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L'UTILISATION DE LA NANOPARTICULE DU VIRUS DE LA MOSAÏQUE DE LA PAPAYE DANS LE TRAITEMENT D'UNE INFECTION VIRALE CHRONIQUE : EFFET DE L'INTERFÉRENCE DES ANTICORPS ET DE LA RÉGULATION DE LA VOIE DE SIGNALISATION DU TLR7

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RÉSUMÉ

Bien que la vaccination prophylactique soit un des meilleurs moyens de prévenir les infections, la vaccination thérapeutique reste encore en développement. L'utilisation de virus atténués ou inactivés comme plateforme vaccinale n'est pas toujours possible dû à des problèmes de sécurité. Une des alternatives sécuritaires à la vaccination classique repose sur l'utilisation de nanoparticules virales, qui permettent d'imiter l'agent pathogène sans risques d'infections pour l'hôte. Nous avons auparavant démontré que l'utilisation prophylactique de nanoparticules du virus de la mosaïque de la papaye (PapMV) permet de protéger la souris d'infections virales et bactériennes grâce à l'activation de la réponse immunitaire humorale et cellulaire. Nous avons aussi démontré le potentiel du PapMV en tant qu'immunothérapie dépendante de l'interféron de type I (IFN-I) dans le traitement d'un modèle de cancer localisé. Nous nous sommes donc intéressés au fort potentiel de production de l'IFN-α du PapMV dans le traitement d'une infection virale chronique tel que celle provoquée par la souche Cl13 du virus de la choriomeningite lymphocytaire (LCMV). Le traitement de souris infectées au LCMV Cl13 par deux immunisations systémiques de PapMV n'induit aucun changement dans les titres viraux de LCMV Cl13 ou dans la réponse immunitaire montée contre ce virus. De plus, la production d'IFN-α suite à une immunisation au PapMV est complètement abolie chez les souris infectées. Afin de mieux isoler les mécanismes responsables de cette tolérance au PapMV, nous avons déterminé l'influence d'un prétraitement au PapMV sur des immunisations subséquentes de PapMV. Une immunisation au PapMV précédée d'un prétraitement au PapMV est incapable d'induire la production d'IFN-α pendant au moins 50 jours suivant la deuxième immunisation et la réponse immunitaire est grandement affectée suite a ce régime d'immunisation. Nous avons démontré que deux mécanismes distincts seraient responsables de cet effet de tolérance. La tolérance à court terme résulterait partiellement de la dégradation d'IRAK1, une composante essentielle de la cascade de signalisation du TLR7, alors que l'inhibition à long terme semble être principalement due à l'interférence des anticorps spécifiques au PapMV. Nous avons donc identifié certains défis importants dans l'utilisation de nanoparticules virales dans le traitement systémique d'infections virales chroniques et nous discutons d'alternatives à ces défis.

Mots clés : Vaccination thérapeutique ; nanoparticules virales ; virus de la mosaïque de la papaye ; interféron alpha ; cellules dendritiques plasmacytoïdes ; virus de la choriomeningite lymphocytaire ; anticorps

ABSTRACT

Although vaccination has been an effective way of preventing infections ever since the 18th century, the generation of therapeutic vaccines and immunotherapies is still in development. Several challenges impede the development of these therapeutic approaches such as safety issues. One safe alternative to classical vaccination methods gaining recognition is the use of nanoparticles, whether synthetic or naturally derived. We have recently demonstrated that the papaya mosaic virus-like (PapMV) nanoparticle can be used as a prophylactic vaccine against various viral and bacterial infections through the induction of protective humoral and cellular immune responses. Moreover, PapMV is also very efficient when used as an immune adjuvant in an immunotherapeutic setting at slowing down the growth of aggressive mouse melanoma tumors in a type I interferon (IFN-I)-dependent manner. In the present study we were interested in exploiting the capacity of PapMV to induce robust IFN-I production as treatment for the chronic viral infection model lymphocytic choriomeningitis virus (LCMV) Clone 13 (Cl13). Treatment of LCMV Cl13-infected mice with two systemic administrations of PapMV was ineffective, as shown by the lack of changes in viral titers and immune response to LCMV following treatment. Moreover, IFN-α production following PapMV administration was almost completely abolished in LCMV infected mice. To better isolate the mechanisms at play, we determined the influence of a pre-treatment with PapMV on secondary PapMV administration. Pre-treatment with PapMV led to the same outcome as an LCMV infection in that IFN-α production following secondary PapMV immunization was abrogated for up to 50 days while immune activation was also dramatically impaired. We showed that two distinct and overlapping mechanisms were responsible for this outcome. While short-term inhibition was partially the result of IRAK1 degradation, a crucial component of the TLR7 signaling pathway, long-term inhibition was mainly due to interference by PapMV-specific antibodies. Thus, we identified a possible pitfall in the use of virus-like particles for the systemic treatment of chronic viral infections and discuss mitigating alternatives to circumvent these potential problems.

