlighted its dangerousness (*figure 2*).

How did ZIKV, considered as an obscure and low-pathogenic mosquito-borne flavivirus for more than 60 years, emerge from its sylvatic forest existence in Africa and Asia to cause major epidemics throughout the Pacific and the Americas? Several not mutually exclusive possibilities have been proposed: First, with respect to the evolution of ZIKV strains, results from recent studies combining reverse genetics with mathematic models have provided evidence that ZIKV has acquired amino-acid substitutions around the same time as the detection of congenital Zika syndrome and other birth defects [16]. Second, it can be assumed that the intensification of the globalization process, associated with a modern lifestyle, could amplify an epidemic through travel of naive, non-naturally immunized individuals. Third, environmental elements, in partic-

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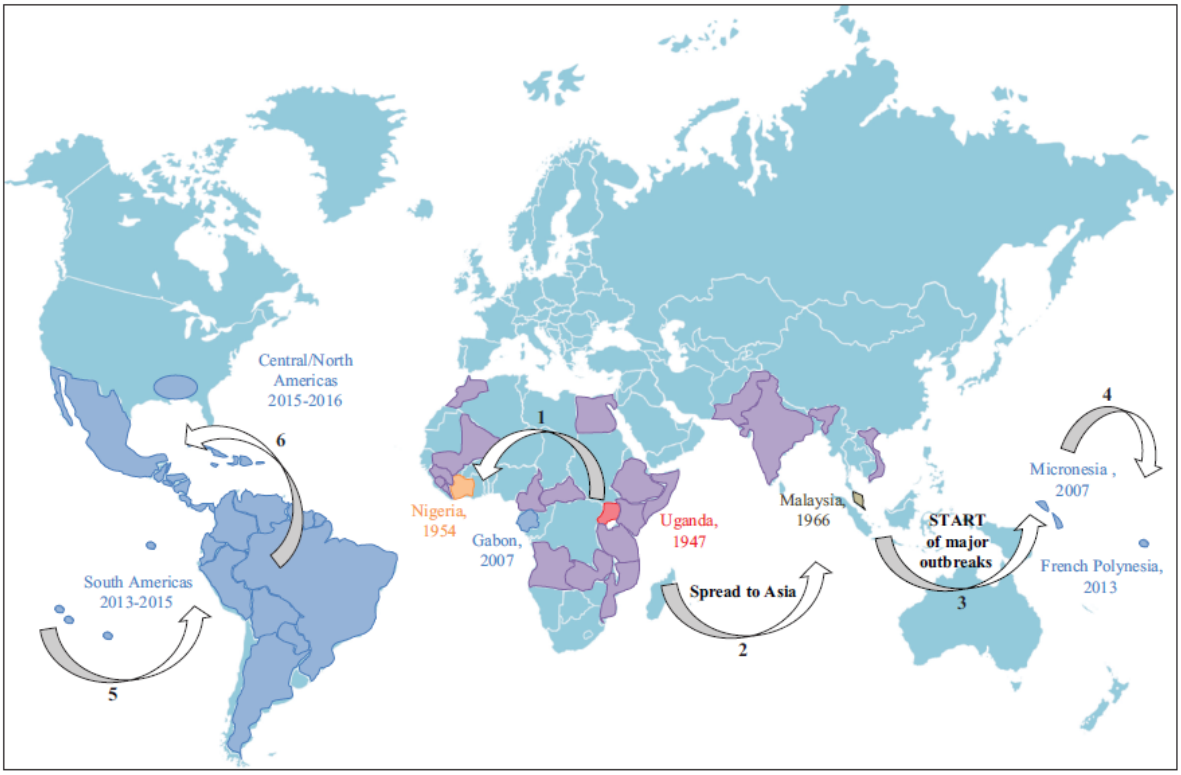

Figure 1

ZIKV spread from Africa to the Americas. (1) 1947: First documented in monkeys in Uganda. (2) 1960: First documented human cases in Nigeria. (3) 1970s: First cases in Asia. (4) 2007: Epidemic on island of Yap, Micronesia; (5) 2013-2015: Epidemic on French Polynesia and then through South America; (6) 2014-2016: ZIKV appears in northern Brazil and spreads through Central and North America.

ular climate change, are likely of critical importance for the survival and spread of mosquitoes.

Several mosquito species belonging to the Aedes genus have been identified as potential transmission vectors for ZIKV, and more especially *Ae aegypti* and possibly *Ae albopictus*, for their wide and increasing spread [17, 18]. As previously described for other flaviviruses, viral dissemination occurs via the skin, at the site of the mosquito bite, into the extracellular space of the dermis [19]. The initial step in the life cycle of flaviviruses is attachment of the virion to host-cell entry factor(s), including DC-SIGN, AXL, and TYRO3 that have been shown to be important for mediating ZIKV infection [19, 20], underscoring the pantropic nature of ZIKV. Following entry, ZIKV replicates in tissue macrophages and dendritic cells that traffic the virus to the draining lymph nodes and other lymphoid tissues. Increased number of macrophages is thus recruited that further amplify viral replication [21]. The "cytokine cascade" engaged during this early process of ZIKV infection will be discussed in this review.

THE "CYTOKINE CASCADE" IN ZIKA FEVER

The innate immune response is the first line of host defense against a viral infection. Multiple host pattern recognition receptors expressed on innate immune cells, including Toll-like receptors (TLR) and retinoic acid-inducible gene I (RIG-I)-like receptors, detect different pathogen-associated molecular patterns and trigger antiviral responses by producing type I interferons (IFNs), in particular IFN-β and multiple subtypes of IFN-α, that mount a rapid and potent innate defense against a number of viruses [22, 23]. Production of type I IFNs is initiated through recognition of pathogen-associated molecular patterns (PAMPs), generated during viral infection [24]. Binding of type I IFNs to their receptor, composed of two subunits (IFNAR1 and IFNAR2), activates the Janus kinases, Jak1 and Tyk2, as well as the signal transducers of transcription, STAT1 and STAT2, resulting in the upregulation of hundreds of IFN-stimulated genes (ISGs), whose activity restricts viral replication through a broad range of mechanisms [25]. The importance of type I IFNs in host antiviral innate immunity is highlighted by the diversity of viral strategies to evade these responses [26], some of which have been demonstrated for DENV, WNV, and YFV. It seems that the flavivirus nonstructural NS5 protein, which encodes both the viral methyltransferase and the RNA-dependent RNA polymerase, required for viral RNA synthesis, plays a key role in the inhibition of IFN-signaling by different mechanisms, depending on the flavivirus. WNV NS5 targets the host prolidase protein to prevent surface expression of IFNAR1 [27]. By contrast, YFV NS5 binds directly to STAT2, to prevent its binding to the IFN-stimulated responsive elements (ISRE) present upstream of ISGs [28], whereas DENV NS5 recruits the host E3 ubiquitin

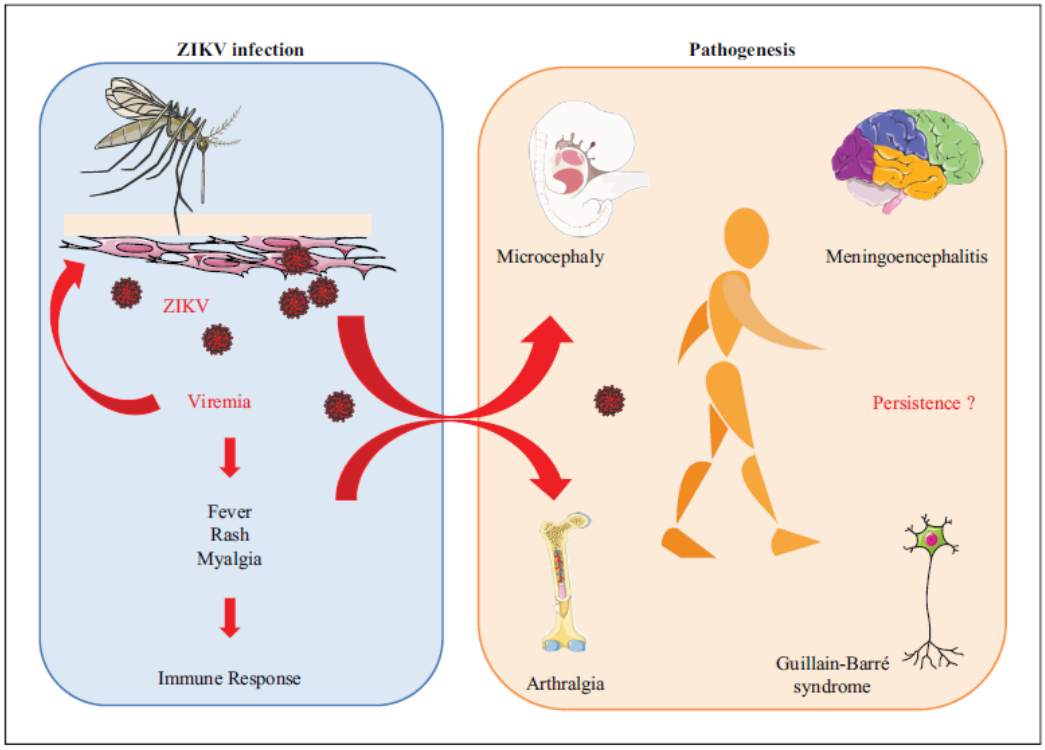

Figure 2

Virus dissemination, immune responses, and clinical manifestations in ZIKV-infected patients. ZIKV is transmitted through the bite of a female Aedes mosquito. Following infection of permissive cells in the dermis, such as endothelial cells, fibroblasts and macrophages and the virus replicates rapidly. Locally produced viral particles are transported through the circulatory system to secondary lymphoid organs, then disseminated to different organs. The acute phase of infection is associated with a high release of type 1 IFNs, followed by the up- or down-modulation of many other cytokines and chemokines, in association with the development of specific cellular immune responses. The infection by ZIKV can be accompanied by a spectrum of diseases, ranging from self-limiting meningo-encephalitis to congenital birth defects, like microcephaly, or Guillain-Barré syndrome.

ligase UBR4 to degrade STAT2 [29]. In ZIKV, NS5 expression results in proteasomal degradation of the IFN-regulated transcriptional activator STAT2, however, unlike DENV, via a UBR4-independent process [30]. This ability to strongly inhibit type I IFN responses has been proposed to favor a mild infection that allows flaviviruses to persist in the host and cause long-term defects [31].

As flavivirus NS5 proteins exhibit a remarkable, albeit virus-specific, functional convergence in their IFN type I antagonism, it is likely that ZIKV can also evade type III IFN (IFN-λ) signaling through STAT2 degradation via NS5. IFN-λ is a key cytokine produced abundantly at mucosal sites by epithelial and myeloid cells in response to viral infection [32]. Its induction and subsequent action at the epithelial layer of the vagina depends on the hormone-dependent stage of the estrous cycle [33]. Furthermore, IFN-λ protects trophoblasts, a layer of barrier cells in the human placenta from ZIKV infection [34, 35]. The ability of ZIKV to escape from the action of IFN-λ could contribute to its capacity to cross the placenta during pregnancy and cause neuronal disease in the developing fetus (see below).

Type I and type III IFNs are not the sole cytokines that are targeted by ZIKV. A longitudinal study from a Singapore cohort of ZIKV-infected patients revealed high levels of inflammatory cytokines and chemokines, such as GM-CSF, IFN-γ, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-9, IL-17, IL-22, CXCL10, CCL2, and CCL5, that were specifically identified in the acute phase of viral infection [36]. Most of these factors were previously described in two independent studies of Brazilian patients acutely infected by ZIKV [37, 38], making them useful markers for acute ZIKV infection. However, the over-expression of three of these pro-inflammatory mediators, IFN-γ, CXCL10, and CCL5, appears to be sufficient for generating an effective anti-ZIKV response, and consequently, a mild disease (*figure 3*). IFN-γ is unique in its action because it coordinates the transition from innate to adaptive immune responses by supporting macrophage activation and recruitment of other immune cells, like T_H1 lymphocytes, to the site of infection [39]. The expression of IFN-γ is limited to cells of the immune system and mainly to natural killer (NK) cells. These cells are a key element of the innate immune system, and represent a first-line defence against a variety of viral infections. They play both antiviral and regulatory roles via the release of soluble factors and operate via a balance of inhibitory and activating signals that enable them to detect and lyse virus-infected target cells [40-42]. To date, few data on the involvement of NK cells in ZIKV infection have been reported in the literature [43-45]. Our own data, obtained in a cohort of Gabonese patients, point to an early and transient

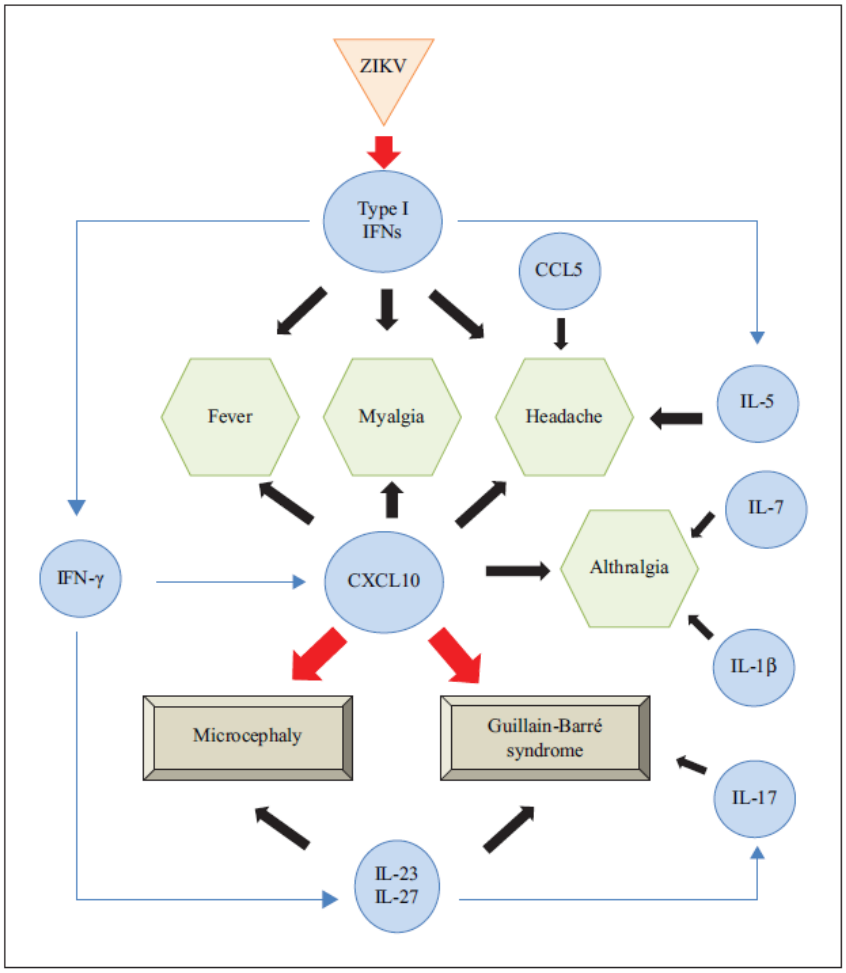

Figure 3
Model cytokine/chemokine pathways in the development of clinical symptoms mediated by ZIKV infection.

accumulation of specific NK cells, called adaptive NK cells, following an acute infection by DENV-2 that was absent in healthy donors [46]. This "clonal" expansion of adaptive NK cells, also described in other viral infections, such as Chikungunya, and cytomegalovirus [47, 48], is associated with a functional decoupling, in which IFN-γ production seems sufficient to control a DENV infection, in the absence of cytotoxicity (Petitdemange, Maucourant et al. Personal data). Upregulation of IFN-γ production by NK cells was also observed in WNV infection [49] and preliminary data suggested that NK cells from ZIKV-infected patients also produced high level of IFN-γ [43]. Interestingly, ZIKV infection has been reported to induce the expression of many IFN-γ-stimulated genes that, likely due to NS5-mediated depletion of STAT2, shift the STAT1-STAT2 balance toward STAT1, resulting in more STAT1 homodimers available to preferentially induce the transcription of IFN-γ-stimulated genes [50]. However, other groups have shown that pretreatment of human foreskin fibroblast with IFN-γ restricts ZIKV replication [19], possibly through the initiation of an inhibitory feed-back mechanism. Thus, the interplay between IFN-γ and ZIKV pathogenesis needs further investigation.

Monocytes, one of the first targets infected in the peripheral blood, also play a key role during the acute phase of infection by ZIKV, as highlighted by the high levels of GM-CSF and CCL2 produced early after infection [44]. CCL2 associated with IL-8 and CXCL10 was recently correlated with high viremia in symptomatic patients [36]. During the later phase of ZIKV infection, concentrations of IL-1ß, IL-2, and CCL2 in the sera, produced during the acute phase, were not decreased in ZIKV-infected patients during the later phase of infection, in contrast to DENV fever. However, in both infections, very high concentration of IL-10 was detected tardily [51-53]. It is of note that contrasting results were reported in these different studies for several other proinflammatory cytokines, like TNF-α and IL-6. Several hypotheses could explain these discrepancies, but the marked difference seems to be the studied populations. It seems that acute ZIKV infection in resident to an endemic area displays a modest proinflammatory systemic immune-activation profile, compared to non-residents individuals [38]. This suggests that constant exposure to ZIKV, and possibly other environmental factors, may affect the immunological inflammatory impact of infection. However, most studies to date have focused on direct

measurements of cytokines and chemokines in the peripheral blood compartment and have failed to interrogate the whole of the immune cascade in the context of the infecting pathogen and the rapidly changing immune environment in tissues.

In this context, it is also important to point out that immune cross-reactivity with other flaviviruses could be beneficial and result in cross-protection [54]; on the other hand, humoral cross-reactivity can also exacerbate disease through the process of antibody-dependent enhancement (ADE), of which DENV is the prototypic model [55]. Primary DENV infection results in a mild, acute disease with production of efficacious neutralizing antibodies, in which virus-antibody complexes are recognized by the Fc Receptor, internalized and destroyed. Problems may arise when a second DENV infection of a different serotype occurs, as the antibodies produced during the first infection can recognize and bind the second infecting strain, but with sub-neutralizing capability. For DENV, ADE has been associated with lower levels of innate immune mediators, such as nitric oxide or type I IFNs, and high production of IL-10 [55]. Whereas experimental evidence has demonstrated both a preventive, as well as a pathogenicity-enhancing role, of preexisting DENV antibodies in ZIKV infections, to date, ADE has not been confirmed in ZIKV [56, 57]. Because most countries with confirmed ZIKV cases are also endemic for DENV, there is a higher probability that ZIKV infection and immune response intensity may be amplified, owing to preexisting DENV cross-reactive antibodies; this should be a concern, particularly during vaccine development.

CYTOKINES ASSOCIATED WITH ZIKA-RELATED DETRIMENTAL NEUROGENESIS

Recent widespread outbreaks had brought ZIKV into spotlight, in particular because of the presumed causal relationship between infection and adverse fetal microcephaly. This has prompted the WHO to declare a Public Health Emergency of International Concern in February 2016 [58], and to advocate research into possible causal relationships and underlying mechanisms of ZIKV-induced neurologic disorders.

Fetal abnormalities

Pregnancy is a sophisticated biological process that relies on maternal-initiated immunosuppression toward the growing fetus particularly [59]. Because of the temporal and geographical overlap between the emergence of fetal microcephaly and the outbreak of ZIKV, the hypothesis was formulated that fetal microcephaly was caused by ZIKV infection during pregnancy [60, 61]. In the United States, there have been approximately 2,500 pregnant women infected with ZIKV and 116 infants born with ZIKV-associated birth defects since 2015. However, the majority of birth defects were reported in Brazil, which accounted for almost 400,000 cases of ZIKV infections and approximately 1,700 cases of neonates with confirmed microcephaly in 2015 [58].

