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Complement Component 3 Regulates IFN-α Production by Plasmacytoid Dendritic Cells following TLR7 Activation by a Plant Virus–like Nanoparticle

Marie-Ève Lebel,* Marie-Pierre Langlois,* Jean-François Daudelin,[†] Esther Tarrab,* Pierre Savard,[‡] Denis Leclerc,[§] and Alain Lamarre*

The increasing use of plant viruses for the development of new vaccines and immunotherapy approaches poses questions regarding the mechanism by which the mammalian immune system recognizes these viruses. For example, although natural Abs (NA) and complement are key components of the innate immune system involved in the opsonization, phagocytosis, and destruction of microorganisms infecting mammals, their implication in plant virus recognition and immunogenicity is not well defined. In this study, we address the involvement of NA and the complement system in the activation of innate immunity through engagement of TLR7 with papaya mosaic virus (PapMV)-like nanoparticles. We demonstrate that NA, although binding to PapMV, are not involved in its recognition by the immune system. On the other hand, C3 strongly binds to PapMV nanoparticles and its depletion significantly reduces PapMV's interaction with immune cells. Unexpectedly, however, we observed increased immune cell activation following administration of PapMV to complement-depleted mice. TLR7 activation by PapMV in the absence of C3 induced higher IFN-α production, resulting in superior immune cell activation and increased immunotherapeutic properties. In conclusion, in this study we established the involvement of the complement system in the recognition and the phagocytosis of PapMV nanoparticles and identified an unsuspected role for C3 in regulating the production of IFN-α following TLR7 activation. ***The Journal of Immunology*****, 2017, 198: 292–299.**

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The use of plant viruses for vaccination and cancer immunotherapy has gained interest in recent years (1). We and others have shown that plant viruses can be engineered to express vaccine epitopes, resulting in the development of specific humoral and cellular immune responses in vaccinated hosts (2–6). In addition, plant viruses are highly immunogenic in mammals making them effective adjuvants, vaccines, and immunomodulators (2, 4, 7–10). More specifically, in our work with papaya mosaic virus (PapMV)-like nanoparticles, we demonstrated that the ssRNA contained within PapMV engages the TLR7 pathway in plasmacytoid dendritic cells (pDCs) resulting in strong IFN-α production. IFN-α then activates a number of immune cells, which promote the development of protective $CD8^+$ T cell responses in the context of bone marrow-derived dendritic cell (BMDC) immunization in mice (8). Moreover, the immunomodulatory properties of PapMV advantageously alter the tumor microenvironment, increasing the anti-tumoral immune response generated and consequently slowing down tumor growth in the stringent B16 melanoma model (7). Importantly, used in conjunction with other immunotherapy treatments such as DC vaccination and PD-1 blockade, PapMV greatly potentiates their therapeutic effect (7). Therefore, plant viruses such as PapMV constitute promising tools in the fight against infectious diseases and cancer. However, the immunological process by which the mammalian immune system recognizes and interacts with plant viruses is not fully understood.

Initial recognition of the microorganisms infecting mammals is achieved mainly through interaction with components of the innate immune system such as natural Abs (NA) or the complement system. NA and complement proteins bind conserved motifs expressed on the surface of pathogens or dying cells favoring their clearance from the bloodstream (11, 12). These immune effectors are therefore crucial for the initial containment of viral as well as bacterial infections wherein they function by directly neutralizing or eliminating pathogens; this function forms immune complexes that are recruited to the spleen, which prevents infection of other organs such as the brain (13) and primes the adaptive immune response (14–16). Recognition of pathogens by the complement system can be done in three different ways: the classical, alternative or lectin pathway. The classical pathway is initiated by the binding of C1q to the Fc portion of Abs opsonizing pathogens whereas in the alternative pathway, the C3 directly binds to the surface of the microorganism. Finally, the lectin pathway involves the fixation of mannose binding lectin to carbohydrates on the

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; CVF, cobra venom factor; DC, dendritic cell; KO, knockout; MFI, mean fluorescence intensity; NA, natural Ab; PapMV, papaya mosaic virus nanoparticle; pDC, plasmacytoid dendritic cell; p.i., postinfection; poly(I:C), polyinosinic-polycytidylic acid; VSV, vesicular stomatitis virus.

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membranes of pathogens. Although initiation takes place in different ways, activation of the complement system invariably leads to the formation of C3 convertase and cleavage of C3. Thereafter, a proteolytic cascade leads to the different effector activities of the complement system. Conversely, elimination of the central protein C3, following injection of cobra venom factor (CVF), blocks the activation of the three pathways. Thus, NA and complement are crucial to the initial control of microorganisms infecting mammals (12, 16). However, their role in plant-virus recognition has not been studied. Because plant viruses are not that different to mammalian viruses, it is likely that these elements of the innate immune system are also involved in their recognition. This could have a significant impact on their distribution in the body and on the development of the adaptive immune response, and thus the effectiveness of vaccines and immunotherapy approaches that use plant viruses.

In this study, we evaluate the role of NA and the complement system following immunization with PapMV nanoparticles used in vaccines and immunotherapies currently in development. Using B cell-deficient JHT mice and CVF, we demonstrate that NA do not significantly contribute to PapMV's immunogenicity. On the contrary, C3 binds to the surface of PapMV and its absence reduces PapMV phagocytosis. Unexpectedly, however, complement depletion enhances immune cell activation and IFN-α production following PapMV administration, resulting in a significant increase in its immunotherapeutic properties.

Materials and Methods

Ethics statement

This study was performed in accordance with the Canadian Council on Animal Care guidelines. All animal experiments were reviewed and approved by the Institut National de la Recherche Scientifique institutional animal care committee.

Mice

Female 6–10 wk-old C57BL/6 mice were purchased from Charles River Laboratories. *Tlr7* knockout (KO), *Myd88* KO and *C3* KO mice were purchased from The Jackson Laboratory. JHT mice were kindly provided by Dr. Rolf Zinkernagel, Zurich University, Switzerland. Where indicated, complement depletion was performed by injecting 20 μg CVF (Quidel) i.p. 24 h before the start of the experiment.