Keywords: Therapeutic vaccination; viral nanoparticles; papaya mosaic virus; interferon alpha; plasmacytoid dendritic cells; lymphocytic choriomeningitis virus; antibodies

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LISTE DES ABRÉVIATIONS

ADN : Acide désoxyribonucléique

ARN : Acide ribonucléique

BCG : Bacille Calmette-Guérin

BMDC : Cellules dendritiques dérivées de la moelle osseuse

BMpDC : Cellules dendritiques plasmacytoïdes dérivées de la moelle osseuse

cDC : Cellules dendritiques conventionnelles

CMH : Complexe majeur d'histocompatibilité

CPA : Cellules présentatrices d'antigènes

CpG : 5'-C-phosphate-G-3'

CTL : Lymphocytes cytotoxiques ($CD8^+$)

DC : Cellules dendritiques

EMCV : Virus de l'encéphalomyocardite

HSP : Protéines de choc thermique

IFNAR : Récepteur de l'interféron α/β

IFN-I : Interféron de type 1

IKKi : Sous unité iota de la kinase inhibitrice du facteur nucléaire kappa-B

IRAK1 : Kinase associée au récepteur de l'interleukin-1 1

IRF 3/7 : Facteur régulateur de l'interféron

kDa : Kilodalton

LCMV : Virus lymphocytaire de la chorioméningite

LPS : Lipopolysaccharide

MAPK : Protéine kinase activée par un facteur mitogène

MCMV : Cytomégalovirus murin

MyD88 : Gène de la réponse primaire de la différentiation myéloïde 88

NEMO : Modulateur essentiel de NF-κB

PAMP : Motifs moléculaires associés aux pathogènes

PapMV : Pseudoparticule du virus de la mosaïque de la papaye

pDC : Cellules dendritiques plasmacytoïdes

PRR : Récepteur de reconnaissance de motifs moléculaires

Sca-1 : Antigène de cellules souches

SLE : Lupus érythémateux disséminé

TAK1 : Kinase activée par le facteur de croissance transformant beta

TBK1 : Kinase liant TANK 1

TCR : Récepteur des cellules T

TIR : Récepteur Toll-IL-1

TIV : Vaccin trivalent contre l'influenza

TLR : Récepteur de type Toll

TNF-α : Facteur de nécrose tumoral alpha

TRIF : Adapteur contenant le domaine TIR induisant l'interféron-β

VHB : Virus de l'hépatite B

VHS : Virus de l'Herpès Simplex

VIH : Virus de l'immunodéficience humaine

VLP : Pseudoparticule virale

VPH : Virus du papillome humain

CHAPITRE 1 : ÉTAT DES CONNAISSANCES

1 LA RÉPONSE IMMUNITAIRE

Le système immunitaire constitue le meilleur moyen de défense du corps humain contre l'intrusion de pathogènes. Lors d'une infection virale ou bactérienne, l'hôte met en place plusieurs mécanismes de défense se déroulant en deux grandes étapes soit la réponse immunitaire innée et la réponse immunitaire adaptative. Bien que la réponse immunitaire soit vaste et complexe, seulement quelques composantes seront ici développées.