Microcephaly results from any insult that disturbs early brain growth, and can be caused by genetic variations, teratogenic agents, or other congenital infections [62]. One potential mechanism for the observed microcephaly is the capacity of ZIKV to preferentially infect human neural progenitor cells and to trigger their apoptosis [63]. The capacity of the microglia to interact with ZIKV-infected tissues could also contribute to further spreading of the virus in the developing brain [64]. Activation of microglia leads to the production of pro-inflammatory cytokines, like TNF-α, IL-1β, IL-6, IL-12, and cytotoxic molecules, such as nitric oxide that aggravate inflammatory damage [65, 66]. In an extensive multiplexing analysis of 69 cytokines from a large cohort of pregnant women, high expression of CXCL10, in addition to CCL8 and CCL2, was associated with ZIKV-induced abnormal birth [67].

The majority of mediators modulated by ZIKV in pregnant women was also involved in the recruitment of monocytes and NK cells, like type I IFNs, IL-12, CXCL10, CCL8, or CCL2 [68, 69]. An excessive infiltration of both cell subsets at the maternal-fetal interface has previously been linked to pregnancy complications, such as preeclampsia and preterm birth [70, 71]. Instead, at the human implantation site, the predominant population of immune cells consists of uterine NK (uNK) cells and macrophages, which may comprise about 90% of all leukocytes, that are important for the control of placentation [72]. It is thought that the main function of uNK cells is to produce cytokines, such as TNFα, TGFβ, and IFNγ, as well as IL-1β and IL-10 [73]. Such production is regulated by inhibitory and activating receptors binding to HLA class I on trophoblast cells, but their role during ZIKV infection remains totally elusive. Interestingly, Foo *et al* [74] have recently shown that ZIKV infection promoted the dramatic expansion of nonclassical $CD14^{lo}CD16^{+}$ monocytes and an apparent production of the IL-10 in blood from the first and second trimesters of pregnancy; this cytokine produced by inflammatory monocytes and NK cells is known to promote viral persistency and to dampen host defenses [75], and can be detected at high level in the amniotic fluid of pregnant ZIKV patients who had microcephalic fetuses or neonates [76]. Production of IL-10 is certainly a marker of a counter anti-inflammatory response that has been termed "immunoparalysis." Downregulation of systemic inflammation by IL-10 in ZIKV may be conceptually beneficial in controlling systemic responses to local infection, but also detrimental with the development of fetal abnormalities in ZIKV-infected pregnant women.

Together, these observations suggest that excess production of certain factors, like IL-10 and CXCL10, driven by specific monocytes and uNK cells in $ZIKV^{+}$ pregnant women could contribute to neuronal damage affecting the developing fetal brain and the development of microcephaly.

Guillain-Barré syndrome

Convincing evidence has also associated ZIKV infection with the development of Guillain-Barré syndrome

(GBS), an infrequent autoimmune disorder characterized by progressive muscle weakness of limbs and areflexic paralysis [77]. GBS is the most common cause of neuromuscular paralysis and, in rare cases, may lead to death. Its worldwide incidence is approximately 1 case per 100,000 people. The first challenge to the apparently benign nature of ZIKV infection occurred during the outbreak in French Polynesia, which began in October 2013, when 45 individuals developed GBS [78]. In 2015, the WHO reported 1708 cases of GBS in Brazil, although this must be interpreted with caution because several cases were not tested for ZIKV infection [79]. The reasons for the increase in the incidence of GBS in Brazil, but also El Salvador, and Suriname are unknown, particularly because potentially other viral pathogens might be involved in particular DENV or CHIKV that are co-circulating in these countries, during the same period. Importantly, GBS associated with ZIKV infection was found to be associated with a higher morbidity during the acute phase, compared with GBS triggered by other etiologies [66]. This suggests that the immune response specific to ZIKV infection could be partially implicated in the symptomology of the GBS.

Pro-inflammatory cytokines play various roles in the pathogenesis of GBS, such as TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-17, IL-18, and IL-23, but also anti-inflammatory mediators, like TGF-β, IL-4 and IL-10, as well as IL-27, that exerts both pro-inflammatory and anti-inflammatory effects, [69, 80]. It seems that IL-23 and IL-27, two members of the IL-12 family, are more particularly associated with the recovery of GBS [81], whereas CXCL10 has been implicated in GBS pathogenesis [82] (*figure 3*). Thus, it was hypothesized that high levels of CXCL10 in ZIKV patients may contribute to neuronal damage affecting the developing fetal brain and potentially targeting peripheral nerves in Guillain-Barré syndrome as well [66].

CONCLUDING COMMENTS

Across the world, infectious diseases remain a real threat, accounting for approximately half of all deaths each year. Tuberculosis, malaria, AIDS, influenza, as well as endemic and (re)emerging flavivirus infections, like ZIKV, all contribute to morbidity and mortality. Economic development, urbanization, and environmental degradation gather pace, whereas the structure of societies changes, creating a "perfect storm" for the future spread of ZIKV, leading to new challenges in the future. Against this backdrop and the absence of an effective vaccine against ZIKV infection, although actively sought [83, 84], increasing interest has focused on the development of drugs that target the cytokine response following ZIKV infection. The virus/host interaction is a complex interplay between pro- and anti-viral components that ultimately determines the spread or halt of virus infections in tissues. Integrating the data listed above in this review reveals the role for certain key cytokines in the pathology of ZIKV (*figure 3*). High production of CXCL10 and IL-10 is associated with several aspects of ZIKV-related detrimental neurogenesis, including microcephaly and/or GBS. Thus, it should be important to try to target these mediators in order to reduce the collateral damage initiated by the host immune response to ZIKV. As previously shown, CXCL10 neutralization by specific antibodies or genetic deletion in $CXCL10^{-/-}$ mice protected against cerebral malaria infection and inflammation [85], and passive transfer of anti-CXCL10 antibodies reduced inflammatory leukocyte recruitment across the blood-brain barrier. Furthermore, statin medications commonly used for cholesterol control have been shown to decrease CXCL10 and to be effective in Crohn's disease [86, 87]. However, to date, successful targeting of the immune system during an acute infection has proved to be extraordinarily difficult and largely unsuccessful. A reason could be that we still do not totally understand the delicate nature of the rapid changes of the cytokine response during an acute infection, and until we do, it is unlikely that we will be able to develop rational therapies that target the exact phase of the immune cascade and administrate those therapies at the time they are needed.

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Figura 4 – Artigo 1: Zika vírus in the eye of the cytokine storn
Fonte: (MOUCOURANT et al. 2019)

5 **CAPÍTULO 2 – ARTIGO 2**

A avaliação do fenótipo e a função das células NK por citometria de massa (CyTOF) foi realizada e os resultados foram descritos no estudo intitulado “NK Cell Responses in Zika Virus Infection Are Biased towards Cytokine-Mediated Effector Functions”, publicado no Journal of Immunology, apresentado no capítulo 2 desta tese.

The Journal of Immunology

NK Cell Responses in Zika Virus Infection Are Biased towards Cytokine-Mediated Effector Functions

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Christopher Maucourant, Gabriel Andrade Nonato Queiroz, Aurelien Corneau, Luana Leandro Gois, Aida Meghraoui-Kheddar, Nadine Tarantino, Antonio Carlos Bandeira, Assia Samri, Catherine Blanc, Hans Yssel, Maria Fernanda Rios Grassi and Vincent Vieillard

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NK Cell Responses in Zika Virus Infection Are Biased towards Cytokine-Mediated Effector Functions

Christopher Maucourant,*[,1] Gabriel Andrade Nonato Queiroz,[†,‡,1] Aurelien Corneau,[§,1] Luana Leandro Gois,[†,‡] Aida Meghraoui-Kheddar,*[,2] Nadine Tarantino,* Antonio Carlos Bandeira,[†,¶] Assia Samri,* Catherine Blanc,[§] Hans Yssel,* Maria Fernanda Rios Grassi,[†,‡] and Vincent Vieillard*

Zika virus (ZIKV) is a mosquito-borne flavivirus that has emerged as a global concern because of its impact on human health. ZIKV infection during pregnancy can cause microcephaly and other severe brain defects in the developing fetus and there have been reports of the occurrence of Guillain-Barré syndrome in areas affected by ZIKV. NK cells are activated during acute viral infections and their activity contributes to a first line of defense because of their ability to rapidly recognize and kill virus-infected cells. To provide insight into NK cell function during ZIKV infection, we have profiled, using mass cytometry, the NK cell receptor-ligand repertoire in a cohort of acute ZIKV-infected female patients. Freshly isolated NK cells from these patients contained distinct, activated, and terminally differentiated, subsets expressing higher levels of CD57, NKG2C, and KIR3DL1 as compared with those from healthy donors. Moreover, KIR3DL1$^+$ NK cells from these patients produced high levels of IFN-γ and TNF-α, in the absence of direct cytotoxicity, in response to in vitro stimulation with autologous, ZIKV-infected, monocyte-derived dendritic cells. In ZIKV-infected patients, overproduction of IFN-γ correlated with STAT-5 activation ($r = 0.6643$; $p = 0.0085$) and was mediated following the recognition of MHC class 1–related chain A and chain B molecules expressed by ZIKV-infected monocyte-derived dendritic cells, in synergy with IL-12 production by the latter cells. Together, these findings suggest that NK cells contribute to the generation of an efficacious adaptive anti-ZIKV immune response that could potentially affect the outcome of the disease and/or the development of persistent symptoms. *The Journal of Immunology*, **2021, 207: 1–11.**

Zika virus (ZIKV) is a mosquito-borne flavivirus related to Dengue virus (DENV), yellow fever virus, and West Nile virus (1). During the first decades since its first discovery in the 1950s in Africa (2), ZIKV received little attention, remaining confined to the equatorial belt in Africa and Asia. The early clinical picture of natural human ZIKV infection was mainly associated with self-limiting symptoms, including fever, rash, and conjunctivitis. Recently however, ZIKV has emerged as a global concern because of its pandemic potential and its impact on human health with more than 500,000 infected individuals between 2015 and 2016 (3). In 2016, the World Health Organization has declared this virus a public health emergency of international concern because to its ability to cause Guillain-Barré syndrome in adults and birth defects, in particular microcephaly, in newborns from infected women (4–6).

ZIKV is primarily transmitted to humans by the bite of infected mosquitoes from the *Aedes aegypti* family (7). Viral entry into permissive dendritic cells (DC) facilitates the traffic of ZIKV to draining lymph nodes and other lymphoid tissues through DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), one of the main cellular receptors for this virus (8). In response, the infected host produces early type I IFNs, to mount a rapid and potent innate defense against ZIKV (9). This first innate immune response is responsible for the recruitment of monocytes to the inflammatory site, permitting the priming of NK cell activity during arbovirosis (10, 11).

NK cells are critical effectors of the innate immune response and represent a first line of defense against a variety of viral infections, including those caused by arboviruses (12–15). There is abundant proof for an early control of infectious disease by NK cells and further evidence for a direct role of these cells in protection against

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C.M., H.Y., M.F.R.G., and V.V. designed the study; L.L.G., A.C.B., A.S., and M.F.R.G. were in charge of patient care, clinical data, and biological samples; C.M., G.A.N.Q., L.L.G., and N.T. performed experiments; A.C., A.M.-K., and C.B. designed and performed the mass cytometry analyses; V.V. wrote the first draft of the manuscript with critical input from H.Y. All authors approved the final version of the manuscript.

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Abbreviations used in this article: DC, dendritic cell; DC-SIGN, DC-specific ICAM-3–grabbing nonintegrin; DENV, Dengue virus; MIC, MHC class I–related chain; Mo-DC, monocyte-derived DC; RQ, relative quantification; SB, staining buffer; SPADE, spanning-tree progression analysis of density-normalized events; TP1, timepoint 1; TP2, timepoint 2; viSNE, visualization of *t*-distributed stochastic neighbor embedding; ZIKV, Zika virus.

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viral infections comes from studies of patients with selective NK cell deficiencies, a condition that leads to the development of fulminant viral infections (16, 17). The importance of these cells is also underscored by the development of escape mechanisms by many viruses (i.e., HIV, herpes, and hepatitis viruses) to thwart NK cell responses (18). NK cell activity is shaped by the integration of signals that arise from the binding of a vast array of receptors present on the surface of these cells. They include not only inhibitory receptors, like KIR-L and NKG2A, but also activating receptors, such as the natural cytotoxicity receptors (NKp30, NKp44, and NKp46), NKG2D, and NKG2C. Although ligands for inhibitory receptors are mostly MHC class I molecules, those for activating receptors, exemplified by MHC class I–related chain (MIC)–A, MIC-B, and ULBP-1 to -6 for the NKG2D receptor, are diverse and their expression is upregulated under conditions of cellular stress, in particular during viral infection thereby allowing NK cells to specifically eliminate harmful infected host cells (19). When activating signals predominate, NK cells produce an array of proinflammatory cytokines, most importantly IFN-γ and TNF-α, in parallel with the initiation of their cytotoxic functions (20–22). Despite substantial progress in understanding ZIKV-specific immune responses in individuals, little is known about the role of NK cells in the pathogenesis of Zika fever. With respect to their activity in response to infection with other flaviviruses, we and others have observed that NK cells become activated and rapidly expand following infection of the host with DENV (10, 14, 23). Moreover, DENV type 2 was found to inhibit glycogen synthase kinase 3 activity and subsequently induce MIC-A expression in monocyte-derived DC (Mo-DCs), resulting in a specific increase in IFN-γ and TNF-α production in the absence of cytotoxicity, by autologous NK cells (15). It has previously been shown that ZIKV inhibits NK cell cytotoxicity by upregulating the expression of MHC class I molecules in response to IFN-β (24), whereas a protective role of NK cells against ZIKV infection was reported in mice (25).

In the present work, we have conducted a detailed phenotypic and functional analysis of NK cells by mass cytometry during acute infection of patients with ZIKV and have characterized in vitro the role of NK cell receptors and their ligands in the context of autologous ZIKV-infected Mo-DCs.

Materials and Methods

Patients, healthy controls, and sample preparation

Peripheral blood samples from 17 ZIKV-infected patients were obtained during the ZIKV outbreak that occurred in the Salvador area (Brazil) between March 2016 and December 2016. Samples from patients who had developed symptoms included fever, arthralgia, and asthenia and who visited the emergency room of a medical center in Salvador (Bahia, Brazil) were collected at the admission (timepoint 1 [TP1]) and 13–19 d later (timepoint 2 [TP2]). Molecular diagnosis of ZIKV infection was confirmed for each of the patients using PCR, as described (26). Ten healthy volunteers were used as negative controls. Patients and controls were PCR negative for chikungunya, yellow fever virus, and DENV infections. PBMCs were isolated from blood samples by standard density centrifugation, and then frozen at −150°C.

The study was conducted in accordance with the principles of the Declaration of Helsinki, as well as French statutory and regulatory law, and received approval from the Institutional Review Board of FIOCRUZ (protocol numbers 1.159.814 and 1.593.256/CAAE 55882016.6.0000.0040). Patients received information about research to be performed on their biological samples and provided written informed consent to participate.

Staining and mass cytometry acquisition

The 40 mAbs used for PBMC labeling were in-house conjugated to metal isotopes using MaxPAR Ab Conjugation Kits (Fluidigm), following the manufacturer's recommendations (Supplemental Table I) indicated by a star. Conjugated Abs were diluted to 100 times the working concentration in PBS Ab Stabilization Solution (Candor Bioscience) and stored at 4°C.

For each sample, PBMCs were thawed rapidly, incubated with a 50 μM solution of 127-IdU Cell-IDTM (Fluidigm) for 25 min at 37°C, followed by the addition of 2.5 μM 103Rh Cell-ID-Intercalator (Fluidigm) for 5 min at 37°C. Cells were then washed with staining buffer (SB; 1xPBS with 0.5% BSA and 0.02% sodium azide), and surface markers were stained following a multistep protocol. After Fc blocking, cells were incubated with a first Ab mix targeting chemokine receptors during 15 min, then with a second Ab mix targeting the other surface markers for 30 min at room temperature. After the 45 min of incubation, cells were washed with SB, fixed for 15 min with paraformaldehyde (Sigma-Aldrich) at a final concentration of 2%, permeabilized with methanol (Sigma-Aldrich) for 10 min and then intracellular stained for 60 min at +4°C. Cells were washed twice with SB and incubated overnight in 2% paraformaldehyde with 1:4000 the iridium intercalator [pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine; Fluidigm] at 4°C, and frozen at −80°C, as described (27, Meghraoui-Kheddar, A. B.G. Chousterman, N. Guillou, S.M. Barone, S. Granjeaud, H. Vallet, A. Cornau, K. Guessous, A. Boissonnas, J.M. Irish, and C. Combadière, manuscript posted on bioRxiv, DOI: 10.1101/2020.05.29.123992).

Before acquisition, cells were thawed rapidly, resuspended in distilled-deionized water at 10^6 cells per mL and mixed with 4-Element EQ Beads (Fluidigm) and passed through a cell strainer cap with 35-mm pores (BD Biosciences). Cell events were acquired on the CyTOF Helios Mass Cytometer (Fluidigm) and CyTOF software version 6.7.1014 (Fluidigm) at the "Plateforme de Cytométrie de L'hôpital Pitié-Salpêtrière (CyPS)," Paris, France. Cytometry standard files produced on the CyTOF Helios were normalized using MatLab Compiler software normalizer based on the signals of the 4-Element EQ beads (Fluidigm) using MatLab Compiler software as recommended by the software developers.

Mass cytometry data analysis

After beads have been removed by an exclusion gate, intact single cells were gated on the basis of iridium intercalator DNA staining and live cells were selected on the basis of 103Rh before cell subsets analysis. The different analyzes were performed only with the samples with at least 2000 events.

To identify the main circulating immune populations, cell clustering was performed using Spanning-tree Progression Analysis of Density-normalized Events (SPADE) (28) in Cytobank platform (29), applying a down-sampling of 10% and targeting 50 nodes based on all the markers of the panel (Supplemental Table I). Identification of metaclusters, representing immune cell populations, were expert driven based on the median expression of all the markers in each node (Supplemental Table I).

Then, after the concatenation of samples from the same condition into a single group, a visualization of *t*-distributed stochastic neighbor embedding (viSNE) analysis was performed using the Cytobank platform based on the expression of the NK cells following markers: CD56, CD57, CD16, NKG2A, NKG2C, KIR2DL1, KIR2DL2L3, KIR3DL1, HLA-DR, IFN-γ, pSTAT-5, and Ki-67.

The viSNE (implementation of *t*-SNE) (30) was used to rearrange, by an unsupervised approach, cells in the different groups of the study in a common two-dimensional map, according to their expression profile of markers (Supplemental Table I).

A citrus analysis was performed using markers previously cited to identify the specific NK signature timepoint of the study.

To check discoveries from the unsupervised analysis, manual gating was done using a classical gating strategy to identify NK cells: $CD33^{-}CD14^{-}$ $CD3^{-}CD56^{+}$ and the NK cells markers previously cited to be used in our unsupervised analyze. Then data were collected using GraphPad PRISM and a statistical analysis was done as discussed in the specific section of the materials and methods.

Cell isolation and generation of Mo-DCs

Monocytes were positively purified using $CD14^{+}$ beads (Miltenyi Biotec) from fresh PBMC isolated from buffy coat samples of healthy donors (Etablissement Français du Sang), and cultured for 6 d in the presence of 50 ng/ml IL-4 and 100 ng/ml GM-CSF (BioTechne). Successful differentiation of Mo-DCs was verified by the presence of CD1a (HI149), DC-SIGN (DCN46), CD40 (5C3) (all from BD Pharmingen), and HLA-DR (Immu-357; BioLegend) expression and the absence of CD14 (61D3; eBioscience) expression, as previously described (15).

Autologous NK cells were purified from the same healthy donors by negative selection using the NK Cell Isolation Kit (Miltenyi Biotec) (routinely purity > 98%) and subsequently cultured in RPMI supplemented with 10% human AB serum and 1,000 IU/ml of Proleukin-2 (Chiron) for 6 d. The purity was assessed using anti-CD45 (J.33; Coulter), anti-CD3 (UCHT1; eBioscience), and anti-CD56 (N901; Coulter) mAbs.