PapMV nanoparticles

PapMV nanoparticles used in this study were kindly provided by Folia Biotech and produced as described in our previous study (9). LPS contamination was always below 50 endotoxin units per milligram of protein and considered negligible. For some experiments, PapMV was coated with polyclonal Abs by incubation at 4°C overnight with rabbit anti-PapMV immune serum.

Cell culture

B16-OVA cells were kindly provided by Dr. Richard Vile (Mayo Clinic, Rochester, MN) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 5 mg/ml of G418 to select for OVA expression. BMDC were generated as described previously (8).

Virus

Vesicular stomatitis virus (VSV) was propagated and titrated on Vero cells. To evaluated immune cell activation, IFN-α production, and virus recruitment to the spleen, mice were infected by the i.v. route with 2×10^6 PFU VSV Indiana. Blood samples were taken at 7, 14, and 24 h postinfection (p.i.) for IFN-α quantification and mice were euthanized at 24 h p.i. to evaluate immune cell activation and at 2 h p.i. for virus titration.

Immunization

PapMV administration was done by i.v. injection with 100 μg. For BMDC-OVA immunization experiments, 100 μg of PapMV or 100 μl of vehicle (Tris 10 mM) were injected i.v. 6 h before i.v. immunization with 1.25×10^6 mature BMDC-OVA or unloaded BMDC. OVA-specific $CD8^+$ T cell responses were analyzed at day 7 postimmunization in the spleen and peripheral lymph nodes. For some experiments, *C3* KO mice were injected with 10 μg C3a (R&D systems) 20 min before PapMV administration. Functionality of C3a was tested by measuring IL-6 secretion by human PBMCs as per the manufacturer's recommendations. R837 (100 μg; Invivogen) and polyinosinic-polycytidylic acid [poly(I:C)] (100 μg; GE Healthcare Life Sciences) were injected i.v. 24 h after CVF (20 μg i.p.; Quidel) or PBS injection.

Plasmacytoid dendritic cell depletion

The hybridoma cell line producing mAb 927, specific for the mouse bone marrow stromal Ag 2 (BST2), used for pDC depletion, was kindly provided by Dr. Marco Colonna (Washington University School of Medicine). Depletion was done as described previously (17). Briefly, mice were injected (i.p.) with 500 μg of purified Ab 927 or an isotype control 24 and 48 h before PapMV administration. Depletion leads to a reduction of at least 60% of pDC numbers in the spleen as measured by flow cytometry.

Intracellular IFN-α staining

For flow cytometry analysis of IFN-α production following PapMV administration, mice were euthanized 4 h after i.v. injection of 400 μg PapMV and immune cells were isolated from the blood and the bone marrow. Intracellular staining for IFN-α (RMMA-1) (PBL Assay Science) was performed after a 4 h incubation period at 37°C in the presence of brefeldin A (10 μg/ml) using fixation/permeabilization buffer (BioLegend), according to the manufacturer's instructions.

Flow cytometry analysis and Abs

Flow cytometry analysis of mouse surface Ags was performed with the following Abs: anti-CD69 (H1.2F3), -CD86 (GL1), -CD11c (N418), -CD11b (M1/70), -F4/80 (BM8), -CD8α (53-6.7), -CD19 (6D5), -CD45R/B220 (RA3-6B2), -CD317 (PDCA-1) (927), -CD44 (IM7) (BioLegend). Staining was performed for 20 min at 4°C. PE-coupled OVA-H-2Kb tetrameric complexes were generated as previously described (18) and staining was performed at 37°C for 15 min. Intracellular staining for IFN-γ (XMG1.2) (BioLegend) and granzyme B (NGZB) (eBioscience) was performed after a 5 h restimulation with OVA (2 μg/ml) in the presence of brefeldin A (10 μg/ml) using fixation/permeabilization buffer (BioLegend), according to the manufacturer's instructions. Flow cytometry analyses were performed on a BDLSR Fortessa flow cytometer (BD) and data were analyzed using the FlowJo software (Tree Star).

PapMV distribution experiment

PapMV was labeled with Alexa 647 using the Molecular Probes Protein Labeling Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Alexa 647-labeled PapMV was injected i.v. and mice were sacrificed 2 h postimmunization for flow cytometry analysis.

Cytokine analyses

IFN-α levels in sera, lymph nodes, and spleen homogenates from immunized mice were determined by ELISA following the manufacturer's instructions (PBL IFN Source). Production of other cytokines and chemokines was evaluated in the blood of mice 6 h after PapMV injection using a Luminex screening assay kit (R&D Systems) detecting FGF Basic, GM-CSF, IFN-γ, IL-10, IL-12, IL-13, IL-17, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α, and VEGF following the manufacturer's instructions and are reported in picogram per milliliter of serum.

Lung nodule establishment assay

Mice were injected i.v. with 100 μl Tris 10 mM or 100 μg PapMV 6 h before tumor inoculation with 5×10^5 B16-OVA and euthanized on day 17 post inoculation. Lungs were perfused with 5 ml PBS and conserved in Fekete's solution for nodule visualization.

Complement proteins and IgM detection by ELISA

To evaluate the binding of complement proteins or IgM to PapMV and VSV, ELISA plates were coated with 5 μg/ml of PapMV, VSV, IgM (positive control) or BSA (negative control) and freshly harvested serum from C57BL/6 or JHT mice was used as a source of IgM or complement proteins. Serum was diluted one in three in PBS $Mg^{2+}Ca^{2+}$ when used as a source of complement proteins whereas serial 2-fold dilutions of serum starting with a one in four dilution in PBS were used for the IgM binding assay. After 20 min incubation at 37°C, detection of IgM and complement proteins was done with biotinylated anti-IgM, anti-C3-related proteins

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(C3/C3b/iC3b/C3dg), anti-C1q or anti-C4-related proteins (C4/C4b/C4d) antibodies (Cedarlane), followed by streptavidin-HRP (Southern Biotech). Results were normalized on the level of binding to the IgM positive control and background signal detected in BSA-coated wells was subtracted from all samples. Percentage of binding was calculated by considering that the level of binding on IgM-coated plates was 100%. C3a levels in serum (collected in EDTA + Futhan to limit spontaneous proteolytic cleavage of complement) 30 min following PapMV administration by i.v. injection were measured by ELISA using purified and biotinylated rat anti-mouse C3a (BD Biosciences) followed by streptavidin-HRP (Southern Biotech). OD values were obtained by reading at 490 nm.