1.1 La réponse immunitaire innée

Les médiateurs de la réponse immunitaire innée sont les premiers à intervenir lors d'une infection. Les cellules impliquées dans cette première étape de la réponse immunitaire vont reconnaître des motifs simples et répétés que l'on retrouve chez plusieurs agents pathogènes. Ces motifs moléculaires associés aux pathogènes (PAMP) sont reconnus par des récepteurs de motifs moléculaires (PRR) que l'on retrouve en surface ainsi qu'à l'intérieur des cellules immunitaires. La liaison de ces ligands à leurs récepteurs respectifs engendre des cascades de signalisation qui mènent à la production de médiateurs pro-inflammatoires qui agissent à titre d'alarme afin de prévenir les cellules immunitaires avoisinantes et distantes du danger existant. La réponse immunitaire innée s'organise rapidement et permet de contenir les débuts d'une infection. Bien que différents acteurs soient impliqués à différentes étapes dans la réponse immunitaire innée, seulement les récepteurs de type Toll et les cellules dendritiques plasmacytoïdes seront ici abordés.

1.1.1 Les récepteurs de type Toll

Avant d'être identifiée chez l'humain ou chez la souris, la protéine Toll a grandement été étudiée chez *Drosophila melanogaster,* où un rôle dans le développement de l'embryon lui a été attribué (O'Neill *et al.*, 2013). C'est une homologie entre ce récepteur et le récepteur de l'IL-1 (IL-1R) qui a démarré les recherches sur les récepteurs de type Toll chez l'humain et la souris. Douze récepteurs sont aujourd'hui connus chez la souris (1-9, 11-13) et 10 chez l'humain (1-10) (O'Neill *et al.*, 2013). Ces récepteurs constituent une classe de PRR à eux seuls et sont distribués à travers différentes cellules du système immunitaire inné. Les ligands extracellulaires tel que les protéines, les sucres ou les lipides sont reconnus par des récepteurs retrouvés à la surface tel que les TLR1, TLR2, TLR4, TLR5 et TLR6 alors que le matériel génétique des agents pathogènes

est reconnu dans les endosomes par les TLR3, TLR7/8 et le TLR9 (Blasius *et al.*, 2010). Deux voies de signalisation peuvent être déclenchées suite à l'interaction du ligand avec son récepteur. La première voie de signalisation, qui est adoptée par tous les TLR à l'exception du TLR3, requiert la protéine adaptatrice MyD88 (Lester *et al.*, 2014). Lorsqu'un PAMP extracellulaire est reconnu par son récepteur, la protéine adaptatrice MyD88 est recrutée au domaine TIR du récepteur, ce qui déclenche le recrutement d'un complexe protéique composé d'IRAK4, IRAK1 et de TRAF6 (Brubaker *et al.*, 2015, L. Li *et al.*, 2000, S. Sato *et al.*, 2002). Ce complexe va ensuite interagir avec différents intermédiaires afin de transmettre le signal et induire translocation de NF-κB et de AP-1 au noyau (Brubaker *et al.*, 2015), résultant en la production de cytokines pro-inflammatoires (Figure 1.1). En plus de la production de cytokines et de chimiokines, la liaison du matériel génétique aux récepteurs intracellulaires entraîne la production d'IFN-I. La formation du complexe IRAK4-IRAK1-TRAF6 est suivie de la phosphorylation d'IRF7, qui à son tour va transloquer au noyau et ainsi induire la production d'IFN-I (Honda *et al.*, 2006, Kawai *et al.*, 2004, M. Sato *et al.*, 1998) (Figure 1.1). La seconde voie de signalisation, qui n'est adoptée que par le TLR3 et à l'occasion par le TLR4, emploie la protéine adaptatrice TRIF, qui joue le rôle de protéine adaptatrice au même titre que MyD88 (Figure 1.1) (O'Neill *et al.*, 2013). Bien que le TLR4 adopte la voie de signalisation dépendante de MyD88, l'emploi de la voie TRIF-dépendante est aussi possible lors de la stimulation de ce TLR, ce qui permet l'expression d'une plus grande variété de cytokines (Kawai *et al.*, 2001, Kawasaki *et al.*, 2014). Suite à la stimulation du TLR3 ou du TLR4, la protéine adaptatrice TRIF est recrutée au domaine intracellulaire du récepteur et interagit avec TRAF6 et TRAF3. L'interaction avec TRAF6 permet le recrutement de la protéine kinase RIP-1, qui va ensuite interagir avec TAK1 pour activer NF-κB et la cascade de MAPK afin d'induire la production de cytokines pro-inflammatoires (Kawasaki *et al.*, 2014, Narayanan *et al.*, 2015). L'interaction de la molécule adaptatrice TRIF avec TRAF3 engendre pour sa part le recrutement de TBK1, IKKi et de NEMO afin d'induire la phosphorylation d'IRF3, qui va ensuite dimériser et transloquer au noyau pour induire l'expression de gènes de l'IFN-I (Figure 1.1) (Gay *et al.*, 2014, Kawasaki *et al.*, 2014). Même si les voies de signalisation semblent redondantes entre les récepteurs, les gènes transcrits en fin de cascade diffèrent selon les ligands et les récepteurs, menant ainsi à la production d'une variété de cytokines et de chimiokines.