Preparation of ZIKV, infection of Mo-DCs, and coculture with autologous NK cells

The primary PF-25013-18 strain of ZIKV, kindly provided by Dr. Dorothée Missé (MIVEGEC, IRD Montpellier, France), was propagated in Vero cells (no. CCL-81; American Type Culture Collection) and harvested after 5 d of culture at 37°C. Cell debris was removed by centrifugation and aliquots were stored at −80°C, as previously described (31). Virus stock was titrated after extraction of cellular RNA from cell-supernatant using MirVanaTM miRNA Isolation Kit (Ambion). Quantitative PCR was next performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and MESA GREEN Quantitative PCR Master Mix (Eurogentec), using FLR ZIKV forward 5′-AGGACAGGCCTTGACTTTTC-3′ and reverse primer 5′-TGTTCCAGTGTGGAGTTC-3′. The level of gene expression was normalized to GAPDH. The copy of RNA (molecules per microliter) was determined using a dsDNA copy number calculator (http://cels.uri.edu/gsc/cndna.html), and estimated $\sim 5 \times 10^8$ copies per mL.

After 6 d of culture, 2×10^6 differentiated Mo-DCs were infected for 2 h at 37°C with 100 μl of virus stock (5×10^7 copies). Cells were washed twice to remove cell-free virus and cultured at a density of 10^6 cells per milliliter in 24-well plates RPMI supplemented with 10% FCS. As control, noninfected and Mo-DCs treated overnight with 10 μg/ml of LPS were used. Infected cells were fixed and permeabilized with Cytofix and Cytoperm (BD Pharmingen) during 30 min at 4°C, intracellularly stained with 5 μg/ml 3H5-1 antiflavivirus mAb (MilliporeSigma), specific to the envelope E glycoprotein of the flavivirus, then washed and stained with 1/500 of anti-mouse IgG1-FITC (Beckman Coulter). During this time, autologous NK cells were trashed or cultivated to be used in the functional assay. Staining was acquired on Gallios Cytometer (Beckman coulter) and analyzed using FlowJo software v09 (TreeStar).

Flow cytometry analyzes of Mo-DCs

Mo-DCs were first preincubated for 15 min at 4°C with an Fc blocking reagent (Miltenyi Biotec) to block nonspecific Fc receptor binding. Differentiation into Mo-DCs was assessed by flow cytometry with CD45-KO (J.33), HLA-DR-ECD (Immu-357) from Beckman Coulter, MIC-A–PE (159207; R&D Systems), MIC-B–PE (236511; R&D Systems), CD14-EF 780 (61D3; eBiosciences), and CD1a-allophycocyanin (HI149), CD83-PC7 (HB15e), and CD86-PB (GL1) from BioLegend. Isotype-matched Igs served as negative controls. The expression of ligands for NK cell receptors on Mo-DCs was assessed using specific mAbs: cells were first incubated with 1 μg/ml of fusion proteins (NKp30-Ig or NKp46-Ig, 1849-NK, and 1850-NK, respectively), anti–ULBP1-2-3 (170818, 165903, and 166510, respectively), or anti–MIC-A/-B (159207) mAbs from R&D Systems, for 2 h at 4°C. Cells were washed in PBS-BSA 0.5% and then stained using anti-human IgG1-PE at 1/50 dilution (Jackson ImmunoResearch) for Ig-fusion proteins, and anti-mouse hu-IgG2a-PE (Beckman Coulter) for anti–ULBP1-2-3 and anti–MIC-A/-B mAb or directly stained with allophycocyanin–anti–HLA-A,B,C (G46-2.6) mAbs for 1h at 4°C, as previously described (15, 32). Cells were acquired on a Gallios Ccytometer (Beckman Coulter). Flow cytometry data were analyzed using FlowJo software v. 9 (TreeStar).

NK cell degranulation and intracellular production of cytokines

For in vitro functional analysis, purified NK cells were incubated with autologous Mo-DCs at an E:T cell ratio from 1:1 in the presence of an anti-CD107a mAb (H4A3; Becton Dickinson) for 1 h, to measure degranulation. Cells were thereafter incubated for 4 h in the presence of Golgi Stop (4 μL per 6 mL of culture) and Golgi Plug (1 μL per mL of culture) from BD Biosciences and then were stained using anti–CD45-KO (J.33; Coulter), anti–CD3 (UCHT1; eBioscience) and anti-CD56 (N901; Coulter) mAbs. Cells were fixed, permeabilized using a Cytofix/Cytoperm Kit (BD Bioscience) during 30 mins at 4°C and then intracellularly stained for IFN-γ and TNF-α production, as described (23). In some experiments, Mo-DCs were preincubated with 10 μg/ml of 6D4 anti–MIC-A/-B (BioLegend) (2001; Groh), 10 μg/ml of W6/32 anti–HLA class I–blocking mAbs, 2.5 μM of Apilimod (formerly STA-5326), a specific inhibitor of IL-12 synthesis (33), or 10 μg/ml of LPS (both from Sigma-Aldrich).

Cells were acquired on a Gallios Cytometer (Beckman Coulter) and analyzed using FlowJo Software V09 (TreeStar).

For the ex vivo functional analysis, PBMC from seven randomly selected patients were rested during 2 h after thawing and then have been cultivated with K562 or not in the same condition as discussed for the in vitro functional analysis.

Real-time PCR analysis of IL-12 p35 and IL-12 p40

mRNA was extracted using the mirVana Kit (Thermo Fisher Scientific) and treated by DNAse (Ambion DNA-free; Invitrogen). Reverse transcription was then conducted with 200 ng mRNA using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The real-time PCR was performed with the Applied Biosystems 7300 Real Time PCR system in the presence of SYBR-green. with 20 ng of cDNA and 10 μM of sense and reverse primers. After an initial step at 50°C for 2 min and 95°C for 10 min, a total of 40 cycles was performed. Each cycle consisted in 95°C for 15 s and 60°C for 1 min. At the end, a final cycle was performed at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. The oligonucleotide sequences used for IL-12 p35, IL-12 p40, and RSP14, respectively: sense primers: CTCCTGGACCACCTCAGTTTG, CGGTCATCTGCCGCAAA, TGAAACCTTCCCTGGCCT-ACG; reverse primers: GGTGAAGGCATGGGAACATT, TGCCCATTCGCTCCAAGA, and GCTGCTGTGAGGGCTGGAGCTC. Data were analyzed with the double δ Ct (ΔΔCt) method using the 7300 Software System (Applied Biosystems) to determine relative quantification (RQ) of IL-12 p35 and IL-12 p40 gene expression: $RQ = 2^{(-\Delta\Delta Ct)}$ with ΔΔCt = ΔCt (sample) − ΔCt (calibrator); ΔCt (sample) = Ct (sample in test) − Ct (RSP14 in test); ΔCt = Ct (sample in calibrator) − Ct (RSP14 in calibrator), as previously described (15).

Statistical analysis

The nonparametric Mann–Whitney *U*, Kruskal–Wallis, and Wilcoxon tests were used as appropriate for the comparison of continuous variables between groups. Statistical analysis was performed using PRISM software (GraphPad).

Results

Proportion of immune cell subsets in ZIKV-infected patients analyzed by mass cytometry

We developed a mass cytometry 40 markers panel for a high-dimensional analysis of PBMCs subsets of ZIKV-infected patients and healthy controls. Surface markers were chosen to identify myeloid cells, T and B lymphocytes, and NK cells in addition to differentiation, activation, and functional markers (http://cels.uri.edu/gsc/cndna.html).

To establish the cellular heterogeneity of PBMCs, an SPADE clustering was performed using the same number of total live single cells of 17 ZIKV-infected patients, and eight healthy donors. The resulting 50 SPADE nodes were then grouped into five metaclusters corresponding to the canonical immune cell populations: $CD14^+$ myeloid cells, $CD3^-CD56^+$ NK cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells (Fig. 1A).

Analysis of metaclusters revealed that the frequencies of each cell population was conserved in PBMCs from ZIKV-infected patients at admission (TP1) and 13–19 d later (TP2), as compared with healthy donors (Fig. 1B), despite an interindividual degree of variability in ZIKV-infected patients (Fig. 1C). These data were confirmed with individual viSNE analyzes (data not shown), and suggest that distribution of the major immune cell subsets remains similar in donors and ZIKV-infected patients.

Major phenotypic features of NK cells from ZIKV-infected patients

Given the importance of NK cells in the early stage of infection, we performed a detailed analysis of the expression pattern of NK cells receptors in ZIKV-infected patients during the acute and postfebrile phase of infection. A viSNE dimensional reduction combined to SPADE revealed that several NK cell markers from $ZIKV^+$ patients are indistinguishable from those from healthy donors, irrespective of the time-point tested (data not shown). Notably NK cells from $ZIKV^+$ and healthy donors expressed similar levels of CD16, NKG2A, KIR2DL1, KIR2DL2/DL3, CCR2, CXCR5, as well as exhaustion markers PD1, CTLA4, and Tim-3 (Fig. 2A and data not shown). However, NK cells from $ZIKV^+$ patients were found to express higher levels of CD57, NKG2C, and KIR3DL1, as compared with their healthy counterparts (Fig. 2A–C). Notably, overexpression of NKG2C was mainly associated with CD57 (Fig. 2B). These data were corroborated with a more systematic investigation using citrus algorithm, in which the three major specific metaclusters of TP1 and TP2 were $CD57^+NKG2C^+KIR3DL1^-$, $CD57^+NKG2C^-KIR3DL1^+$, and $CD57^+NKG2C^-KIR3DL1^-$ differentiated cell subsets (Supplemental Fig. 1).

To further characterize these responding NK cells during the acute phase of infection, markers of activation and cellular

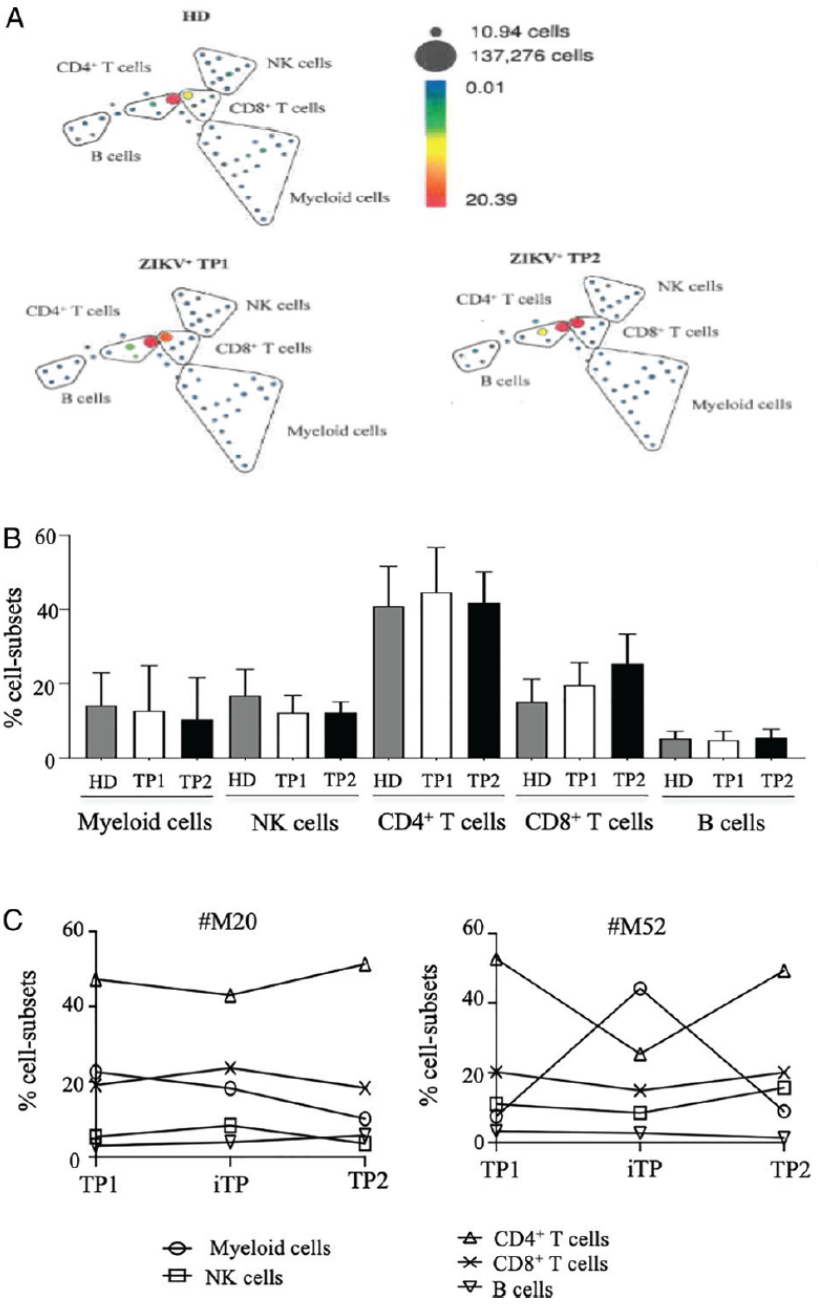

FIGURE 1. Impact of ZIKV on various cell-populations. (**A**) SPADE representations of cell-subsets distribution in the peripheral blood of ZIKV-infected patients. Cell-subsets are shown in mean of all healthy donors (HD) and ZIKV-infected patients ($ZIKV^+$) at the different timepoints (TP1 and TP2) after onset of the symptoms. For this general population SPADE, the channels used are as follows: CD19, CD4, CD16, CD11b, CD123, CD56, CD8, CD14, CD11c, CD33, HLA-DR, CD163, and CD3. (**B**) Distribution of cell-subsets in ZIKV-infected patients, compared with HDs. Data are done in frequency of $CD45^+$ cells from SPADE analysis. (**C**) Kinetic study of cell-subsets from two representative ZIKV-infected patients (no. M20 and no. M52).

proliferation were tested. A high percentage of NK cells from $ZIKV^+$ patients expressed the proliferation marker Ki-67 during TP1, compared with heathy donors, which subsequently declined throughout TP2 (Fig. 3A, 3B). A similar pattern was observed regarding HLA-DR–expressing cells, and cells coexpressing Ki-67 and HLA-DR (Fig. 3A, 3B). Importantly, HLA-DR was significantly overexpressed in both $NKG2C^+$ and $KIR3DL1^+$ NK cell subsets, whereas overexpression of Ki-67 was only observed in the $KIR3DL1^+$ NK cell subsets at TP1 (Fig. 3C). It seems, however, that expression of HLA-DR and Ki-67 decreases over time (Fig. 3C).

These data suggest that ZIKV infection is associated with the presence of activated and proliferating NK cell subsets expressing NKG2C or KIR3DL1.

ZIKV infection induces production of IFN-γ by NK cells

Given our finding that NK cells are activated and present specific phenotypic characteristics during acute ZIKV infection, we subsequently evaluated their functional capacities. Mass cytometry analysis revealed that the intracellular production of IFN-γ by NK cells was the most significant functional marker that distinguished ZIKV-infected patients from healthy donors ($p = 0.038$) (Fig. 4A, 4B). Although IFN-γ production was increased in $NKG2C^+$ NK cells from ZIKV-infected patients, the great majority of IFN-γ–producing cells expressed KIR3DL1. The latter population was increased in a statistically significant manner in $ZIKV^+$ patients ($p < 0.0001$), compared with healthy donors (Fig. 4C, 4D). Consequently, KIR3DL1 expression and IFN-γ production were also positively correlated in NK cells

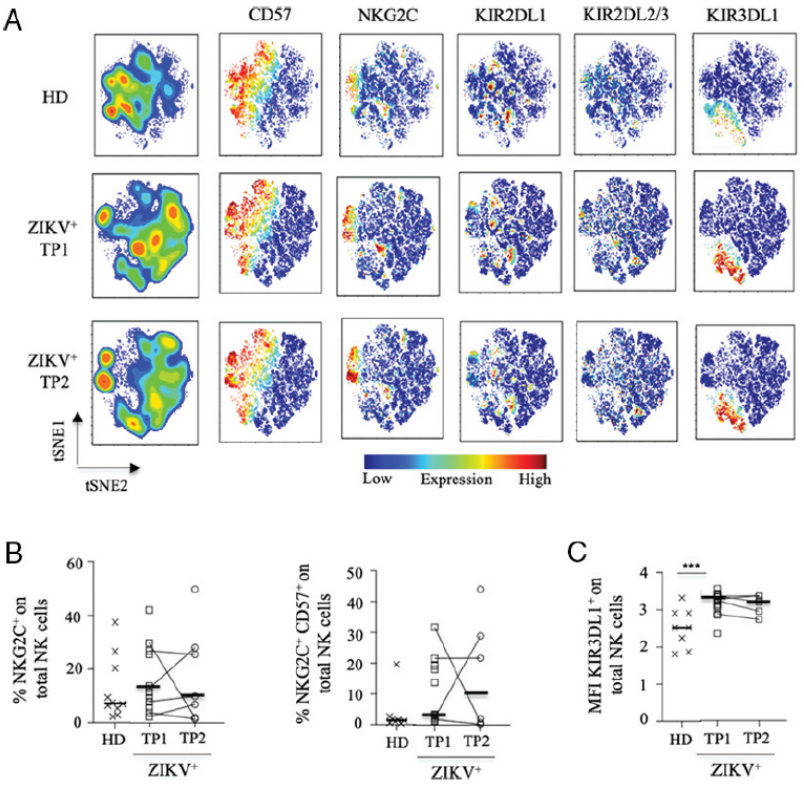

FIGURE 2. Expansion of adaptive NK cells from ZIKV-infected patients. (**A**) tSNE plots representing NK cells density in healthy donors (HD) and ZIKV-infected patients ($ZIKV^+$) PBMCs collected at the different timepoints (TP1 and TP2) after onset of symptoms in addition to NK cell receptors expression patterns in these groups. Plots represent merged files of each group of individuals. The settings used for the viSNE run were as follow: equal event sampling (12,468 events each), markers (CD16, CD56, CD57, NKG2A, NKG2C, KIR2DL1, KIR2DL2L3, KIR3DL1, IFN-γ, Ki-67, and pSTAT-5), iterations (7500), perplexity (30), and θ (0.7). (**B**) Frequency of $NKG2C^+$ and $CD57^+NKG2C^+$ NK cells (or NKG2C and CD57/NKG2C-expressing NK cells). (**C**) Mean of fluorescence intensity (MFI) of $KIR3DL1^+$ expression. Black lines represent the median of 10 HD, 15 TP1, and 7 TP2. An unpaired Mann–Whitney *U* test was performed between HD and TP1 groups and a paired Wilcoxon test was performed between TP1 and TP2 groups. ***$p < 0.0001$.

from $ZIKV^+$ patients at TP1 ($r = 0.8143$, $p = 0.0004$) (Fig. 4E). Interestingly, the frequency of IFN-γ production was significantly increased in KIR3DL1-expressing NK cells, as compared with $KIR3DL1^-$ cells in ZIKV-infected patients ($p < 0.0001$), whereas expression in NK cells from healthy donors remained close to baseline levels (Fig. 4F).

For validation, we next assessed the functional activity of NK cells by a standard flow cytometry assay in PBMC from ZIKV-infected patients. Fig. 5A shows that intracellular production of IFN-γ and TNF-α production by NK cells was significantly increased in $ZIKV^+$ patients, as compared with healthy controls. In contrast, the capacity of NK cells to release cytotoxic granules, as demonstrated by the expression of CD107a in the presence of K562 target cells, was not increased in ZIKV-infected patients, as compared with healthy donors (Fig. 5A).