PapMV ssRNA quantification by quantitative PCR

Spleen samples were homogenized in TRIzol (Life Technologies) using a Polytron homogenizer and total RNA was extracted following the manufacturer's recommendations. Two microgram was reverse-transcribed with Superscript II using random hexamers (Life Technologies) according to the supplier's instructions. Quantitative PCR was performed with the following primers and probes. For *Gapdh*: Taqman gene expression assay ID: Mm99999915_g1. In the case of PapMV ssRNA, sequences used were as follows: (Forward) 5′-CTCACTCCGGGCTGTTATTC-3′, (Probe) 5′-FAM-AGAGGCCTACAGAGACATTAAGCTCACT-MGB-3′ and (Reverse) 5′-CTGCTACTGCTACTGGATGTG-3′. Each reaction was performed in triplicate using a real-time cycler ABI Prism 7500 (Life Technologies). PapMV ssRNA copy numbers were determined using a standard curve established using serial dilutions of pure PapMV RNA. Results were normalized on *Gapdh* expression and were presented as the number of PapMV ssRNA copies by microgram of total RNA.

Statistical analysis

For pairwise comparisons, data were analyzed for statistical significance using the Student *t* test. Data from more than two groups were analyzed with a parametric ANOVA test. Tukey's post tests were used to compare differences among groups. Statistical significance was determined as $p < 0.05$.

Results

The complement system is involved in the recognition of PapMV by immune cells

To evaluate the implication of NA and the complement system in the immunogenicity of PapMV, we first analyzed their binding to PapMV in comparison with VSV, an ssRNA virus infecting mammals for which the involvement of NA and complement during infection is well characterized (14, 15). We first performed ELISA with serum from C57BL/6 and found that natural IgM binds to PapMV at levels similar to VSV (Fig. 1A). As expected, sera from B cell deficient JHT mice did not contain any natural IgM binding to either virus. Next we evaluated the association between various complement proteins and PapMV in vitro and observed a strong association between C3 and PapMV whereas no significant binding to VSV could be detected (Fig. 1B). In contrast, C1q and C4 bound only slightly to PapMV in comparison with their stronger association with IgM or VSV. Interestingly, the use of serum from JHT mice as source of complement proteins led to similar levels of C3 binding to PapMV compared with Ab sufficient serum. In fact, levels of C3 detected on PapMV in the absence of NA were significantly higher. On the contrary and consistent with the mechanisms of action of C1q and C4, absence of NA reduced their binding to both PapMV and VSV (Fig. 1B). Furthermore, increased levels of anaphylatoxin C3a, produced after C3 cleavage, were measured in serum by ELISA shortly after administration of PapMV to C57BL/6 mice (Fig. 1C). Although absolute C3a levels could be overestimated using this method due to spontaneous proteolytic cleavage during blood harvesting and handling, these results suggest that PapMV's surface promotes C3 cleavage in an Ab-independent fashion consequently releasing C3a and C3b molecules, the latter then directly associating with PapMV.

We next determined the role of NA and complement proteins on the recognition of PapMV by immune cells by administrating PapMV to C57BL/6 and JHT mice in the presence or absence of CVF-treatment to inactivate the complement system. We observed that in the absence of NA in JHT mice, there is no modification in the distribution of PapMV to the spleen when compared with C57BL/6 mice whereas there is a significant reduction in VSV titers (Fig. 1D). However, a decrease in the amount of PapMV ssRNA in the spleen was observed following inactivation of the complement system by CVF injection, especially in the absence of NA, similar to VSV infection (Fig. 1D). This effect was confirmed by flow cytometry using A647-labeled PapMV (Fig. 1E, Supplemental Fig. 1A, 1B). Proportions of DCs and B lymphocytes from spleen, blood, and lymph nodes associating with PapMV were significantly reduced in CVF-treated mice. Although proportions of macrophages positive for PapMV did not dramatically decrease in the CVF treated group (Fig. 1E, Supplemental Fig. 1A, 1B), the mean fluorescence intensity (MFI) was significantly lower (Fig. 1F) indicating that the number of macrophages interacting with PapMV is not different, but the amount of PapMV associated with macrophages is lower upon CVF treatment. Finally, the absence of NA bore no impact on the interaction of PapMV with DCs and macrophages. In conclusion, in contrast to VSV, NA are not involved in the interaction of PapMV with immune cells whereas complement proteins bind both viruses, although using different pathways, leading to their recognition by various cells of the immune system.

Complement depletion increases PapMV-induced immune cell activation and IFN-α production