Tel que mentionné précédemment, différents types de TLR permettent de reconnaître un large éventail de ligands. Tous les TLR ne sont pas retrouvés chez tous les types cellulaires. En effet, certains TLR ne sont retrouvés que dans une population définie (Tableau 1.2). La variété de TLR présents dans le système immunitaire permet donc à l'hôte de se protéger contre plusieurs différents pathogènes.

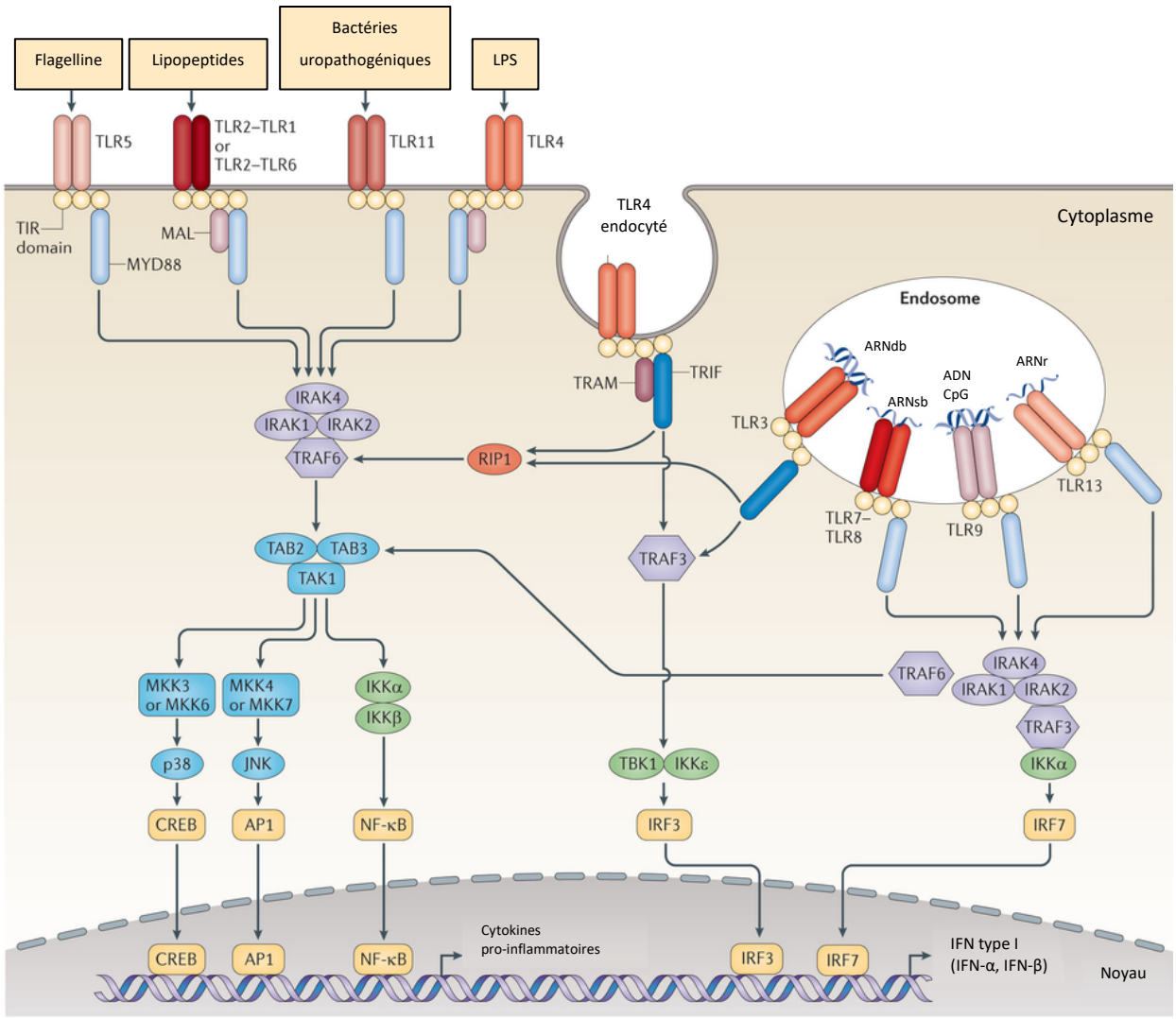

Figure 1.1 : La signalisation des TLR

Voies de signalisation murines des TLR extracellulaires (TLR1, TLR2, TLR 4, TLR5, TLR6, TLR11) ainsi que des TLR endosomaux (TLR3, TLR4, TLR7, TLR8). Les voies de signalisation des TLR extracellulaires mènent principalement à la production de cytokines pro-inflammatoires alors que les voies de signalisation des TLR endosomaux peuvent aussi induire la production d'IFN de type I. Tous les TLR nécessitent la protéine adaptatrice MyD88 afin d'assurer la transmission du signal excepté le TLR4, qui peut aussi utiliser la molécule adaptatrice TRIF, et le TLR3, qui requiert exclusivement la protéine TRIF pour le relai du signal. Figure modifiée à partir de (O'Neill *et al.*, 2013).

Tableau 1.1 : Ligands des TLR3, TLR4, TLR7 et TLR9

TLR	Cellules l'exprimant	Localisation cellulaire	Ligands	Exemple de ligands	Références