To confirm these functional results, we also used an in vitro model of activated NK cells cocultured with autologous ZIKV-infected Mo-DCs. At 48 h postinfection by ZIKV, up to 48% of Mo-DCs were infected (Supplemental Fig. 2A). Except for the DC-SIGN receptor, the expression of most of the costimulatory molecules were upregulated in both bystander and infected Mo-DCs (data not shown). In line with data observed in ZIKV-infected patients (Fig. 5A), NK cell degranulation was not enhanced in NK cells cocultured with autologous ZIKV-infected Mo-DCs (Fig. 5B). In contrast, infected Mo-DCs induced enhanced production of IFN-γ and TNF-α in a statistically significant manner ($p = 0.016$ for both cytokines) by autologous NK cells, as compared with noninfected Mo-DCs (Fig. 5B). Importantly, the production of IFN-γ and TNF-α by autologous NK cells was strongly inhibited in experiments carried out with *trans*-well chambers (Supplemental Fig. 3A), suggesting that cell-cell contact is necessary for cytokine production by NK cells.

IL-12 p35 (14.3 ± 3.4-fold increased) and p40 (15.7 ± 1.5-fold increased) gene expression was significantly increased in Mo-DCs infected by ZIKV, as compared with noninfected cells, similarly to that observed after LPS treatment (Fig. 6A). Consistently, the intracellular production of IFN-γ and TNF-α by NK cells was strongly decreased after coculture with ZIKV-infected Mo-DCs pretreated with 2.5 μM of Apilimod (Fig. 6B). To gain inside in the signaling pathway, we determined phosphorylated STAT-5 (pSTAT-5) levels within NK cells from ZIKV-infected patients. As shown in Fig. 6C, pSTAT-5 levels were strongly increased in $ZIKV^+$ NK cells, as compared with those from healthy individuals, in a time-dependent manner (Fig. 6D), and statistically correlated with IFN-γ production by these cells ($r = 0.6643$, $p = 0.0085$) (Fig. 6E). Notably, pSTAT-5 was significantly increased in NK cells expressing NKG2C ($p = 0.050$) or KIR3DL1 ($p = 0.002$) (Supplemental Fig. 3B). To confirm the specific induction of pSTAT-5 by NK cells in the presence of ZIKV, we tested in vitro the expression of other activated STAT proteins (pSTAT-1, -3, and -6). Fig. 6F shows that only pSTAT-5 expression is increased after cocultured with autologous ZIKV-infected Mo-DC.

In conclusion, the production of cytokines induced by ZIKV is associated with the activation of the STAT-5 signaling pathway in NK cells and the production of IL-12 by the infected Mo-DCs.

Expression of ligands for NK cell receptors in ZIKV-infected Mo-DCs

We next sought to identify ligands for NK cell receptors on Mo-DCs that could contribute to the phenotypic and functional alterations of NK cells during acute ZIKV infection. Postinfection, a slight but distinct increase in the expression of MHC class I molecules, the ligands of KIR, was observed in Mo-DCs (Fig. 7A, 7B). Importantly, the frequency of MIC-A and MIC-B (MIC-A/B), two ligands of NKG2D, were significantly induced in ZIKV-infected Mo-DCs, as compared with LPS-treated and noninfected control

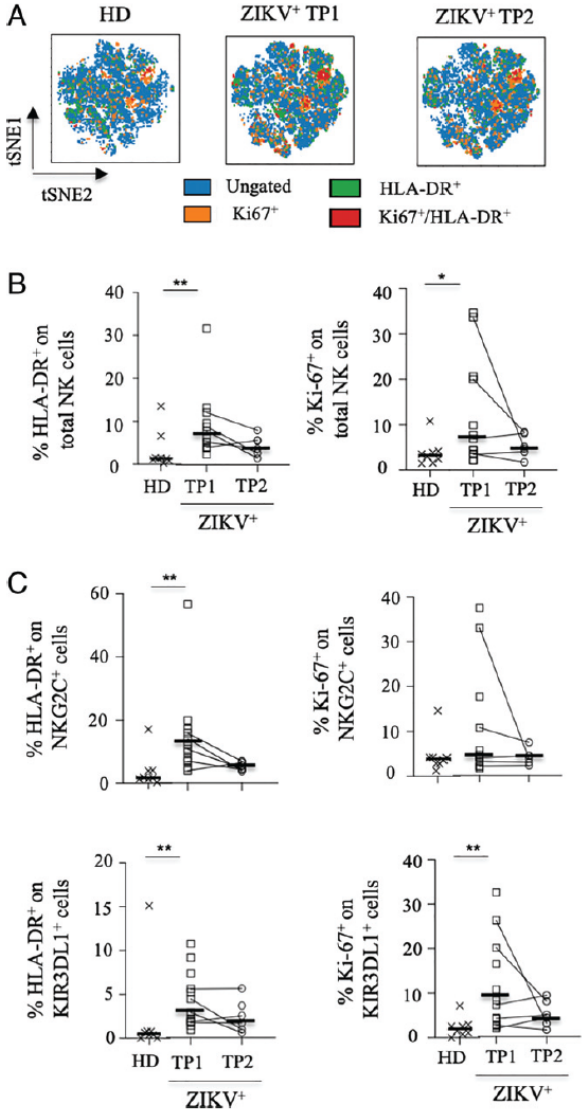

FIGURE 3. Cellular activation of NK cells from ZIKV-infected patients. (**A**) tSNE visualization of the cell activation (HLA-DR) and cell proliferation (Ki-67) markers in NK cells from healthy donors (HD) and ZIKV-infected patients ($ZIKV^+$) at the different timepoints (TP1and TP2) after onset of the symptoms. Plots represent merged files of each group of individuals. The parameters of viSNE are noted in the legend of Fig. 2B. (**B**) Frequency of HLA-DR and Ki-67 expression on total $CD3^-CD56^+$ NK cells. Black lines represent the median. (**C**) Frequency of HLA-DR– or Ki-67–expressing $NKG2C^+$ and $KIR3DL1^+$ NK cell subsets from HD (n = 8) and ZIKV-infected patients ($ZIKV^+$) at different timepoints (TP1, n = 13; TP2, n = 6) after onset of the symptoms. Black lines represent the median. An unpaired Mann–Whitney U test was performed between HD and TP1 groups, whereas a paired Wilcoxon test was performed between TP1 and TP2 groups. $^*p < 0.01$, $^{**}p < 0.001$.

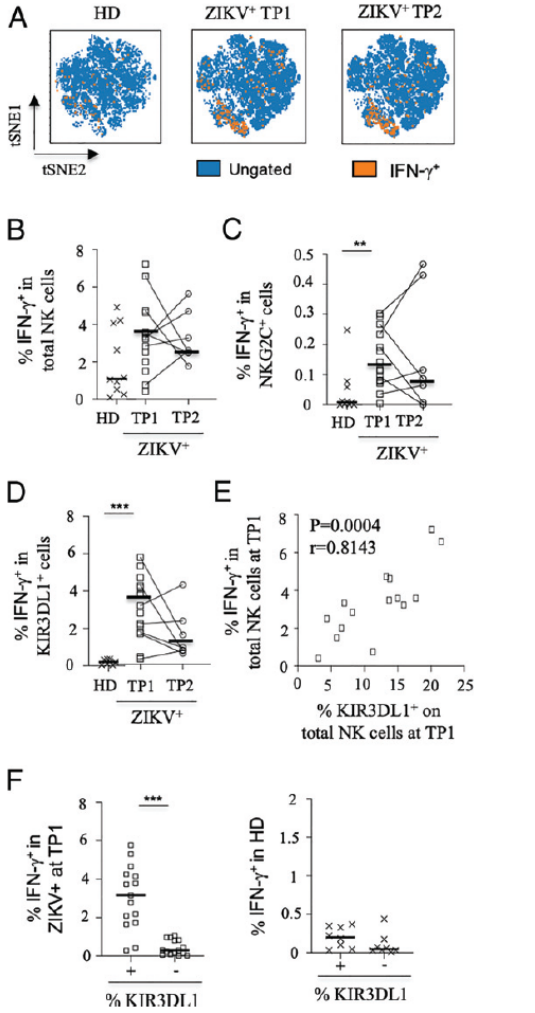

FIGURE 4. Intracellular production of IFN-γ in NK cells from ZIKV-infected patients. (**A**) tSNE visualization of IFN-γ–producing NK cells from healthy donors (HD) and ZIKV-infected patients at the different timepoints (TP1 and TP2) after onset of the symptoms. Plots represent merged files of each group of individuals. (**B**) Frequency of intracellular production of IFN-γ in total $CD3^-CD56^+$ NK cells. (**C**) Frequency of IFN-γ–positive $CD3^-CD56^+$ NK cells expressing NKG2C. (**D**) Frequency of IFN-γ–positive $CD3^-CD56^+$ NK cells expressing KIR3DL1. Data were obtained from HD (n = 8) and ZIKV-infected patients ($ZIKV^+$) at different timepoints (TP1, n = 15; TP2, n = 7) after onset of the symptoms. Black lines represent the median. An unpaired Mann–Whitney U test was performed between HD and TP1 groups, whereas a paired Wilcoxon test was performed between TP1 and TP2 groups. $^{**}p < 0.001$, $^{***}p < 0.0001$. (**E**) Correlation between IFN-γ production of total $CD3^-CD56^+$ NK cells and frequency of KIR3DL1 expression by NK cells from ZIKV-infected patients at TP1. (**F**) Frequency of intracellular production of IFN-γ in $CD3^-CD56^+$ NK cells expressing or not KIR3DL1 in HD and ZIKV-infected patients ($ZIKV^+$) at TP1. Data are extrapolated from the Citrus analysis. Black lines represent the median. An unpaired Mann–Whitney U test was performed between $KIR3DL1^+$ and $KIR3DL1^-$ NK cells. $^{***}p < 0.0001$.

cells (Fig. 7A, 7B); upregulation of MIC-A expression is, however, more significant (p = 0.001) than that of MIC-B (p = 0.025) in ZIKV-infected Mo-DCs, as compared with noninfected control cells (Fig. 7B). In contrast, Mo-DCs infected by ZIKV were indistinguishable from those of noninfected controls subjects in terms of cell surface expression of other cellular ligands for major NK-activating receptors, including ULBP family proteins for NKG2D, and ligands of NKp30 and NKp46 (Supplemental Fig. 3C). Consistently, 73 to 93% of the ZIKV-infected cells expressed MIC-A/B (Supplemental Fig. 2B), and the level of NKG2D expression was downmodulated after a 5-h period of coculture with ZIKV-infected autologous Mo-DCs, as compared with noninfected target cells. In contrast, cell surface expression levels of NKp30 and NKp46, two

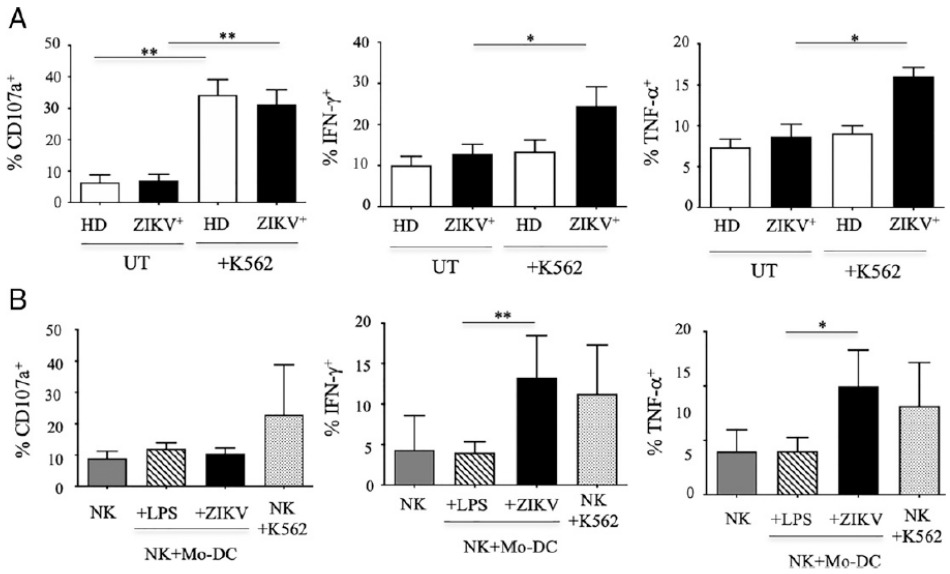

FIGURE 5. Functional activity of NK cells mediated by autologous ZIKV-infected Mo-DCs. (**A**) In vivo degranulation and production of IFN-γ and TNF-α by NK cells from healthy donors (HD; open bars) and ZIKV-infected patients (ZIKV$^+$; black bars) at TP1. Experiments are performed with untreated PBMC (UT) or in the presence of K562 target cells (+K562) at an E:T cell ratio of 1/1. Data are shown for five HDs and five ZIKV-infected patients. (**B**) In vitro degranulation and production of IFN-γ and TNF-α by purified untreated NK cells (gray bars), and NK cells cocultured with LPS-treated (+LPS; hatched bars) or ZIKV-infected (+ZIKV; black bars) autologous Mo-DCs. Experiments in the presence of K562 target cells (dotted bars) served as positive controls. Data are shown for seven different individuals. *$p = 0.05$, **$p = 0.001$.

other major activating NK receptors, remained unchanged following coculture with ZIKV-infected target cells (Supplemental Fig. 2C).

Together, these data strongly suggest that MIC-A/B plays a key role in the control of ZIKV infection by NK cells.

Engagement of MIC-A/B with NKG2D triggers cytokine production by NK cells

To gain insight into the mechanism(s) by which ZIKV induces cytokine production by NK cells in concert with MIC-A/B–expressing ZIKV-infected Mo-DCs, experiments were performed in the presence or absence of neutralizing anti–MIC-A/B mAbs. The production of IFN-γ and TNF-α was significantly decreased in the presence of neutralizing anti-NKG2D (Fig. 7C), and anti–MIC-A/B (Supplemental Fig. 3D) mAbs, at levels close to those observed with NK cells alone or cocultured with noninfected Mo-DCs. In contrast, in the presence of a neutralizing anti–MHC class I mAb, the production of IFN-γ and TNF-α remained unchanged (Supplemental Fig. 3D). Of note, the presence of neither anti–MIC-A/B nor anti–MHC class I mAbs modified the level of degranulation of autologous NK cells (data not shown).

To evaluate a possible additive/synergetic effect of IL-12 production and MIC-A/B expression by ZIKV-infected Mo-DCs on the intracellular cytokine production by autologous NK, we tested the combination of a treatment with the IL-12/IL-23 antagonist Apilimod and a neutralizing anti-NKG2D mAb. Stimulation of NK cells with ZIKV-infected target cells in the presence of both compounds resulted in a lower frequency of IFN-γ– and TNF-α–producing cells, as compared with cells treated with either Apilimod or anti-NKG2D mAb (Fig. 7C).

To further understand the relevance of these in vitro data, we finally assessed cytokine production by NK cells from ZIKV-infected patients in the presence or absence of neutralizing anti–MIC-A/B mAbs. Levels of both IFN-γ and TNF-α production were significantly decreased in NK cells from ZIKV-infected patients after treatment with anti–MIC-A/B mAbs, as compared with untreated cells, at similar levels as those observed in healthy donors (Fig. 7D).

Together, these data suggest that overexpression of MIC-A/B by Mo-DCs is a key element in the induction of cytokine production by NK cells, both in vitro and in patients infected by ZIKV.

Discussion

NK cells are known to rapidly respond during diverse acute viral infections in humans including those by arboviruses like chikungunya, yellow fever, and DENV (14, 23, 34, 35). However, the role of NK cells in the control of anti-ZIKV immunity needs to be better defined in view of the recent emergence and rapid spread of the Zika fever epidemic and the severe consequences of congenital ZIKV infection. Using high-dimensional mass cytometry, flow cytometry, and in vitro coculture assays in this study, we provide the first evidence, to our knowledge, that ZIKV infection may selectively shape the NK cell repertoire. Results revealed a massive activation of NK cells associated to the presence of adaptive CD57$^+$NKG2C$^+$ phenotype, as previously described in acute chikungunya virus and DENV infections (23, 34, 36). A limitation of this study is the absence of data concerning the seroprevalence of CMV with respect to the modulation of adaptive NK cells in ZIKV$^+$-infected patients; however, it was reported that 96.3% of the adolescents in the city of Belém in Brazil presented IgG Abs to CMV (37), suggesting that data on adaptive NK cells can be analyzed independently of the CMV serostatus.

In contrast to the overall NK cell repertoire, which contains a random distribution of KIRs in healthy donors, skewing of KIR repertoire toward self-specific KIRs has previously been observed in patients infected by some viruses (38). In this study, we show that NK cells from ZIKV$^+$ patients are preferentially associated with KIR3DL1, whereas an expansion of KIR2DL1$^+$-adaptive NK cells for DENV has been reported (23). The mechanism underlying the expansion of educated NK cells bearing self-specific KIRs remains

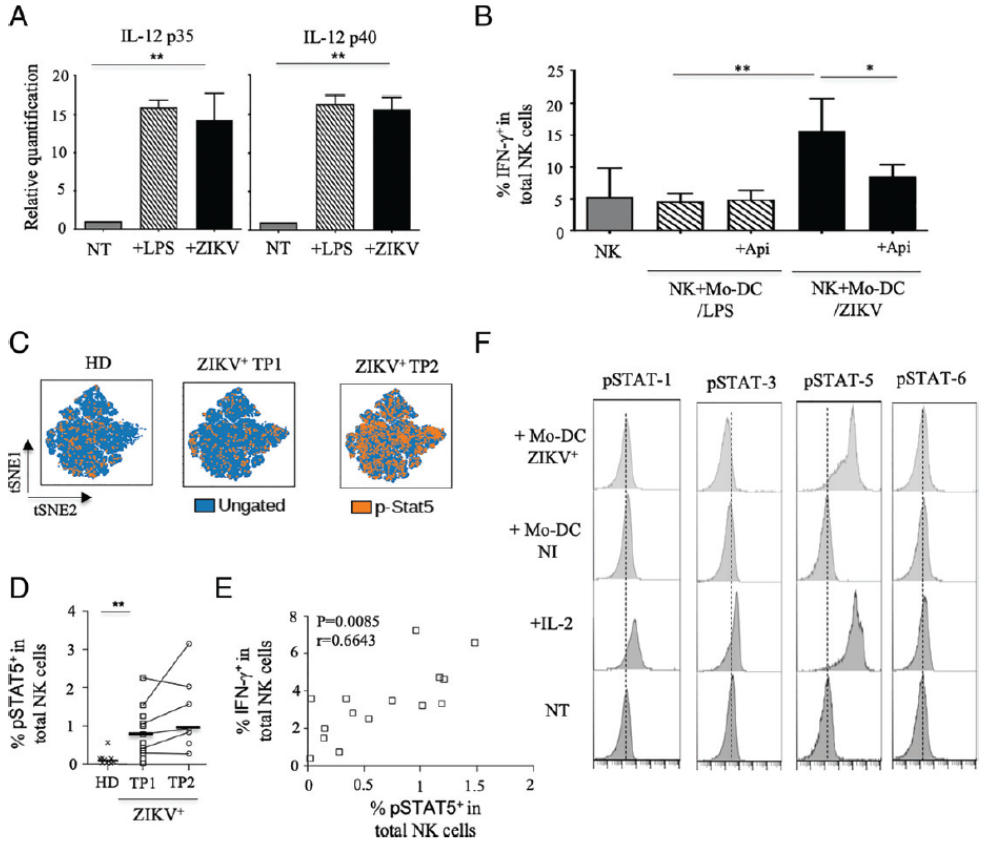

FIGURE 6. Cytokine production by NK cells from ZIKV$^+$ patients is associated to STAT-5 activation. (**A**) Quantification of IL-12 p35 and IL-12 p40 by real-time PCR in Mo-DCs infected by ZIKV. The bar graph shows the enrichment (*n*-fold) of RQ of IL-12 p35 (left panel) and IL-12 p40 (right panel) gene expression in noninfected (NT; gray bars), LPS-treated (+LPS; hatched bars) or ZIKV-infected (+ZIKV; black bars) Mo-DCs from three different individuals. (**B**) Intracellular production of IFN-γ and TNF-α by NK cells after pretreatment of target cells with 2.5 μM of Apilimod (+Api). Experiments are performed with purified NK cells (UT, gray bars) or NK cells cocultured with LPS-treated (NK + Mo-DC/LPS; hatched bars) or ZIKV-infected autologous Mo-DCs for 48 h (NK + DC/ZIKV, black bars). Data are shown in mean ± SD of seven experiments. *$p = 0.05$, **$p = 0.001$. (**C**) tSNE visualization of latent transcription factor STAT-5 activated by tyrosine phosphorylation (pSTAT5) in NK cells from healthy donors (HD) and ZIKV-infected patients (ZIKV$^+$) at different timepoints (TP1 and TP2) after onset of the symptoms. Plots represent merged files of each group of individuals. The settings used for the viSNE run were as follow: equal event sampling (44,400 events each), channels (CD16, CD56, CD57, NKG2A, NKG2C, KIR2DL1, KIR2DL2L3, KIR3DL1, CD38, HLA-DR, PD1, Tim3, CTLA4, IFN-γ, and Ki-67), iterations (7500), perplexity (30), and θ (0.7). (**D**) Frequency of pSTAT-5 expression in CD3$^-$CD56$^+$ NK cells from HD ($n = 10$) and ZIKV-infected patients (ZIKV$^+$) at different timepoints (TP1, $n = 15$; TP2, $n = 7$) after onset of the symptoms. Black lines represent the median. An unpaired Mann–Whitney *U* test was performed between HD and TP1 groups, whereas a paired Wilcoxon test was performed between TP1 and TP2 groups. (**E**) Correlation of intracellular production of IFN-γ and pSTAT-5 expression in total NK cells from ZIKV-infected patients at TP1. Data are extrapolated from the Citrus analysis. (**F**) In vitro expression of activated STAT (pSTAT-1, -3, -5, and -6) in NK cells cocultured with autologous Mo-DCs infected by ZIKV from an HD. Data were obtained using the "BD Phosflow Monocyte/NK cell Activation Kit" (catalog no. 562089). Untreated (NT) and IL-2–activated cells were used as controls. Dotted lines represent the median value observed in untreated NK cells.