Because complement inactivation decreases the phagocytosis of PapMV and its recruitment to the spleen, we evaluated the impact of its depletion on immune cell activation following PapMV administration. Unexpectedly, the reduced interaction of PapMV with immune cells in CVF-treated mice led to a higher activation of immune cells in the spleen and lymph nodes, whereas the lack of NA had no impact (Fig. 2A, 2B, Supplemental Fig. 1C). A significant increase in the expression levels of the costimulatory molecule CD86 on DCs and activation marker CD69 on $CD8^+$ T cells and B lymphocytes was observed following PapMV immunization in complement-depleted C57BL/6 mice, whereas CVF injection alone had no effect (Fig. 2A, Supplemental Fig. 1C). We have previously demonstrated that activation of immune cells following immunization with PapMV is dependent on the production of IFN-α (8). We therefore evaluated the production of this antiviral cytokine following administration of PapMV to CVF-treated mice. Fittingly, IFN-α production was also drastically increased in the serum, peripheral lymph nodes, and spleen of complement-inactivated mice (Fig. 2C). Furthermore, intracellular IFN-α production assessed in bone marrow and blood by flow cytometry revealed that pDCs are the only immune cell type producing the cytokine in response to PapMV administration whereas CVF treatment drastically increased this production (Supplemental Fig. 2). The secretion of IL-6, IP-10, and MCP-1 produced in response to IFN-α in the context of PapMV administration as detected by a multiplex Luminex assay was also significantly higher in CVF-treated mice (Fig. 2D). Other cytokines and chemokines tested (see *Materials and Methods*) were either not produced following PapMV administration or not significantly modulated by CVF injection. In addition, in the absence of complement, IFN-α production remained dependent on MYD88, TLR7, and pDCs, but not on NA (Fig. 2E), consistent with what we have previously shown (8). To assess whether this effect on IFN-α production was restricted to PapMV, CVF-treated mice were infected with VSV. Similarly, inactivation of the complement system increased IFN-α secretion of mice infected with VSV although to a lesser level possibly due to the inability of VSV to activate the alternative complement pathway (Fig. 2F). We next

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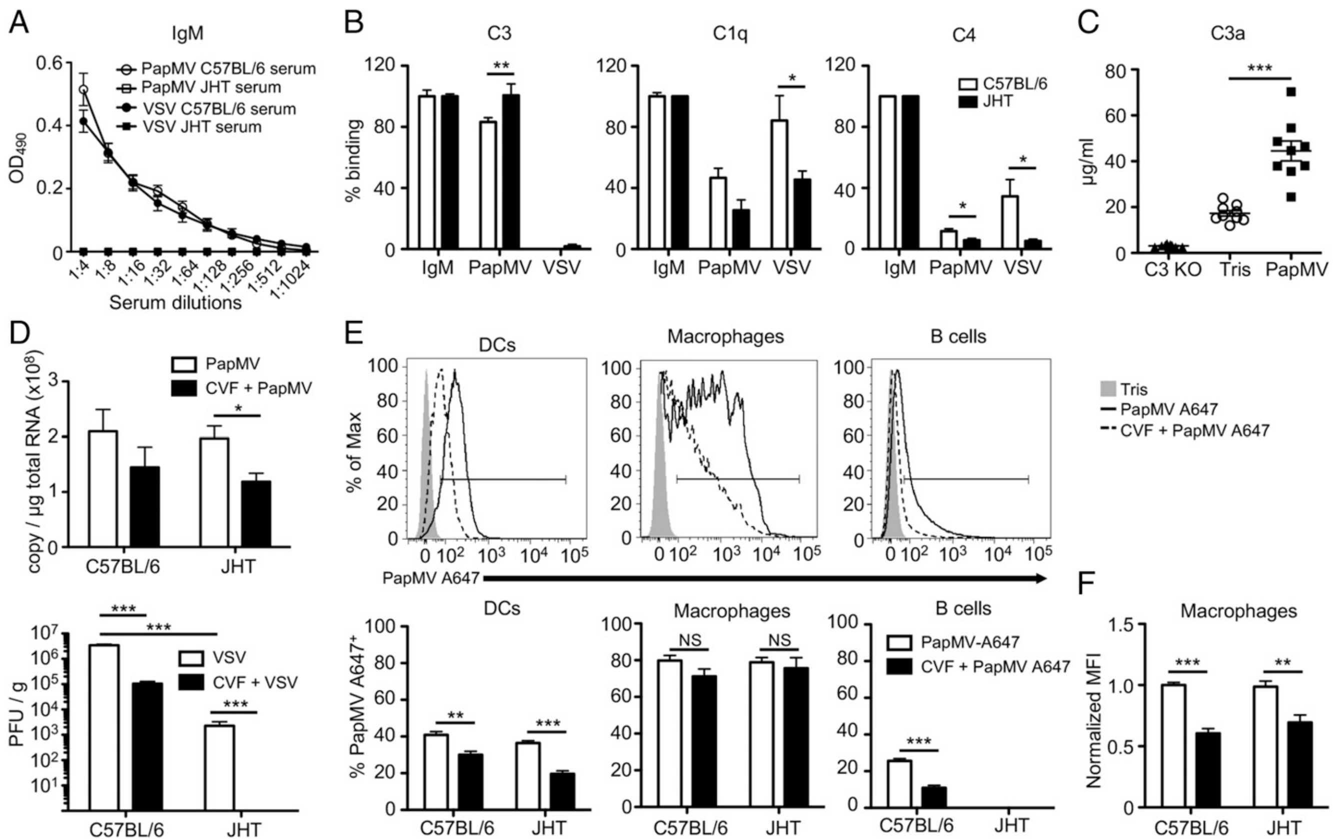

FIGURE 1. Complement proteins bind PapMV and are implicated in its phagocytosis. Evaluation by ELISA of the binding of IgM (**A**), C3, C1q, and C4 (**B**) to PapMV and VSV. (**C**) Determination of C3a production 30 min after PapMV or Tris injection in C57BL/6 mice and *C3* KO by ELISA. (**D**) Quantification of PapMV ssRNA copy numbers (upper panel) and VSV titers (lower panel) in the spleen 2 h post injection in C57BL/6 and JHT mice pretreated (black bars) or not (white bars) with cobra venom factor (CVF). (**E**) Upper panels, Representative overlay histograms showing PapMV nanoparticles associated with DCs, macrophages and B cells after i.v. injection of PapMV-A647 in C57BL/6 mice treated (dash lines) or not (bold lines) with CVF in comparison with Tris-injected mice (filled histograms). Lower panels, Proportion of PapMV-A647^{+} DCs, macrophages and B cells in the spleen 2 h after PapMV-A647 administration. (**F**) Normalized A647 mean fluorescence intensity (MFI) of PapMV^{+} macrophages in C57BL/6 and JHT mice treated or not with CVF. Results are shown as the mean ± SEM. Compilation of three independent experiments with eight to nine mice per group, *$p < 0.05$, **$p < 0.01$. NS, not significant.

evaluated the effect of complement depletion on IFN-α production following administration of two non-particulate TLR agonists (Fig. 2G). Although i.v. injection of the TLR7 agonist R837 did not lead to IFN-α production in the serum of treated mice, CVF treatment prior to R837 injection did not significantly increase this production in contrast to what is observed with PapMV. Similarly, no significant increase in IFN-α production upon administration of the TLR3 agonist poly(I:C) in CVF-treated mice could be observed. These results suggest that increased IFN-α production in response to PapMV or VSV administration in CVF-treated mice results from a modification of the viral nanoparticle itself and not through other immunomodulatory effects.

Absence of C3 is responsible for the increased IFN-α production and immune cell activation following PapMV immunization

CVF administration induces several effects including depletion of C3 and C5 and production of different anaphylatoxins. When analyzing the kinetics of IFN-α production in CVF-treated mice following PapMV administration, we observed that the increase in IFN-α production was inversely correlated with the amount of C3 present in serum (Fig. 3A). Thus, to confirm the implication of C3 in IFN-α production we immunized *C3* KO mice with PapMV. Fittingly, administration of PapMV to *C3* KO mice resulted in elevated IFN-α production and higher expression of CD86 on DC and CD69 on CD8^{+} T cells when compared with C57BL/6 mice (Fig. 3B, 3C). Furthermore, CVF administration to *C3* KO mice had no impact on IFN-α production (Fig. 3B) whereas *C3* KO mice did not produce any IFN-α at steady state (data not shown). Finally, to identify the fragment of C3 that enhanced IFN-α production following TLR7 activation, *C3* KO mice were injected with C3a in combination with PapMV. However, no modification in IFN-α production was observed in this context excluding a role for C3a in the observed phenotype (Fig. 3D). Conversely, when PapMV was incubated with PapMV-specific polyclonal Abs, we noticed a dramatic increase in the binding of C3, C1q, and C4 on PapMV when compared with uncomplexed PapMV (Fig. 3E). Administration of Ab-complexed PapMV to C57BL/6 mice led to a significant decrease in the production of IFN-α whereas no effect was observed in CVF-treated animals (Fig. 3F). These results suggest a role of C3b and possibly C1q and C4 but not C3a in the downregulation of IFN-α production following TLR7 engagement by PapMV ssRNA.

Complement inactivation increases the immunomodulatory properties of PapMV

Considering that the immunomodulatory properties of PapMV are greatly dependent on the production of IFN-α, we evaluated the impact of complement inactivation on the immunomodulatory properties of PapMV. We first assessed the effector CD8^{+} T cell

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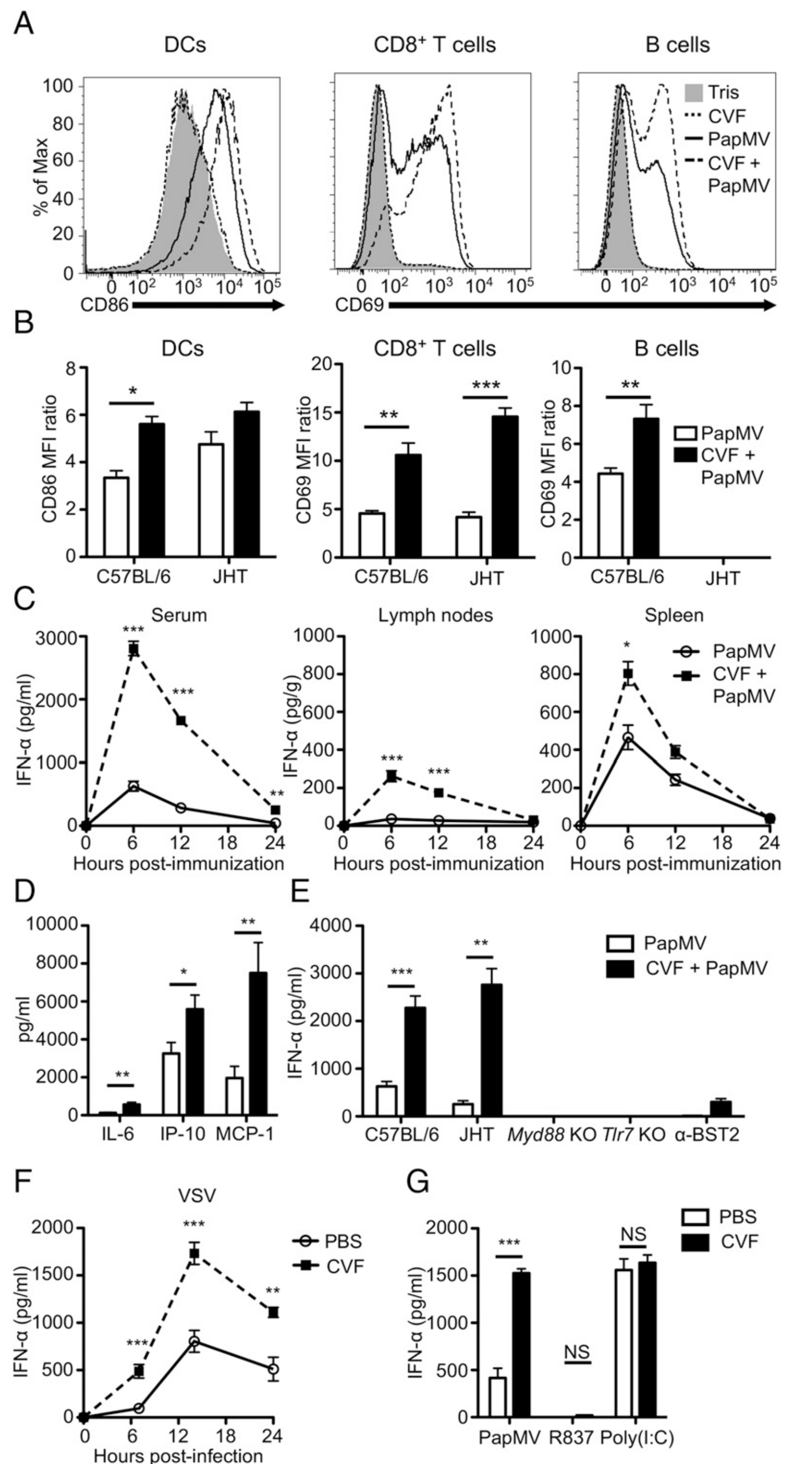