elusive; although it can be hypothesized that HLA-presented viral peptides could modulate KIR/HLA interactions, as previously observed in several other viral infections (39–42). Together, the findings suggest an impact of education of NK cells during the acute phase of ZIKV infection. However, a genetic analysis of the KIR/HLA, combined with phenotypic and functional features of NK cells ZIKV-infected individuals. Another limitation was the low number the samples at the second timepoint (TP2), which reduces the statistical significance of the analysis. Thus, further prospective studies on a larger number of patients are necessary to draw firmer conclusions. Furthermore, it seems that activated and proliferating NK cells decrease over time after ZIKV infection, suggesting that intense NK cell activation and trafficking to and from tissues occurs early in ZIKV, and is associated with subsequent disease progression, providing an insight into the mechanism of clinical deterioration. Thus; it would be interesting to measure the proportion of circulating and resident CD69$^+$CD103$^+$CD49d$^+$ NK cells in different hematopoietic and nonhematopoietic tissues.

Given their functional capacities, it is important to determine whether NK cells play a role in the immune response to ZIKV infection. As previously reported for DENV infections (23, 24), ZIKV appears to impair the ability of NK cells to degranulate in infected patients. This impairment may be linked to high levels of MHC class I expression, as previously shown for other flavivirus infections (13, 24), whereas many other viruses downregulate levels of some MHC class I molecules (43). However, in contrast to DENV, infection by ZIKV does not induce the expression of the HLA-E molecule (44), suggesting that the functional capacity of NK cells is not directly mediated by NKG2C in ZIKV infection, as compared with DENV (14, 23). Consistently, we show that in ZIKV$^+$ patients, the frequency of IFN-γ$^+$ KIR3DL1$^+$ NK cells was significantly higher than that of IFN-γ$^+$NKG2C–expressing NK cells.

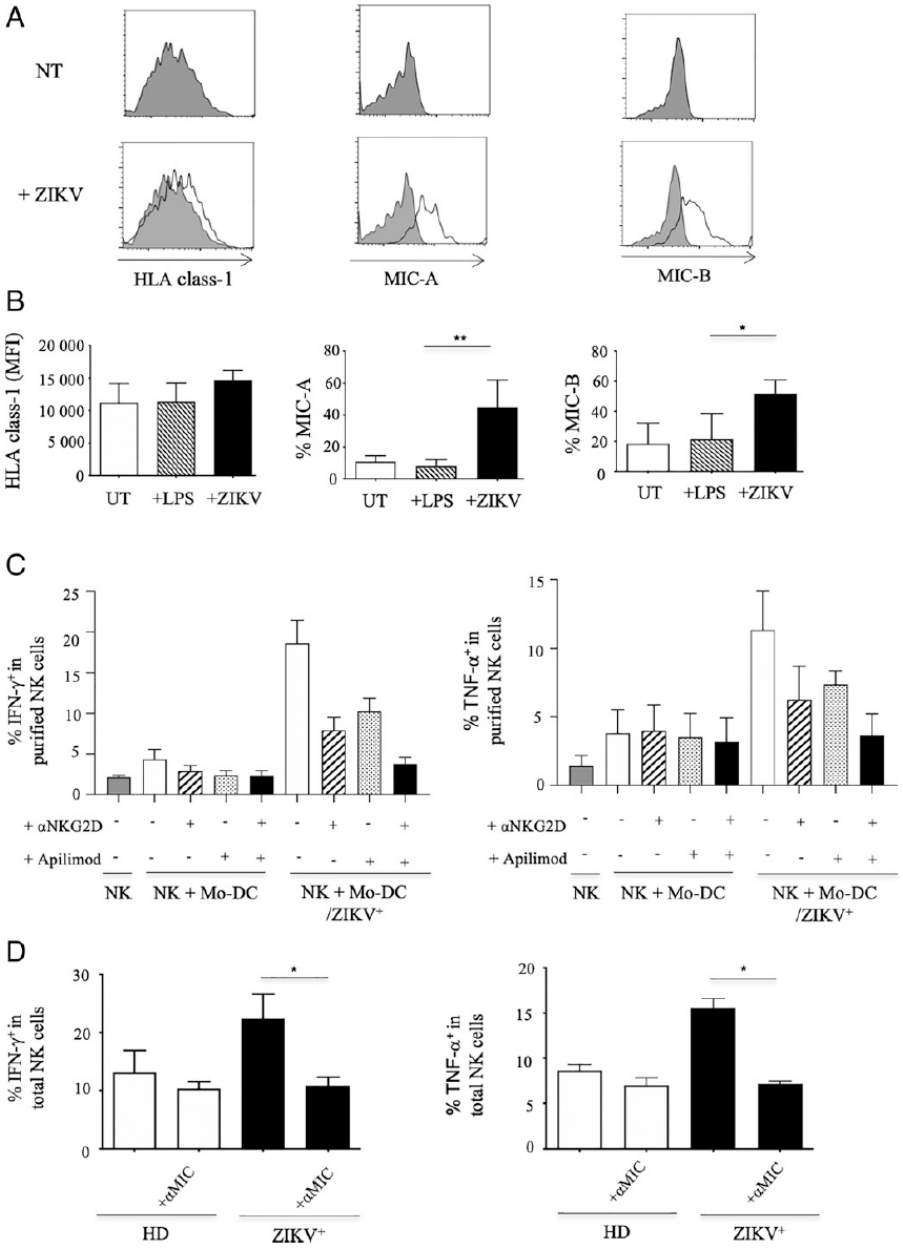

FIGURE 7. Expression of cellular ligands for NK receptors by Mo-DCs infected by ZIKV (**A**) Representative histograms of expression on noninfected (NT), and Mo-DCs infected by ZIKV for 48 h. Positive staining is represented by the open gray line. (**B**) Mean of fluorescence intensity (MFI) of MHC class I molecules expression and frequency MIC-A and MIC-B on untreated (NT, open bars), LPS-treated (+LPS; hatched bars) and ZIKV-infected (+ZIKV; black bars) Mo-DCs. Results are expressed as mean ± SD for five experiments. (**C**) Intracellular production of IFN-γ and TNF-α by NK cells after pretreatment of target cells with 10 μg/ml blocking 1D11 anti-NKG2D (+αNKG2D) mAbs and/or treated with 2.5 μM of Apilimod (+Apilimod). Experiments were performed with purified untreated NK cells (NK, gray bars), or in the presence of autologous Mo-DCs noninfected (Mo-DC) or infected by ZIKV for 48 h (NK + DC/ZIKV$^+$). Results are expressed as mean ± SD for three experiments. (**D**) Effect of pretreatment with 10 μg/ml 6D4 anti–MIC-A/B (+α-MIC) mAbs on the intracellular production of IFN-γ and TNF-α by NK cells from healthy donors (HD) and ZIKV-infected patients (ZIKV$^+$). Results are expressed as mean ± SD for seven experiments. *p = 0.05, **p = 0.001.

Production of cytokines by NK cells, measured by mass cytometry and confirmed after coculture with autologous ZIKV-infected Mo-DCs, is a key element in the control of ZIKV by NK cells. These data are further supported by previous results reporting that ZIKV-infected patients produce high levels of several immune mediators associated with NK cell function, such as IL-18, IFN-γ, and TNF-α (45). The production of IFN-γ and TNF-α by NK cells is also profoundly decreased after treatment of ZIKV-infected Mo-DCs with Apilimod, which selectively and potently inhibits the production of IL-12 (46), whereas IL-12 p35 and p40 transcripts are expressed to a higher extent in ZIKV-infected Mo-DCs. Surprisingly, results obtained from in vitro experiments, as well as from the mass cytometry analysis, also showed a massive phosphorylation of STAT-5 in NK cells from ZIKV-infected patients, confirmed in vitro by coculture with ZIKV-infected Mo-DCs, which strongly correlated with the frequency of IFN-γ$^+$ NK cells. Results obtained from the mass cytometry analysis showed that the phosphorylation of STAT-5 in NK cells from ZIKV-infected patients, confirmed in vitro by coculture with ZIKV-infected Mo-DCs, strongly correlated with the frequency of IFN-γ$^+$ NK cells. It has previously been shown that STAT-5 can upregulate the promoter activity of the IFN-γ–encoding *IFNG* genes (47). Furthermore, IL-2 or IL-12 phosphorylates STAT-5 to drive IFN-γ production in activated cells, which is even enhanced in the presence of IFN-γ (48, 49). These findings suggest that the activation of STAT-5 may be a mechanism through which activated NK cells could increase IFN-γ secretion in the presence of IL-12. Thus, it might be interesting to more precisely determine the signaling pathway(s) that are engaged in the phosphorylation of STAT-5 leading to the production of IFN-γ in ZIKV infection.

The importance of a cell-to-cell cross-talk for cytokine production by NK cells during ZIKV infection also prompted us to analyze, in

detail, the mechanism by which NK cells control infection. In vitro we observed that MIC-A and MIC-B expression was induced on ZIKV-infected Mo-DCs, whereas other ligands of NKG2D (ULBP-1 to 6), and those for NKp30 and NKp46 remained unchanged, as compared with uninfected cells, reminiscent to what has been observed for DENV (15). These data contrast with the, previously reported, small increase in the expression of activating NK cell ligands following ZIKV infection (24), and should be validated in DC from ZIKV-infected patients. The potential explanation for the discrepancies between the latter study and ours might be the difference in target cells; unlike using the A549 tumor lung carcinoma cell line, which expresses basal levels of stress molecules (50, 51), we used primary Mo-DCs from healthy donors in our experiments. MIC-A and MIC-B expression is generally not observed at the surface of normal cells but constitutively expressed in stress situations, like cancer and viral/bacterial infections (52). Furthermore, we observed that NKG2D expression was specifically downregulated in NK cells following coculture with ZIKV-infected target cells. These data are consistent with those of a previous report showing a degradation of the NKG2D receptor from the cell surface following its interaction with MIC-A/B (53). Our data suggest that an additive/synergistic effect of MIC-A/B and IL-12, produced by ZIKV-infected Mo-DCs, can trigger NK cells to produce high levels of proinflammatory cytokines, but fail to induce cytotoxicity.

Collectively, our data reveal an expansion of specific NK cells able to sense ZIKV via the NKG2D/MIC-A/B signaling pathway to induce STAT-5 and the production of proinflammatory cytokines. This specific mechanism could contribute to the generation of an efficacious adaptive anti-ZIKV immune response that may potentially affect both the outcome of the disease and/or the development of persistent symptoms.

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Disclosures

The authors declare no financial or commercial conflict of interest.

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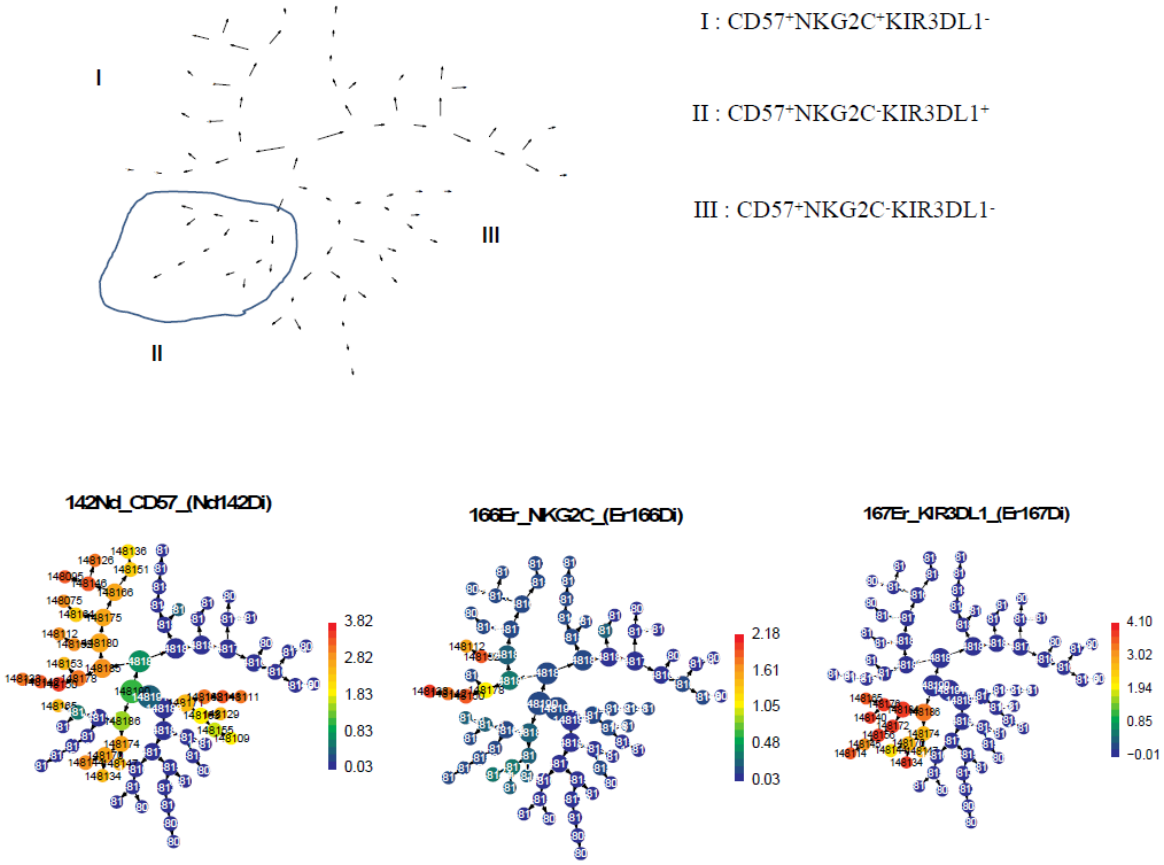
Figura 5 – Artigo 2: Nk cell disponses in zika virus infection are biased towards cytokine – mediated effector functions
Fonte: (MOUCOURANT et al. 2021)

Supplemental TABLE 1. List of markers used in the study

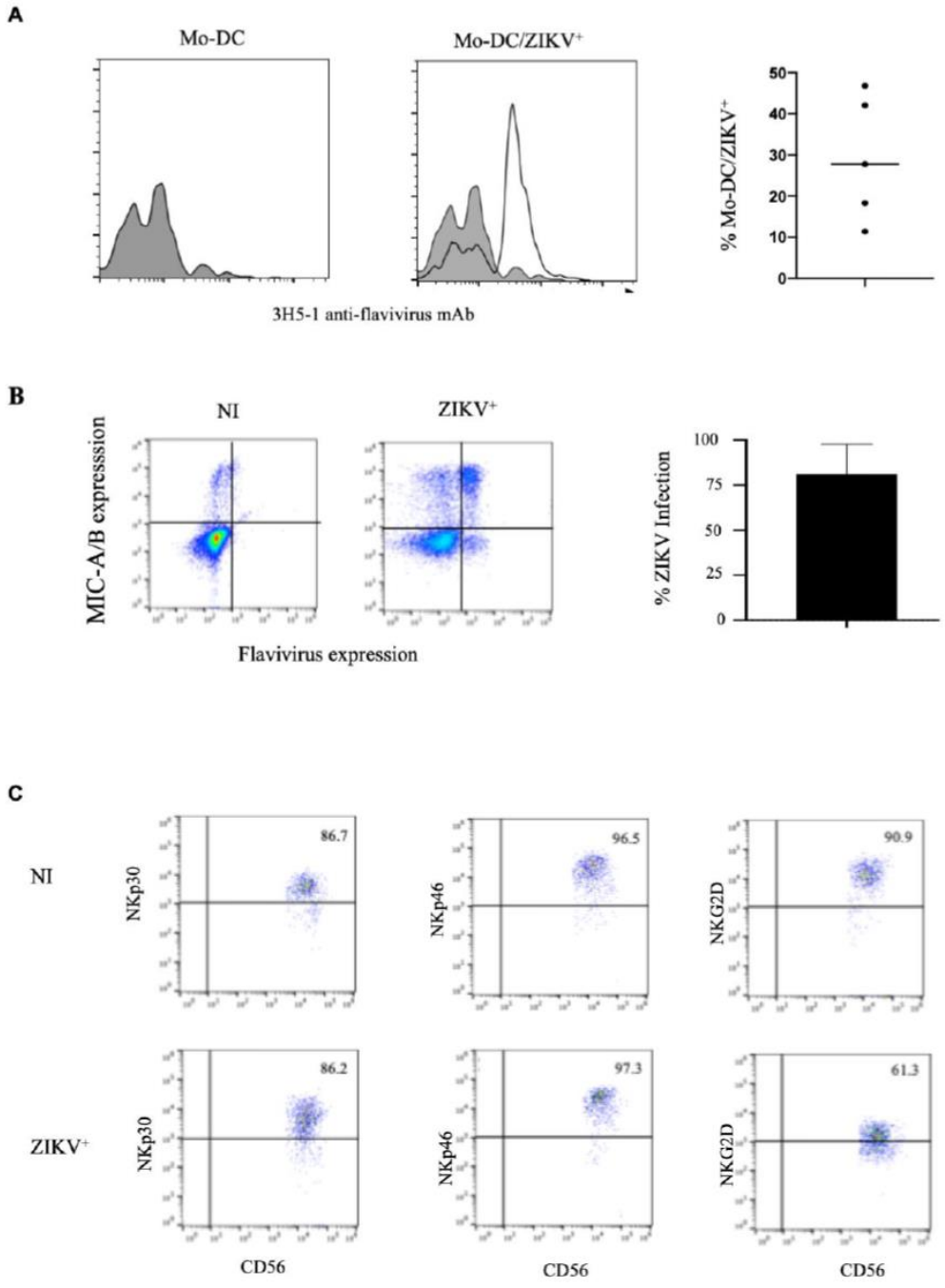
Metal Tag	Target	Clone	Supplier
^{141} Pr	CCR6	GO34E3	Fluidigm
^{142} Nd	CD57	HCD57	Fluidigm
^{144} Nd	CD38	HIT2	Fluidigm
^{145} Nd	CD4	RPA-T4	Fluidigm
^{146} Nd	CD8a	RPA-T8	Fluidigm
^{162} Dy	CD11c	Bu15	Fluidigm
^{155} Gd	CD27	EPR8569	Fluidigm
^{156} Gd	CCR5	NP-6G4	Fluidigm
^{160} Gd	CD14	M5E2	Fluidigm
^{163} Dy	CD33	WM53	Fluidigm
^{164} Dq	HLA-DR*	L243	Fluidigm
^{166} Er	NKG2C*	134591	R&D systems
^{167} Er	KIR3DL1	DX9	Fluidigm
^{171} Yb	CXCR5	51505	Fluidigm
^{209} Bi	CD11b	ICRF44	Fluidigm
^{159} Tb	CCR7	GO43H7	Fluidigm
^{143} Nd	CD45Ra	HI100	Fluidigm
^{149} Sm	CD25	EPR6452	Fluidigm
^{154} Sm	Tim3	F38-2E2	Fluidigm
^{115} Ln	CX3CR1*	2A9-1	Fluidigm
^{148} Nd	CD16	3G8	Fluidigm
^{151} Eu	CD123	6H6	Fluidigm
^{165} Ho	CD163	GHI/61	Fluidigm
^{174} Yb	CD49b	P1E6-C5	Fluidigm
^{175} Lu	PD1	EH12.2H7	Fluidigm
^{150} Nd	CD86	IT2.2	Fluidigm
^{161} Dy	CD80	2D10.4	Fluidigm
^{170} Er	CTLA4	14D3	Fluidigm
^{172} Yb	Ki67	Ki-67	Fluidigm
^{168} Er	IFN-γ	B27	Fluidigm
^{152} Sm	KIR2DL1*	REA248	Miltenyi
^{173} Yb	KIR2DL2/DL3	DX-27	Fluidigm
^{169} Tm	NKG2A*	Z199	Fluidigm
Qdot 605	CD3	UCHT1	Fluidigm
Cisp 194	CD19*	HIB19	Fluidigm
Cisp 198	CD66b*	80H3	Fluidigm
^{176} Yb	CD56	NCAM16.2	Fluidigm
^{89} Y	CD45	HI30	Fluidigm
^{153} Eu	CCR2	RMO52	Fluidigm
^{147} Sm	P-STAT5	pY694 (47)	Fluidigm