FIGURE 2. Complement depletion increases immune cell activation and IFN-α production following PapMV administration. (**A**) Representative overlay histograms of CD86 and CD69 expression on splenic DCs, $CD8^+$ T cells, and B cells 24 h after Tris (filled histograms), PapMV (bold lines), CVF (small dash lines) or CVF + PapMV (large dash lines) administration in C57BL/6 mice. (**B**) Compilation of CD86 expression on splenic DCs and CD69 expression in splenic $CD8^+$ T cells and B cells 24 h after PapMV administration in C57BL/6 or JHT mice preinjected (black bars) or not (white bars) with CVF. Results are presented as a ratio of the mean fluorescence intensity (MFI) of the analyzed sample on the MFI of the Tris sample. (**C**) ELISA quantification of IFN-α production in the serum, peripheral lymph nodes and spleen homogenates of C57BL/6 mice immunized with PapMV and treated (filled squares) or not (empty circles) with CVF 24 h before. (**D**) Evaluation by Luminex of serum IL-6, IP-10 and MCP-1 concentration 6 h post PapMV administration in C57BL/6 mice pretreated (black bars) or not (white bars) with CVF. (**E**) Serum IFN-α production 6 h post PapMV administration in C57BL/6, JHT, *Myd88* KO, *Tlr7* KO and pDC-depleted mice. (**F**) ELISA quantification of IFN-α production in the serum of C57BL/6 mice treated (dash lines) or not (continuous lines) with CVF and infected with VSV. (**G**) Serum IFN-α production 6 h post i.v. administration of PapMV, R837 or poly(I:C) in C57BL/6 mice treated (black bars) or not (white bars) with CVF 24 h before. Results are shown as the mean ± SEM. $n = 3$; two to five mice per group in each experiment, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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response following BMDC immunization. We have shown previously that the use of PapMV as adjuvant for BMDC-OVA immunization increases the proportion of OVA-specific $CD8^+$ T cells (8). In the current study, we observed that whereas administration of CVF alone did not modify the generation of OVA-specific effector $CD8^+$ T cells, injection of PapMV in complement-depleted mice significantly enhanced the proportion of OVA-specific $CD8^+$ T cells in the spleen and lymph nodes 7 d postimmunization relative to PapMV administration in undepleted mice (Fig. 4A). In addition, a greater proportion of $CD8^+$ T cells producing effector molecules such as IFN-γ and granzyme B was obtained by combining CVF and PapMV treatment in conjunction with BMDC-OVA immunization when compared with PapMV treatment alone (Fig. 4B). Importantly, increased IFN-α production and immune cell activation following PapMV immunization in complement-depleted mice also resulted in better protection against B16-OVA melanoma implantation in the lungs (Fig. 4C). Indeed, whereas injection of PapMV 6 h before tumor inoculation drastically reduced B16 nodule formation in the lungs of C57BL/6 mice compared with vehicle-treated controls, lungs from PapMV-treated complement-depleted mice were practically tumor free. Interestingly, contrary to the reported retarded B16 tumor growth in *C3* KO mice (Wang Cancer Disc 2016), CVF injection alone bore no impact on tumor growth probably due to the transient C3

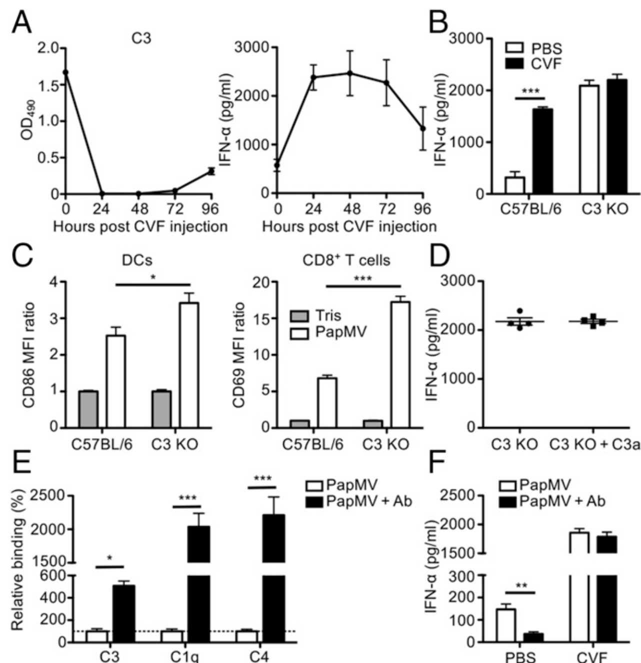

FIGURE 3. Absence of C3 is responsible for the increased IFN-α production and immune cell activation observed following CVF injection. (**A**) Kinetics of C3 depletion following CVF injection (left panel) and IFN-α production at different time intervals between administration of CVF and PapMV (right panel). (**B**) ELISA quantification of IFN-α production in the serum of C57BL/6 or *C3* KO mice injected (black bars) or not (white bars) with CVF, 6 h post PapMV administration. (**C**) CD86 and CD69 expression levels on splenic DCs and $CD8^+$ T cells respectively 24 h post Tris (gray bars) or PapMV (white bars) injection in C57BL/6 or *C3* KO mice. (**D**) Serum IFN-α production following PapMV administration in *C3* KO mice pretreated or not with C3a. (**E**) Evaluation by ELISA of the binding of C3, C1q and C4 to PapMV pretreated (black bars) or not (white bars) with PapMV-specific polyclonal Abs. (**F**) IFN-α production 6 h after administration of PapMV pretreated or not with PapMV specific polyclonal Abs in C57BL/6 treated with PBS or CVF. Results are shown as the mean ± SEM. Compilation of three independent experiments, eight to nine mice per group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

depletion obtained by CVF administration. Taken together, our results suggest that targeting the complement pathway could provide a promising approach to increase the immunotherapeutic properties of PapMV.

Discussion

NA and the complement system play important roles in the control of microorganisms infecting mammals and the development of the adaptive immune response (19, 20). In this study, we further characterized the impact that these elements of the innate immune system bear on the immunogenicity of an engineered nanoparticle derived from a plant virus, PapMV, in comparison with VSV, a mammalian virus for which the importance of NA and the complement system is well described. First, we discovered that natural IgM and the complement proteins C1q, C4, and C3 bind to PapMV. In addition, we demonstrated that although NA are required for C1q and C4 association to PapMV, their absence did not decrease C3 binding. In fact, C3 proteins were detected at slightly higher levels on PapMV in absence of NA. These results suggest that C3 activation leads to direct C3b fixation on PapMV and that absence of NA releases steric hindrance that facilitates C3b binding. Furthermore, a higher level of the anaphylatoxin C3a, produced after C3 cleavage, was found in the serum of mice immunized with PapMV thus confirming the activation of the complement system. In the case of VSV, the classical pathway of the complement system has been shown to participate in the recognition and control of this virus (13, 14, 21). In the first step of this process, NA bind to VSV, upon which C1q is recruited and activates C1r and C1s, thereby cleaving C4, which permits C4b attachment to the virus ultimately leading to C3 cleavage and deposition on the particle. Consistent with these findings, we observed that C3 does not directly interact with VSV in vitro whereas C1q and C4 molecules bind VSV mainly in presence of NA. Our inability to detect direct C3 deposition on VSV might be due to the slower kinetics of the classical complement pathway or to other limiting steps arising in this in vitro experimental setting. However, VSV infection in mice will most likely lead to some level of C3 binding to the viral particle as would be predicted by its ability to activate the classical complement pathway.

Because NA and complement proteins are involved in the opsonization and phagocytosis of microorganisms, we subsequently determined the impact of NA deficiency and complement depletion on the distribution of these viruses to the spleen. Although the absence of NA had no impact on PapMV recruitment to the spleen, this deficiency greatly reduced the targeting of VSV to the lymphoid organ. Phagocytes have Fc receptors that allow them to bind Abs attached to microorganisms, thus leading to their phagocytosis (22, 23). The results we obtained suggest that this process is very important for the recognition of VSV, but not for PapMV. Phagocytic cells also have surface receptors that bind to C1q, C4b and C3b, thus promoting uptake and destruction of virus covered with these components (24). Accordingly, complement depletion reduced PapMV interaction with immune cells and VSV translocation to the spleen. Moreover, we noticed that complement depletion in JHT mice resulted in a greater abrogation of VSV recruitment in comparison with unaltered JHT mice or complement–depleted wild-type mice, indicating synergy between these two components of the innate immune system in VSV control. However, PapMV uptake by immune cells seems solely dependent on C3b. To our knowledge, this is the first demonstration of a role for the complement system in the recognition of a plant virus by the mammalian immune system. Further studies will be required to determine the exact nature of the nanoparticle that led to complement activation.

Although VSV and PapMV are both ssRNA viruses, they differ in some important facets. For example, the VSV capsid is enclosed in an envelope whereas PapMV is a naked virus. This difference could have a significant impact on the interaction of these viruses with immune cells. Here, it is important to note that enveloped viruses, like VSV, can incorporate host cell membrane proteins, such as the complement regulators CD46, CD55, and CD59, during the budding process, thus limiting virus neutralization by complement (25–27). However, activation of the complement system on an enveloped virus can lead to membrane attack complex formation and virus lysis, which is not possible for PapMV (24). In addition, VSV is an infectious virus whereas PapMV is a non-replicative particle. Consequently, although NA and complement can neutralize VSV directly, they will favor phagocytosis of PapMV, which may be beneficial toward the activation of maximal numbers of cells. Surprisingly, however, complement depletion did not reduce immune cell activation following PapMV immunization but actually increased it. Moreover, we discovered that complement-depleted mice produced significantly higher amounts of IFN-α after TLR7 engagement by PapMV ssRNA that resultantly led to greater activation of immune cells. We previously demonstrated that IFN-α production by pDCs was consequent to PapMV's ssRNA detection by TLR7 in an MYD88-dependent pathway (7, 8). In the current study, we observed that IFN-α production in

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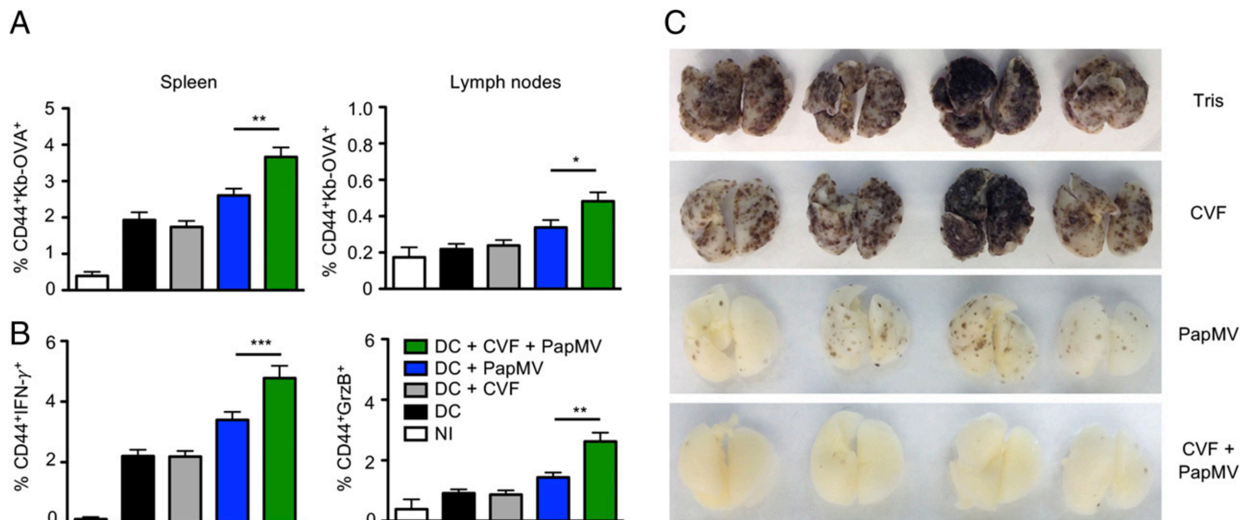