*: in-house mAb conjugated to metal isotopes

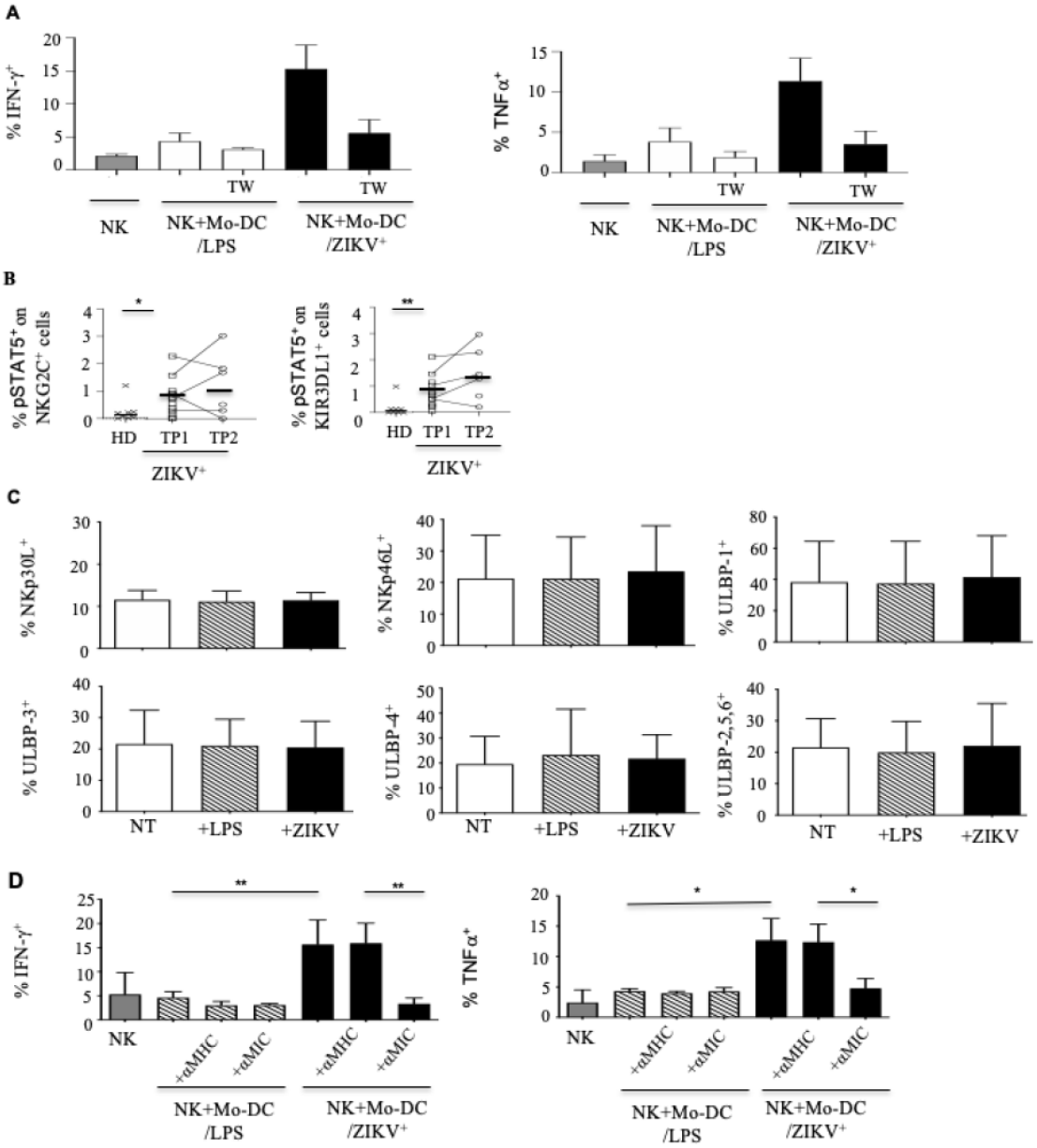


Supplemental FIGURE 1. Citrus analysis of NK cells reveals phenotypic differences in ZIKV-infected patients. Map of hierarchical clusters identified by Citrus algorithm. Purple circles represent meta-clusters of cells that significantly differentially abundant between healthy donors (HD), and ZIKV-infected patients at different timepoints (TP1 and TP2). Blue circles represent clusters below the significance threshold. Three groups of clusters are noted by areas shaded in light colors based on CD57, NKG2C and KIR3DL1 expression. Inside this groups, significantly different cluster are colored in red. The metaclusters in blue show significant differences of an FDR of 0.01 and a metacluster in green shows a significant difference FDR of 0.05.

CITRUS was performed using the Significance Analysis of Microarrays (SAM) correlative association model with the following parameters: Clustering channels: CD56, CD16, CD57, CD38, HLADR, KIR2DL1, KIR3DL1, KIR2DL2L3, NKG2A, NKG2C; Cluster Characterization: "Abundance"; Event sampling: "Equal"; Events sampled per file: "5,000"; Minimum cluster size (%): "1"; Cross Validation Folds: "5"; False Discovery Rate (%): "1".



Supplemental FIGURE 2. Infected Mo-DC downregulates NKG2D ligands expression after infection. **(A)** Representative expression and quantification of ZIKV-infected Mo-DCs in 5 experiments. Positive staining is represented by the black line. **(B)** Representative expression and quantification of ZIKV-infected Mo-DCs expressing MIC-A/B in 5 experiments. **(C)** representative dot-plot analysis of activating receptors (NKp30, NKp46 and NKG2D) on total $CD3^{-}CD56^{+}$ NK cells after 5-hr co-culture with non-infected (NI) or ZIKV-infected ($ZIKV^{+}$) autologous Mo-DCs.



Supplemental FIGURE 3. NK-cell function and pSTAT-5 expression during ZIKV infection. (**A**) Intracellular production of IFN-γ and TNF-α by NK cells after co-culture in the presence of a transwell (+TW). The results are presented as the mean±SD from 3 experiments. (**B**) Frequency of pSTAT5 expression in NK cells expressing NKG2C or KIR3DL1 from healthy donors (HD, n=8) and ZIKV-infected patients (ZIKV$^+$) at different timepoints (TP1, n=13 and TP2, n=6) after onset of the symptoms. Black lines represent the median. **(C)** Frequency (%) of ligands for NKp30 (NKp30L), NKp46 (NKp46L), NKG2D (ULBP-1 to 6) on purified non-infected (NT, open bars), and Mo-DCs infected by ZIKV (Black bars), compared to Mo-DCs treated by LPS (hatched bars). Results are done in mean±SD for 5 experiments. **(D)** Intracellular production of IFN-γ and TNF-α by NK cells after pre-treatment of target cells with 10 µg/mL 6D4 anti-MIC-A/B (+αMIC) or W6/32 anti-MHC Class 1 (+αMHC) mAbs. Experiments are performed with purified untreated NK cells (NK, grey bars), after LPS pretreatment by LPS (NK+Mo-DC/LPS, hatched bars) or in the presence of autologous non-infected Mo-DCs infected by ZIKV for 48 hr (NK+Mo-DC/ZIKV, black bars). Results are done in mean±SD for 7 experiments.

6 **CONSIDERAÇÕES FINAIS**

No primeiro estudo, a nossa revisão buscou avaliar o papel das citocinas e quimiocinas na infecção pelo ZIKAV e suas ligações potenciais à patogênese. Desta forma, percebemos que na infecção pelo ZIKV, a resposta imune inata pode ser essencial para o estabelecimento de mecanismos para controlar a infecção. No entanto, durante a infecção inicial, o ZIKAV pode escapar a resposta imune e modular uma série de eventos que culminam na entrada do vírus principalmente células endoteliais, bem como subtipos de macrófagos M1 e M2. Esta atividade pode ser atribuída à ação de fatores de entrada, como DC-SIGN, AXL, and TYRO3. Após a entrada, o ZIKAV replica-se em macrófagos no tecido e/ou em células dendríticas, que levam o vírus para os nódulos linfáticos e outros tecidos linfóides e a "cascata de citocinas" é ativada ainda durante este processo inicial (HAMEL et al., 2016; NOWAKOWSKI et al., 2016; NGONO & SHRESTA., 2018). Ainda naquele momento, havia escassos relatos sobre o envolvimento de células NK na infecção por ZIKAV relatados na literatura. Para isso, o segundo estudo, buscou avaliar extensivamente através da plataforma de análise de alta tecnologia o fenótipo e a função das células NK por citometria de massa (CyTOF).

Apesar do nosso estudo, nenhum relato usando a técnica de Citometria de Massa (CyTOF) para avaliações da resposta imune inata na infecção pelo ZIKAV foi identificado. A maior parte dos estudos atuais que avaliam a resposta imune no contexto de diversas doenças utilizam a citometria de fluxo como técnica. Ao contrário da citometria de fluxo, que requer compensação para a sobreposição de fluorescência espectros, a citometria de massa (CyTOF) utiliza anticorpos marcados com metais, desta forma, não há interferência da sobreposição espectral entre os canais (BENDALL et al., 2011)

Atualmente, CyTOF permite análise de mais de 45 parâmetros simultaneamente em uma única célula (AMIR et al., 2013). Os citômetros de fluxo convencionais, que utilizam anticorpos conjugados fluorescentes para detectar parâmetros celulares, foram praticamente limitados em cerca de 15-18 parâmetros. O perfil espectral é limitado em sua capacidade e parece haver pouco espaço para forçar mais fluoróforos no perfil espectral em cerca de 18 parâmetros, por este motivo, CyTOF é vantajoso em relação a citometria de fluxo fluorescente tradicional, pois permite que um número significativo de parâmetros seja estudado por célula. O CyTOF também é mais sensível e há menos erros (FLOWJO, 2019). As desvantagens do CyTOF estão principalmente associadas ao fato de que as células são incineradas (portanto, não há recursos de classificação). Além disso, os reagentes são extremamente caros atualmente, mas como

qualquer tecnologia, o preço provavelmente cairá à medida que a tecnologia for adotada (FLOWJO, 2019).

Além da Citometria de massa, nós utilizamos citometria de fluxo e ensaios de cocultura *in vitro* neste estudo. E assim, nós fornecemos a primeira evidência, que a infecção pelo ZIKAV pode o repertório de resposta da célula NK. Os nossos resultados revelaram uma expressiva ativação de células NK, através da presença do fenótipo adaptativo CD57+ NKG2C+ nestas células, como anteriormente descrito em infecções agudas por vírus CHIKV e DENV (PETITDEMANGE et al., 2011; PETITDEMANGE et al., 2016; PAUST et al., 2017).

Nós observamos que as células NK de pacientes com ZIKAV estão preferencialmente associadas a expressão de KIR3DL1. Corroborando com os dados que mostram que embora o repertório geral de células NK em doadores saudáveis, contenham uma distribuição aleatória de KIRs, há uma distorção do repertório KIR em pacientes infectados por alguns vírus, embora o mecanismo associado a esta distorção ainda permaneça indefinido (KULKARNI et al., 2008; PETITDEMANGE et al., 2016). Observa-se também que a infecção pelo ZIKAV parece prejudicar a capacidade das células NK de degranularem. Esta deficiência pode estar ligada a expressão de altos níveis de MHC classe I, como observado em outras infecções por flavivírus, embora muitos outros vírus regulem negativamente os níveis de expressão de MHC de classe I (LOBIGS et al., 2003; GLASNER et al., 2017; MAUCOURANT et al., 2019).

Adicionalmente, nós observamos que a em pacientes com ZIKV, a frequência de células NK IFN-g+ KIR3DL1+ foi significativamente maior do que o de Células NK que expressam IFN-g+ NKG2C+. Além disso, na infecção pelo ZIKV não foi observada a expressão da molécula HLA-E, diferentemente da infecção pelo DENV, desta forma, podendo sugerir, que a capacidade funcional das células NK não é diretamente mediada por NKG2C na infecção por ZIKV, como em comparação com DENV (PETITDEMANGE et al., 2016; DREWS et al., 2018; ZIMMER et al., 2019)

Em relação a produção de citocinas por células NK, nossos resultados foram medidos por citometria de massa e confirmados após a cocultura com Mo-DCs (*Monocyte-derived dendritic cells*) infectados com ZIKV. Estes resultados revelaram que esta resposta é um elemento chave no controle do ZIKAV. Esses dados são corroborados por resultados anteriores que evidenciam a alta expressão de diversos mediadores imunológicos associados a função de células NK, como IL-18, IFN-γ e TNF-α (KAM et al., 2017). Os resultados obtidos também mostraram alta expressão de STAT-5 em células NK de pacientes infectados com ZIKV e se correlacionaram fortemente com a frequência de células NK IFN-γ+, como já observado

anteriormente, onde STAT-5 pode regular positivamente a expressão do IFN-g (GONSKY et al., 2004).

Desta forma, nossos resultados mostram que as células NK podem ter um importante papel na geração de uma resposta imune anti-ZIKAV eficaz que pode potencialmente afetar o desfecho da doença e/ou o desenvolvimento de sintomas persistentes. Entretanto, apesar de realizarmos uma extensa avaliação de marcadores de células NK, tendo em vista ainda o recorrente sazonal ressurgimento e disseminação de casos da infecção pelo ZIKAV e as consequências graves da infecção congênita, são necessário novos estudos que avaliem a genética dos receptores KIR / HLA, demais receptores, combinado com avaliação das características fenotípicas e funcionais das células NK dos indivíduos infectados com ZIKV. Além disso, outros estudos prospectivos em um número maior de pacientes são necessários para entender o papel das células NK no controle da imunidade anti-ZIKV.

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ANEXO

Anexo A - Aprovação do comitê de ética em pesquisa

CENTRO DE PESQUISAS GONÇALO MONIZ - FIOCRUZ/BA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Resposta imune na infecção pelo vírus Zika: impacto na patogênese e aplicações no desenvolvimento de vacinas e imunodiagnósticos

Pesquisador: Maria Fernanda Rios Grassi

Área Temática:

Versão: 1

CAAE: 55882016.6.0000.0040

Instituição Proponente: Centro de Pesquisas Gonçalo Moniz - CPqGM/ FIOCRUZ/ BA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.556.677

Apresentação do Projeto:

A presença do vírus Zika (ZIKV) no Brasil, descrito pela primeira vez em 2015, coincidiu com um importante aumento nos casos de síndrome de Guillan-Barré em adultos e alterações neurológicas em recém-nascidos, incluindo microcefalia. A participação da resposta imune na proteção e/ou desenvolvimento das complicações necessita ser estabelecida. A presente proposta tem como objetivo avaliar elementos da resposta imune inata e adquirida em indivíduos com diferentes formas clínicas da infecção. Em especial pretende identificar assinaturas imunológicas que sejam particulares das diferentes formas de apresentação da doença e, secundariamente, avaliar se existem reações cruzadas aos vírus de chikungunya e da dengue (DENV). Para tal, um consórcio de pesquisadores clínicos (Secretaria de Saúde do Estado da Bahia), virologistas (Universidade Federal da Bahia), imunologistas (Fiocruz-Bahia) e biologistas moleculares e imunologistas (Département d'Immunologie Hôpitaux Universitaires Pitié-Salpêtrière, Université Pierre et Marie Curie – França) está sendo estruturado. A resposta de linfócitos aos peptídeos e proteínas recombinantes de ZIKV, assim como a resposta de células da imunidade inata a antígenos virais serão avaliadas por diferentes metodologias incluindo citometria de massa (CyTOF), que analisa simultaneamente até 30 parâmetros em um único tubo. Além disso, os anticorpos de indivíduos infectados pelo ZIKV serão avaliadas quanto a especificidade antigênica, a capacidade de neutralização e a reatividade

Endereço: Rua Waldemar Falcão, 121
Bairro: Candeal **CEP:** 40.296-710
UF: BA **Município:** SALVADOR
Telefone: (71)3176-2327 **Fax:** (71)3176-2285 **E-mail:** cep@bahia.fiocruz.br

cruzada a antígenos de DENV-4 e chikungunya.

Objetivo da Pesquisa:

2 OBJETIVO GERAL

Investigar a resposta imune no curso da infecção pelo ZIKV em pacientes com diferentes formas clínicas.

2.1 Objetivos específicos

1) Predizer in silico os epítopos T CD8+ imunodominantes do ZIKV, dengue e CHIKV considerando o perfil de moléculas de HLA de classe I da população de Salvador e Brasil;

2) Produzir proteínas recombinantes de ZIKV, DENV e CHIKV em sistema procariótico e/ou eucariótico;

3) Caracterizar os aspectos morfológicos das lesões exantemáticas;

4) Avaliar biomarcadores sorológicos (citocinas, marcadores de fase aguda inflamatória);

5) Determinar o papel de fagócitos mononucleares na patogênese de doenças associadas à infecção pelo ZIKV.

6) Avaliar a resposta linfoproliferativa e produção de citocinas frente às proteínas recombinantes e peptídeos derivados de ZIKV, DENV e CHIKV;

7) Quantificar a proporção de linfócitos TCD8+ específicos de peptídeos de ZIKV, DENV e CHIKV utilizando ELISPOT;

8) Caracterizar o fenótipo de linfócitos T CD4 e T CD8, monócitos, células dendríticas, linfócitos NK, NKT e células linfóides inatas pela avaliação multiparamétrica de marcadoras de função, ativação, memória, produção de citocinas utilizando citometria de massa;

9) Avaliar a especificidade antigênica de clones de linfócitos B imortalizados frente às proteínas recombinantes de ZIKV, DENV e CHIKV;

10) Avaliar a produção de anticorpos neutralizantes para ZIKV e o grau de reatividade cruzada com DENV e CHIKV.