FIGURE 4. Complement inactivation increases the immunomodulatory properties of PapMV. (**A**) Compilation of the proportion of OVA-specific $CD8^+$ T cells in the spleen (left panel) and peripheral lymph nodes (right panel) 7 d post BMDC-OVA immunization. (**B**) Proportion of IFN-γ– (left panel) and granzyme B– (GrzB) (right panel) producing $CD8^+$ T cells in the spleen 7 d after BMDC-OVA immunization. (**C**) Pictures of lungs taken 17 d after B16-OVA i.v. injection. (A and B) were gated on $CD8^+$ T cells. $n = 3$; four mice per group, results are shown as the mean ± SEM, $*p < 0.05$, $**p < 0.01$.

CVF-treated mice immunized with PapMV is still dependent on TLR7, MYD88, and pDCs, suggesting that complement inactivation does not modify how PapMV triggers IFN-I production. Taken together, these results indicate that the mechanism by which CVF treatment increases IFN-α production following PapMV administration is not through a modification of its interaction with different cells of the immune system but through a direct effect on pDCs. Interestingly, this effect was not restricted to PapMV given that a higher IFN-α production was also observed upon VSV infection, indicating that activating both the classical and alternative complement pathways can lead to the observed enhancement of immunogenicity. However, the lack of increased IFN-α production of CVF-treated mice in response to small molecule TLR agonists suggests that the particulate nature of the signal is important for the immunomodulatory effect of CVF administration.

Complement depletion using CVF can lead to various effects such as C3 and C5 depletion as well as anaphylatoxin production, whereas other components of the complement system such as C1q and C4 are left intact. Because C1q and C4 did not interact strongly with PapMV in vitro, they are unlikely to play a major role in the effect observed following CVF injection, although this cannot be ruled out. However, anaphylatoxins such as C3a and C5a could subsequently have a direct influence on cytokine production following TLR activation (28, 29). Interestingly, we observed that the levels of IFN-α after PapMV immunization in CVF-treated mice were inversely correlated with the amount of C3 in the blood. Accordingly, *C3* KO mice recapitulated the effect obtained with CVF showing increased production of IFN-α and immune activation in response to PapMV administration, whereas injection of CVF in this setting had no impact. Although we cannot completely rule out that low levels of C5a generated following PapMV administration in *C3* KO mice could influence IFN-α production by pDCs, these results rather suggest a predominant role for C3 or of its derivatives.

The existence of a crosstalk between complement and TLR signaling pathways is well established (30, 31). They can influence each other and have synergistic or antagonistic effects in regulating inflammatory responses. However, these interactions, which vary depending on cell type, TLR ligand or cytokine studied, are very complex and yet to be fully understood. In most cases, anaphylatoxins C5a and C3a are reported to influence the complement system with respect to the response to MYD88-dependent TLR activation, mainly through MAPK phosphorylation (28, 32) whereby they can either increase or inhibit TLR-induced cytokines (30, 33–35). However, in our study, administration of C3a in *C3* KO mice did not affect IFN-α production following PapMV immunization, suggesting that this anaphylatoxin is not implicated in the regulation of type I IFN secretion. Nevertheless, other complement proteins, such as C1q, C3b, and iC3b, were also reported to impact TLR responses. For example, these proteins can induce PI3K and ERK1/2 signaling, thereby suppressing transcription factors, IRF1 and IRF8, required for IL-12 production by APCs (36, 37). In addition, phagocytosis of apoptotic cells opsonized with C3b is an anti-inflammatory process in which the inhibition of IL-12 production is observed (38, 39). Thus we hypothesize that complement proteins such as C3b and possibly C1q and C4 suppress IFN-α production by pDCs. Accordingly, PapMV complexed with specific polyclonal Abs increased fixation of C3b, C1q and C4 on its surface leading to lower IFN-α production. This also suggests a different capacity between NA- and PapMV-specific Abs to promote or block complement binding and activation as we observed that absence of NA increased binding of C3-related molecules to PapMV. Conversely, C3 removal through CVF administration increased IFN-α–dependent immune activation providing indirect evidence for a role played by C3b in downregulating of IFN-α production following TLR7 activation.

The immunomodulatory properties of PapMV are strictly dependent on IFN-α production (8). As a result, administration of PapMV to CVF-treated mice leads to greater activation of immune cells and provides increased immunomodulatory capacity following BMDC immunization as well as a superior therapeutic effect in the context of B16 metastasis development. Type I IFN is known to increase MHC-I expression on tumors and immune cells, cross-presentation by APCs and T cell responses (40–42); this effector function is therefore congruent with the enhanced immune responses observed in the context of vaccination and cancer immunotherapy upon increase of IFN-α production.

In conclusion, in this study we delineated novel roles of NA and the complement system in the recognition and phagocytosis of a

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plant virus–based nanoparticle. More importantly, we identified an unexpected link between C3 and the innate immune response to this TLR7 agonist. Because PapMV is being developed for various immunotherapy applications, modulation of the complement pathway represents a promising approach to potentially enhance its therapeutic efficacy.

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Disclosures

D.L. is the founder and a shareholder of Folia Biotech. Inc., a Canadian biotechnology company with the mandate to commercialize the PapMV technology. The other authors have no financial conflicts of interest.

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