Avaliação dos Riscos e Benefícios:

Não há considerações sobre potenciais riscos aos participantes da pesquisa no protocolo anexado. No formulário preenchido na Plataforma Brasil aponta-se apenas os riscos inerentes à coleta de sangue, apesar de nos TCLEs anexados referir que "Os investigadores deste estudo tratarão a sua identidade com confidencialidade. Nomes de participantes ou materiais identificando participantes não serão liberados sem permissão escrita, exceto se exigido por lei. Os participantes não serão identificados em nenhuma publicação que possa resultar deste estudo." Os TCLEs anexados

Anexo B - Termo de consentimento livre e esclarecido (TCLE)

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Identificação do paciente:

Título do protocolo de pesquisa: Resposta imune na infecção pelo vírus Zika: impacto na patogênese e aplicações no desenvolvimento de vacinas e imunodiagnósticos

Avaliação do risco da pesquisa: risco mínimo

Duração da pesquisa: 2 anos, após inclusão do último paciente

Contato do Comitê de Ética em Pesquisa (CEP) do Instituto Gonçalo Moniz/FIOCRUZ: Rua Waldemar Falcão, 121, Candeal, Salvador-Ba, CEP: 40296-710, Telefone: 3176-2285.

Contato dos pesquisadores responsáveis:

Dr. Antônio Carlos Bandeira CRM-Ba 8946; Telefone: 3116 3084 / 999376149

Dr. Maria Fernanda Rios Grassi CRM-Ba 9894; Telefone: 3176 2213 / 988287469

A infecção pelo vírus ZIKA pode não causar nenhum sintomas ou levar ao aparecimento de manchas vermelhas pelo corpo, febre baixa, dor nas juntas e inflamação dos olhos. No entanto, alguns casos de complicações da infecção foram relatados, a exemplo de uma fraqueza progressiva das pernas e dos músculos envolvidos na respiração (síndrome de Guillan-Barré) e, em recém-nascidos que foram infectados durante a gravidez, calcificações e diminuição do tamanho do cérebro (microcefalia).

Você está sendo convidado a participar deste estudo porque tem ou teve infecção pelo vírus Zika. Esta pesquisa tem como objetivo investigar o papel da resposta imune em pessoas com a infecção pelo Zika que apresentaram diferentes formas clínicas da doença.Sua participação nesta pesquisa será muito importante, pois ajudará a caracterizar o tipo de resposta imune contra o vírus. Isso poderá nos ajudar a desenvolver mais tarde testes para o diagnóstico, vacinas e medicações.

Nós solicitamos que você nos doe 50 ml de sangue da veia (mais ou menos um xícara de café), para realização dos testes que avaliarão a resposta de defesa contra o vírus. Se você teve o diagnóstico da infecção aguda por Zika, gostaríamos de coletar a mesma quantidade de sangue daqui a 6 meses. Neste caso, faremos a coleta no momento de uma consulta médica de revisão, sem custos para você. Seus gastos com transporte e alimentação para realizar a segunda coleta será devolvido a você. O sangue será coletado por um profissional treinado, porém poderá ocorrer uma pequena dor e um rápido sangramento no local da picada. Oseu braço pode ficar roxo no local, mas esta mancha desaparecerá em 1 ou 2 dias. Seu sangue será testado para HIV, HTLV, HBV, HCV, dengue, CHIKV e sífilisapenas por causa desta pesquisa. Estes exames serão realizados sem nenhum custo e os resultados serão comunicadosa você pelo médico em uma consulta de revisão.

Solicitamos, ainda, que você realize uma consulta médica, na qual serão feitas perguntas sobre os seus dados demográficos (idade, sexo e procedência), sobre sua história médica (doenças que já apresentou) e avaliação da sua condição física (exame físico). Solicitamos também o acesso ao seu prontuário médico para que seja revisado pelos pesquisadores para obtenção das seguintes informações sobre exames laboratoriais e história médica.

A sua participação nesse estudo não lhe trará nenhum benefício direto. No entanto, as informações obtidas a partir desta pesquisa poderão no futuro beneficiar outras pessoas ao contribuir para

a melhor compreensão da resposta de defesa contra o vírus.Você não receberá pagamento pelasua participação neste estudo.As consultas, os procedimentos e testes laboratoriais realizados para esta pesquisa serão fornecidos sem nenhum custo. Os potencias riscos de sua participação nesse estudo são mínimos e estão ligados à coleta de sangue que poderá causar um desconforto leve e passageiro.

Vocêpoderá deixar de participar deste estudo se o pesquisador achar necessária a sua retirada, se você decidir retirar a sua participação ou se o pesquisador e/ou o Comitê de Ética em Pesquisa decidirem pelo término do estudo.

Você terá a garantia que em qualquer momento poderá solicitar informações sobre os procedimentos, riscos e benefícios relacionados a esta pesquisa, inclusive esclarecimento sobre qualquer dúvida. Igualmente, você tem liberdade de se recusar a participar ou retirar o consentimento de sua participação em qualquer momento. A participação é voluntária e a recusa não causará nenhuma perda de benefícios e não irá colocar em risco o seu direito de receber tratamento agora ou no futuro.

Os investigadores deste estudo tratarão a sua identidade com confidencialidade. Os nomes ou materiais identificando participantes não serão liberados sem permissão escrita, exceto se exigido por lei. Os participantes não serão identificados em nenhuma publicação que possa resultar deste estudo. Uma cópia deste consentimento informado será mantida nos seus registros médicos e uma será dada a você.

Você declara que recebeu todas as orientações sobre esta pesquisa e que entendeu que o objetivo deste estudo é uma melhor compreensão a resposta de defesa contra o vírus Zika. Declara também que sabe que este trabalho foi submetido e aprovado pelo Comitê de Ética em Pesquisa Humana.

Estou recebendo uma via deste documento datada e assinada, e não estou abdicando de nenhum dos meus direitos legais.

Autorizo os pesquisadores a guardar as minhas amostras de sangue (soro e células do sangue congeladas) obtidas neste estudo nas instituições de pesquisa sob sua responsabilidade. Caso as amostras não sejam totalmente utilizadas neste estudo, autorizo a utilizá-las em estudos futuros relacionados a este tema, desde que aprovados pelo Comitê de Ética em Pesquisa.

☒ SIM ☐ NÃO

__/__/__ _____

Assinatura do paciente

Impressão datiloscópica do paciente (para indivíduos não alfabetizados):

__/__/__ _____ _____

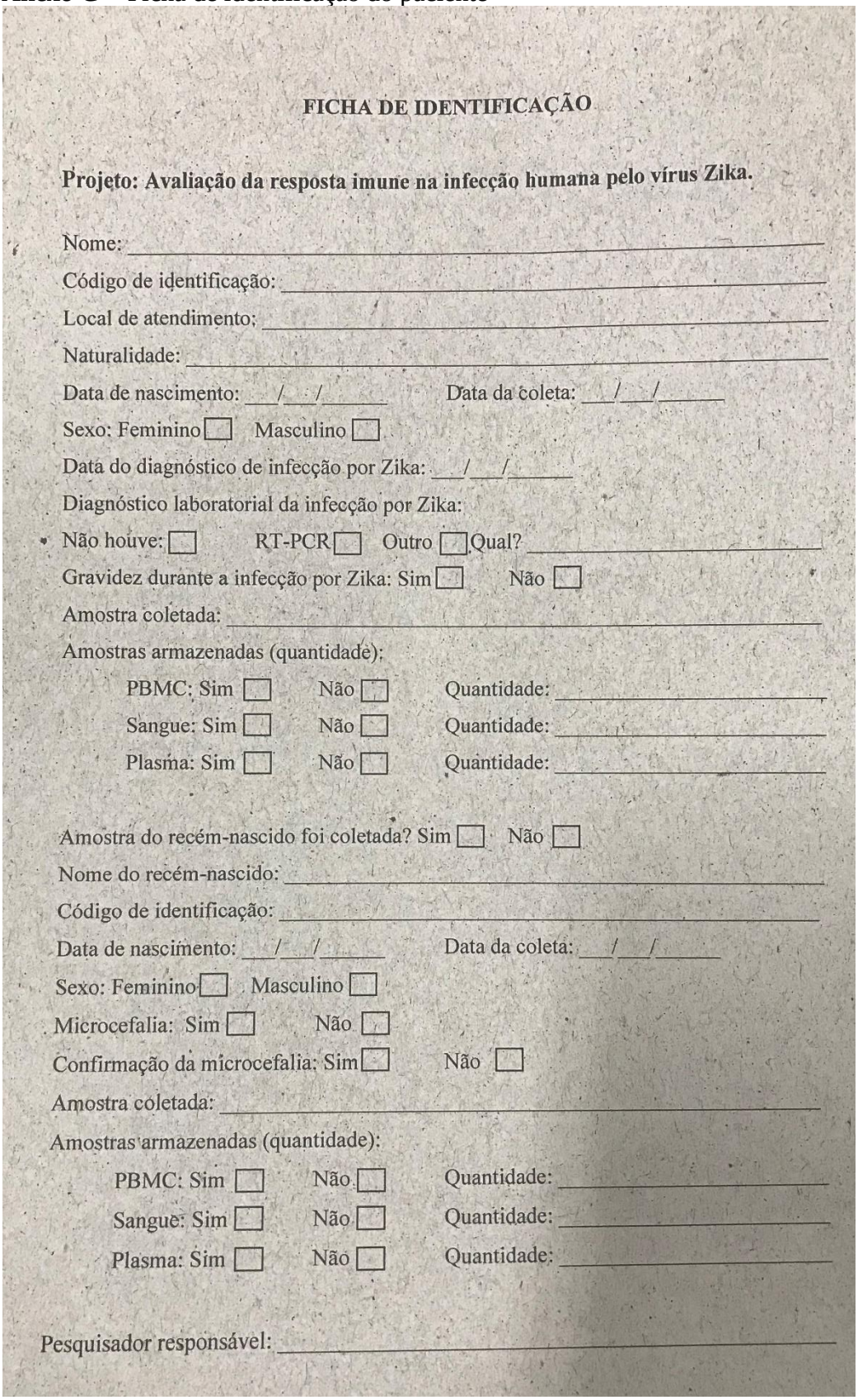
Assinatura do investigador que explicou o termo (carimbar sobre a assinatura)

__/__/__ _____

Assinatura do coordenador do estudo (carimbar sobre a assinatura)

Testemunha: _____

Anexo C - Ficha de identificação do paciente



FICHA DE IDENTIFICAÇÃO

Projeto: Avaliação da resposta imune na infecção humana pelo vírus Zika.

Nome: _____

Código de identificação: _____

Local de atendimento: _____

Naturalidade: _____

Data de nascimento: ___/___/_____ Data da coleta: ___/___/_____

Sexo: Feminino ☐ Masculino ☐

Data do diagnóstico de infecção por Zika: ___/___/_____

Diagnóstico laboratorial da infecção por Zika:

Não houve: ☐ RT-PCR ☐ Outro ☐ Qual? _____

Gravidez durante a infecção por Zika: Sim ☐ Não ☐

Amostra coletada: _____

Amostras armazenadas (quantidade):

PBMC: Sim ☐ Não ☐ Quantidade: _____

Sangue: Sim ☐ Não ☐ Quantidade: _____

Plasma: Sim ☐ Não ☐ Quantidade: _____

Amostra do recém-nascido foi coletada? Sim ☐ Não ☐

Nome do recém-nascido: _____

Código de identificação: _____

Data de nascimento: ___/___/_____ Data da coleta: ___/___/_____

Sexo: Feminino ☐ Masculino ☐

Microcefalia: Sim ☐ Não ☐

Confirmação da microcefalia: Sim ☐ Não ☐

Amostra coletada: _____

Amostras armazenadas (quantidade):

PBMC: Sim ☐ Não ☐ Quantidade: _____

Sangue: Sim ☐ Não ☐ Quantidade: _____

Plasma: Sim ☐ Não ☐ Quantidade: _____

Pesquisador responsável: _____

APÊNDICE

Apêndice A - Relatório de estágio doutoral

INTRODUÇÃO

O Centro de Imunologia e Doenças Infecciosas (*Centre d'Immunologie et des Maladies Infectieuses*/CIMI) está localizado na Faculdade de Medicina Pierre e Marie Curie, no Hospital Pitié-Salpêtrière em Paris-França (Figura 1).

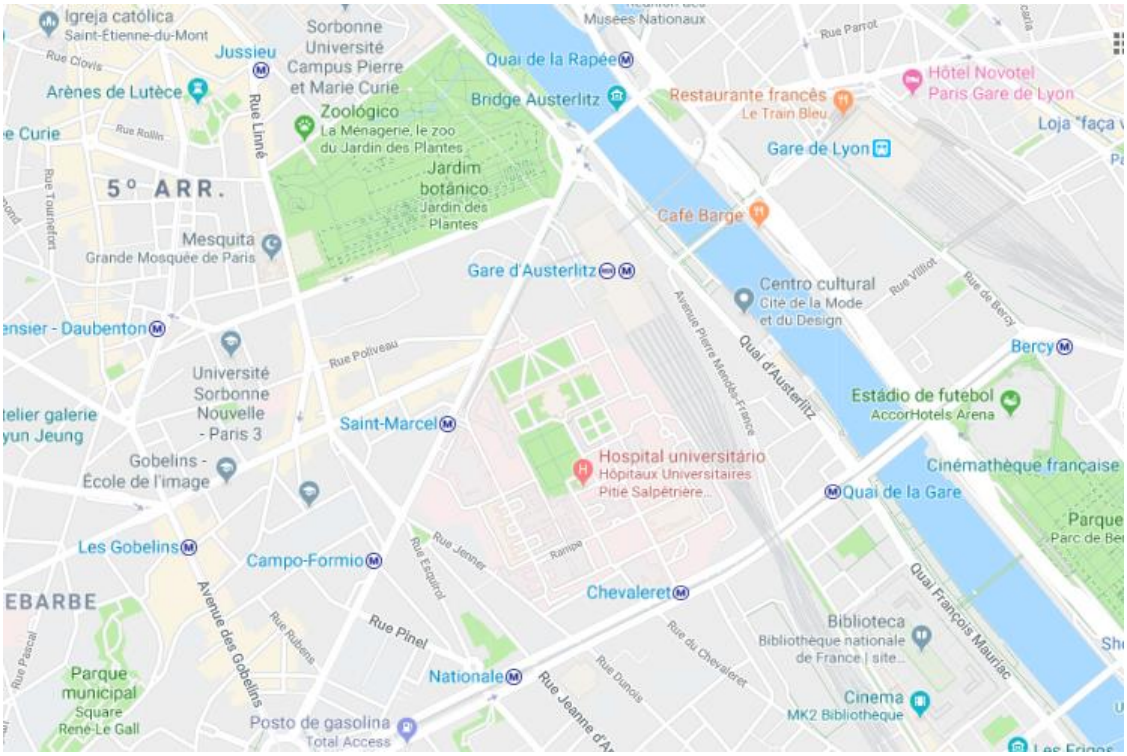


Figura 1- Mapa de localização do Hospital Pitié-Salpêtrière em Paris-França (Fonte: Google Maps, 2019)

O CIMI (Figura 2) é uma unidade de pesquisa criada em janeiro de 2014 pela Universidade Pierre e Marie Curie (UPMC), pelo Instituto Nacional de Pesquisa Médica e de Saúde (INSERM) e pelo Centro Nacional de Pesquisa Científica (CNRS)(CIMI-PARIS, 2019).

Figura 2 – Logotipo do CIMI-Paris(Fonte: CIMI, PARIS -2019).

O CIMI tem diversas vertentes de estudo, principalmente visando compreender as funções do sistema imunológico em condições normais e / ou patológicas devido a agentes infecciosos. O centro reúne experiência científica e clínica em treinamento e pesquisa nas áreas de imunologia, inflamação, microbiologia, interações patógeno-hospedeiro e vacinologia (CIMI-PARIS, 2019).

Por sua localização estratégica, no Hospital Pitié-Salpêtrière em Paris-França, o CIMI possui um ambiente particularmente propício para colaborações clínicas no campo das doenças infecciosas, transplantes, hematologia, imunologia e medicina interna (CIMI-PARIS, 2019).

EIXOS DE PESQUISA

O CIMI reúne pesquisadores que atuam nas diferentes áreas associadas a imunologia e doenças infecciosas, através de pesquisas com uma ampla gama de aspectos associados a respostas imunes e interações hospedeiro / patógeno, bem como a capacidade de microrganismos para superar tratamentos anti-infecciosos. O organograma de áreas do CIMI está disposto na (Figura 3).

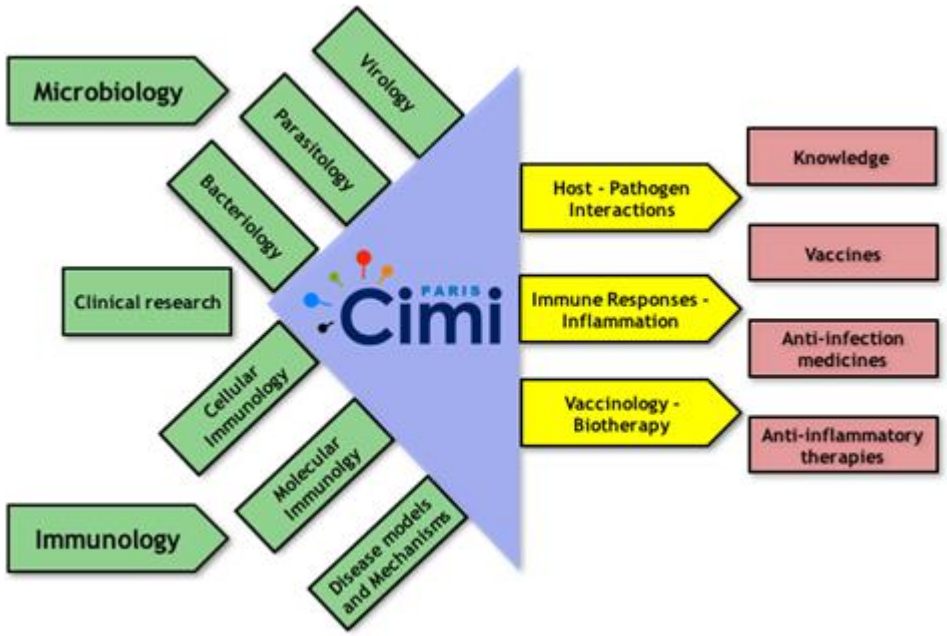


Figura 3 – Organograma de áreas do CIMI-Paris (Fonte: CIMI, PARIS -2019).

EQUIPES

O CIMI-Paris possui diversos grupos de pesquisa, listados abaixo:

- Dinâmica, estrutura e biologia molecular da invasão fúngica (*Dynamique, structure et*

biologie moléculaire de l'invasion fongique). Coordenador: Prof. A. WEINER.

- Imunopatogênese de infecções virais e envelhecimento imune (*Immunopathogénèse des infections virales et vieillissement immunitaire*). Coordenador: Prof. V. APPAY.
- Imunidade e imunogenética antiviral e vacina (*Immunité et immunogénétique antivirale et vaccinale*). Coordenador: Prof. B. AUTRAN.
- Imunidade e Vacinação (*Immunité et Vaccination*). Coordenador: Prof. B. COMBADIÈRE.
- Quimiocinas em patologias inflamatórias (*Les chimiokines dans les pathologies inflammatoires*). Coordenador: Prof. C. COMBADIÈRE e P. DETERRE.
- Imunologia celular e molecular de doenças inflamatórias crônicas (*Immunologie cellulaire et moléculaire des maladies chroniques inflammatoires*). Coordenador: Prof. G. GOROCHOV.
- Imuno-intervenção e Bioterapia (*Immuno-intervention et Biothérapies*). Coordenador: Prof. F. LEMOINE.
- Imunobiologia das infecções virais e apresentação do antígeno (*Immunobiologie des infections virales et présentation de l'antigène*). Coordenador: Prof. A. MORIS.
- Biologia da célula T reguladora e implicações terapêuticas (*Biologie des lymphocytes T régulateurs et implications thérapeutiques*). Coordenador: Prof. B. SALOMON.
- Biologia e Imunologia da Infecção Hepática da Malária (*Biologie et Immunologie de l'infection hépatique du paludisme*). Coordenador: Prof. O. SILVIE.
- Malária: identificação pré-clínica e validação de novos alvos terapêuticos (*Paludisme: identification et validation pré-clinique de nouvelles cibles thérapeutiques*). Coordenador: Prof. D. MAZIER.
- Emergência e disseminação de multirresistência a antibióticos (*Emergence et propagation des multi-résistances aux antibiotiques*). Coordenador. Prof. W. SOUGAKOFF.
- NK e doenças patológicas (*NK et maladies pathologiques*). Coordenador. Prof. V. VIEILLARD.
- Dinâmica, estrutura e biologia molecular da invasão fúngica (Dynamique, structure et biologie moléculaire de l'invasion fongique).

ESTÁGIO DE DOUTORADO NO CIMI PARIS

O presente relatório tem como objetivo descrever o período de estágio do Doutorando Gabriel Queiroz, de 02 de janeiro de 2019 a 01 de fevereiro de 2019, do curso de pós-graduação em Biotecnologia em Saúde e Medicina Investigativa da Fiocruz-BA, no CIMI-Paris.

O estágio fez parte da execução do projeto intitulado "Avaliação da resposta imune das células natural killer (NK) de indivíduos infectados pelo Zika vírus (ZIKV)" e colaboração da equipe do LASP-Fiocruz-BA e do CIMI-Paris.

OBJETIVOS DO ESTÁGIO

- Caracterizar o fenótipo e função das células NK e NKT de indivíduos infectados pelo Zika Vírus com diferentes formas clínicas pela avaliação multiparamétrica com, pelo menos, 25 marcadores.
- Treinar análise de dados de plataforma de tecnológica de citometria de massa (CyTOF).

JUSTIFICATIVA

Até o momento a resposta imune no curso da infecção pelo ZIKV foi pouco avaliada, portanto ainda existem importantes lacunas nesta área de conhecimento. A patogênese destas complicações ainda é desconhecida e não estão claros quais fatores estariam ligados a esses desfechos, a exemplo da carga viral, fatores do hospedeiro, momento da infecção ou presença de outras comorbidades.

As células NK e NKT têm um papel fundamental na resposta imune celular em infecções virais. Os estudos com estas células na infecção pelo ZIKV ainda são restritos e insuficientes em revelar resultados que contribuam com o conhecimento acerca do papel destas células na infecção.

A execução do presente estágio, após as conclusões finais do trabalho de Doutorado, pode contribuir para este entendimento, além de poder contribuir no entendimento da fisiopatologia da infecção e de identificação de possíveis assinaturas imunológicas que possam auxiliar na compreensão do papel da resposta imune inata nesta infecção.

PROJETO E EQUIPE DE PESQUISA

O presente plano de trabalho está vinculado ao projeto ZIKAlliance, intitulado "Avaliação da resposta imune celular na infecção humana pelo vírus Zika", que é financiado pela Comunidade Europeia e tem como objetivo avaliar a resposta imune na infecção pelo vírus Zika. Trata-se de um período de estágio em colaboração entre as equipes brasileiras e a equipe francesa, que possui firme colaboração em trabalhos prévios.

No Brasil, o laboratório de atuação é o Laboratório Avançado de Saúde Pública (LASP), da Fiocruz-BA. Na França, os laboratórios de atuação do estágio de doutorado foram o laboratório de Imunidade e imunogenética antiviral e vacina (*Immunité et immunogénétique antivirale et vaccinale*), coordenado pela Prof. B. AUTRAN e o laboratório de NK e doenças patológicas (*NK et maladies pathologiques*), coordenado pelo Prof. V. VIEILLARD.

NK E DOENÇAS PATOLÓGICAS (*NK ET MALADIES PATHOLOGIQUES*)

O laboratório de NK e doenças patológicas coordenado pelo prof. V. Vieillard, tem sua atividade centrada em torno das células NK, variando de pesquisa básica a aplicações clínicas. Está organizado em torno da identificação e caracterização de novos marcadores envolvidos no controle e / ou atividade de células NK em fisiologia e fisiopatologia. Isso inclui a exploração dos efeitos benéficos e deletérios das células NK em patologias virais. Os modelos estudados são principalmente HIV e infecções emergentes (Chikungunya, dengue e febres hemorrágicas) em patologias tumorais e inflamatórias (CIMI-PARIS, 2019).

Os principais estudos do grupo são associados ao estudo fenotípico de subpopulações NK em sangue periférico e tecidos, estudo polifuncional das células NK no sangue e tecidos periféricos (produção intracitoplasmática de citocinas, lise direta, ADCC) após estimulação e / ou na presença de alvo específico, identificação e caracterização de ligantes do receptor NK, estudo da diferenciação de células NK; cultura a longo prazo e análise genotípica de receptores KIR e seus ligantes (CIMI-PARIS, 2019).

O grupo tem como pesquisas representativas a identificação e caracterização de NKp44L, o ligante celular de um receptor NK ativador, expresso apenas sob estresse. A aplicação de um padrão altamente conservado de HIV a uma aplicação de vacina. A descrição de células "clonais" NK após infecção viral e o papel das células NK após transplante hematopoético alogênico (CIMI-PARIS, 2019).

Além do coordenador do grupo, Prof. Vincent Vieillard, a equipe conta com os pesquisadores patrice debre, Baptiste Hervier e Stéphanie N'guyen. a técnica de laboratório Nadine Tarantino e os estudantes de doutorado Christopher Maucourant e de mestrado Emilie Olami-Omvani.

LABORATÓRIO DE IMUNIDADE E IMUNOGENÉTICA ANTIVIRAL E VACINA (*IMMUNITÉ ET IMMUNOGÉNÉTIQUE ANTIVIRALE ET VACCINALE*).

O laboratório de Imunidade e imunogenética antiviral e vacina coordenado pela Prof. Brigitte AUTRAN, estuda as reações do sistema imunológico às vacinas. Em particular, segue os mecanismos de imunidade humoral e imunidade celular durante a vacinação, particularmente em pacientes imunodeprimidos. As abordagens desenvolvidas enfocam o estudo dos mecanismos de controle imunológico de reservatórios virais do HIV (mecanismos de controle transcricional de células CD4, especialmente aqueles que envolvem o repressor transcricional Blimp-1 em células B). Imunidade antiviral e vacinal e o desenvolvimento de vacinas contra o HIV. Mecanismos de regulação durante a infecção com o vírus oncogênico HHV-8. Câncer e Relações de Imunodepressão. Doenças inflamatórias e comorbidades da infecção pelo HIV. Imunogenética do Vírus do HIV e da Hepatite C: Estudo da associação entre o polimorfismo genético humano e a progressão de infecções virais crônicas por HIV e HCV (CIMI-PARIS, 2019).

OBSERVAÇÃO, PRÁTICA E RESULTADOS OBTIDOS NO CIMI-PARIS

PERÍODO DE ESTÁGIO

O presente estágio teve início no dia 02 de Janeiro de 2019 e foi finalizado no dia 01 de janeiro de 2019.

PACIENTES E CONSIDERAÇÕES ÉTICAS

As amostras de pacientes utilizadas no presente estágio fazem parte do projeto Zika-Alliance e/ou controles não-infectados do CIMI-Paris e foram encaminhadas do Brasil. O projeto ZikaAlliance foi aprovado pelo Comitê de Ética em Pesquisa (CEP) da Fiocruz-BA (CPqGM).

CARACTERIZAÇÃO DO FENÓTIPO E DAS CÉLULAS NK PELA AVALIAÇÃO MULTIPARAMÉTRICA DE MARCADORES DE FUNÇÃO, ATIVAÇÃO, MEMÓRIA, PRODUÇÃO DE CITOCINAS UTILIZANDO CITOMETRIA DE MASSA.

Junto a equipe do Prof. Vincent Vieillard no CIMI-Paris, foi realizado o fenótipo e a função das células NK e de outras células mononucleares do sangue periférico de pacientes dos pacientes infectados por ZIKV com 42 marcadores por citometria de massa (CyTOF)(Figura 4), durante todo o período do estágio. Através desse resultado, pretende-se determinar a assinatura fenotípica das subpopulações de células NK nas diversas formas da doença. A metodologia de execução foi de citometria de massa (CyTOF).

Para esta metodologia foram treinados a experimentação, aquisição e análise de resultados. Foram realizados experimentos com 9 pacientes infectados com ZIKV e 3 controles não infectados. O protocolo da presente metodologia está disponível no caderno de experimentos do Doutorando Gabriel Queiroz.

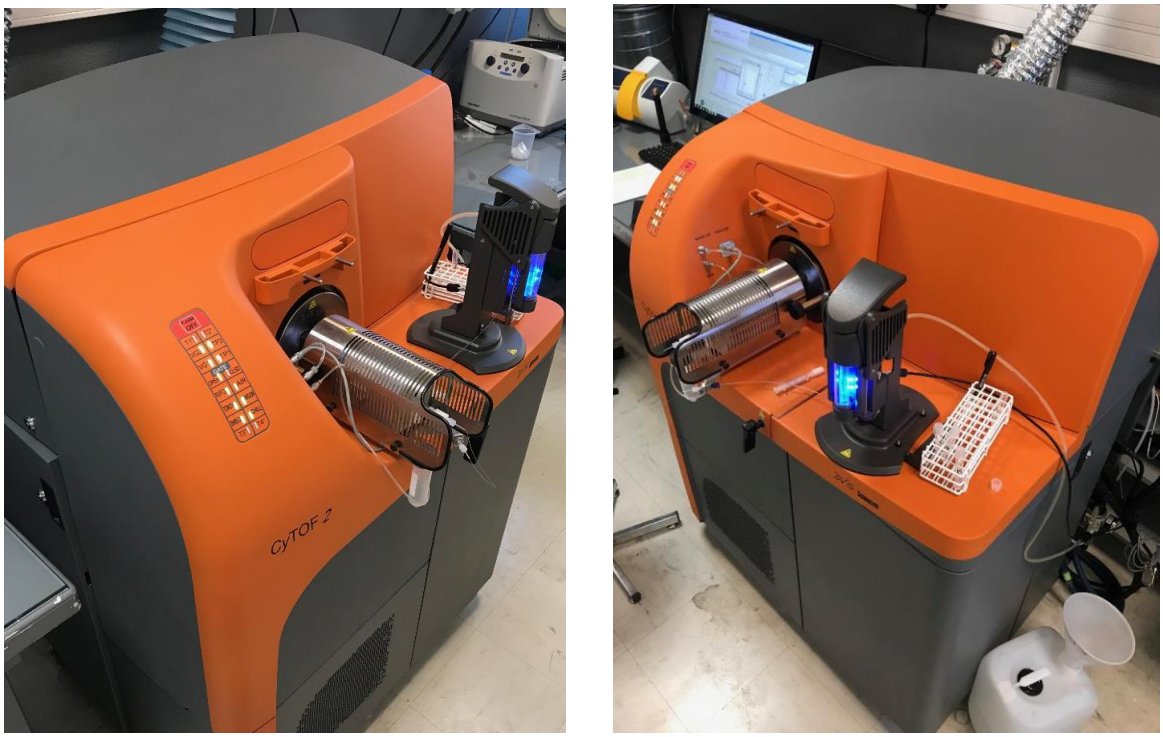


Figura 4 – Cytoff do CIMI-Paris (Fonte: Gabriel Queiroz).

AVALIAÇÕES FUNCIONAIS POR CITOMETRIA DE FLUXO

Junto a equipe do Prof. Vincent Vieillard no CIMI-Paris, foi realizado experimentos para treinamento de de citometria de fluxo. Para esta metodologia foram treinados a

experimentação, aquisição e análise de resultados por citometria de fluxo. O citometro utilizado foi o Beckman Coulter – Gallois (Figura 5). Os resultados obtidos nesses experimentos foram associados a tese de Doutorado do doutorando **Christopher Maucourant, do CIMI-PARIS.** O protocolo da presente metodologia está disponível no caderno de experimentos do Doutorando Gabriel Queiroz.

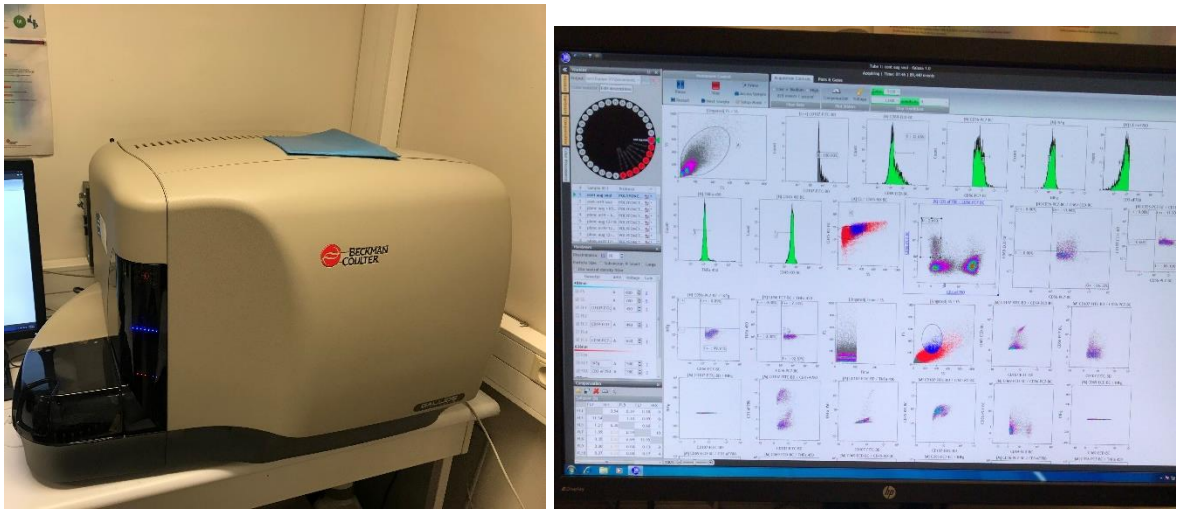


Figura 5 – Citômetro de fluxo do CIMI-Paris (Fonte: Gabriel Queiroz).

ADCC

Junto a equipe do Prof. Vincent Vieillard no CIMI-Paris, foi realizado experimentos para treinamento da metodologia de ADCC - Citotoxicidade mediada por células dependente de anticorpos. Neste experimento foram treinadas apenas o protocolo e a metodologia de execução. O protocolo da presente metodologia está disponível no caderno de experimentos do Doutorando Gabriel Queiroz.

ELISPOT

Junto a equipe da Prof. Brigitte Autran no CIMI-Paris, foi realizado experimento para treinamento da metodologia de ELISPOT. Neste experimento experimento foram treinadas o protocolo e a metodologia de execução e observação e análise dos resultados. O protocolo da presente metodologia está disponível no caderno de experimentos do Doutorando Gabriel Queiroz.

OUTROS ENSAIOS

Além dos ensaios descritos acima, das equipes do Prof. Vincent Vieillard e da Prof. Brigitte Autran no CIMI-Paris. Foram realizados outros ensaios, que foram úteis para revisão e aprimoramento dos protocolos no Brasil. Dentre os diversos ensaios, foram realizados: Descongelamento de células mononucleares do sangue periférico; criopreservação de células mononucleares do sangue periférico; Isolamento de células mononucleares do sangue periférico; criopreservação de células mononucleares do sangue periférico através do gradiente de Ficoll; Separação de células por beads magnéticas e por fim, infecção de monócitos com isolados virais.

OUTROS ASPECTOS RELEVANTES

Além dos ensaios e procedimentos aprendidos, o estágio no CIMI-Paris foi extremamente relevante pelo grande aprendizado teórico-científico, através de leitura de artigos científicos, reuniões científicas semanais, debates e conversas acerca dos temas associados a pesquisa. Além disso, durante todo o período do estágio a linguagem utilizada foi a língua francesa, o que contribuiu para o aprimoramento da leitura, escrita, entendimento e da fala.

OBJETIVOS ALCANÇADOS

É possível perceber que o estágio doutoral no CIMI-PARIS alcançou os objetivos propostos de caracterizar o fenótipo e função das células NK e NKT de indivíduos infectados pelo Zika Vírus com diferentes formas clínicas pela avaliação multiparamétrica com, pelo menos, 25 marcadores e de Treinar análise de dados de plataforma de tecnológica de citometria de massa (CyTOF). Além destes, foram alcançados outros objetivos não previamente determinados:

- Experimentação, aquisição e análise de células mononucleares do sangue periférico de pacientes infectados por ZIKV com 42 marcadores por citometria de massa (CYTOFF).
- Experimentação, aquisição e análise de células mononucleares do sangue periférico por citometria de fluxo.

- Experimentação de ADCC - Citotoxicidade mediada por células dependente de anticorpos.
- Experimentação, aquisição e análise de ensaio de ELISPOT.
- Descongelamento de células mononucleares do sangue periférico.
- Criopreservação de células mononucleares do sangue periférico.
- Isolamento de células mononucleares do sangue periférico através do gradiente de Ficoll;
- Separação de células por beads magnéticas e por fim.
- Infecção de monócitos com isolados virais.
- Leitura de artigos científicos.
- Participação de reuniões científicas.
- Aprimoramento na leitura, escrita, fala e entendimento da língua francesa.

CONSIDERAÇÕES FINAIS

O Estágio no CIMI-PARIS foi de grande relevância, uma vez que um novo ambiente laboratorial dentro de um mesmo contexto de uma mesma pesquisa reforçou técnicas previamente conhecidas, bem como foram observadas novas técnicas de uso. Esta foi uma oportunidade ímpar e o período de estágio foi extremamente significativo, pois foi possível observar a estrutura administrativa e o funcionamento da rotina laboratorial de pesquisa de outro país.

Além disso, todos os objetivos previstos foram alcançados.

AGRADECIMENTOS

Agradecimento a Fiocruz-BA-Brasil, pelo apoio financeiro no custeio de passagens aéreas para realizar estágio doutoral no Departamento de imunologia e doenças infecciosas, do Hospital Universitário Pitié-Salpêtrière, da Universidade Pierre et Marie Curie, Paris, França.

Agradecimento a equipe do CIMI-Paris, pelo apoio financeiro no custeio da hospedagem na Maison des Provinces de France, na Cité Universitaire de Paris, durante todo o período de estágio doutoral no Departamento de imunologia e doenças infecciosas, do Hospital Universitário Pitié-Salpêtrière, da Universidade Pierre et Marie Curie, Paris, França.

Agradecimento a FAPESB, pela bolsa de doutorado no Brasil, que possibilitou a manutenção no período do estágio no Departamento de imunologia e doenças infecciosas, do Hospital Universitário Pitié-Salpêtrière, da Universidade Pierre et Marie Curie, Paris, França.

Agradecimento a Prof. Maria Fernanda Rios Grassi, coordenadora do Laboratório Avançado de Saúde Pública, LASP, na Fiocruz-BA, pela confiança e apoio ao projeto em todos os momentos.

Agradecimento aos Profs. Vincent Vieillard, Prof. Brigitte Aurtran e ao Prof. Hans, do departamento de imunologia e doenças infecciosas, do Hospital Universitário Pitié-Salpêtrière, da Universidade Pierre et Marie Curie, Paris, França, pela gentileza e apoio ao projeto.

Agradecimento aos estudantes Christopher Maucourant e Emilie Olami-Omvani pela companhia diária em todos os momentos de trabalho, nos almoços e em algumas aventuras por Paris.

Agradecimento a toda equipe do CIMI-Paris.

REFERÊNCIAS

CIMI-PARIS. Disponível em: < http://www.cimi-paris.upmc.fr/fr/index.html>. Acesso em 16/02/2019.