TLR3	cDC	Endosome	ARN double brin (génomique ou intermédiaire de réplication)	Poliovirus EMCV VHS	(Abe *et al.*, 2012, Hardarson *et al.*, 2007, Kastenmuller *et al.*, 2014, Lester *et al.*, 2014, O'Neill *et al.*, 2013, Reinert *et al.*, 2012)
TLR4	Macrophages, Monocytes	Surface	Polysaccharides, Protéines de choc thermique, Protéines virales	LPS	(Brubaker *et al.*, 2015, O'Neill *et al.*, 2013, Ohashi *et al.*, 2000, Rassa *et al.*, 2002)
TLR7	pDC, Lymphocytes B (faiblement)	Endosome	ARN simple brin riche en guanosine et uridine	Coronavirus Virus de l'influenza VIH	(Beignon *et al.*, 2005, Cervantes-Barragan *et al.*, 2007, Diebold *et al.*, 2004, Edwards *et al.*, 2003, Hornung *et al.*, 2002, Lester *et al.*, 2014, Swiecki *et al.*, 2015)
TLR9	pDC, Lymphocytes B	Endosome	CpG non méthylés	VHS MCMV	(Hornung *et al.*, 2002, Iwasaki *et al.*, 2004, Krug *et al.*, 2004, Lester *et al.*, 2014, Lima *et al.*, 2010)

La stimulation soutenue des TLR lors d'infections virales ou bactériennes peut mener à une réponse exacerbée chez l'hôte, phénomène dû à la production continue de médiateurs inflammatoires. Afin de limiter les dommages causés par une telle réponse, des mécanismes de protection ont été développés chez les cellules immunitaires, tel qu'observé lors d'une septicémie. Plusieurs effets secondaires découlent d'une telle réponse immunitaire déséquilibrée tel que l'effondrement de certains systèmes anatomiques ainsi que l'induction d'un état d'immunosuppression où les patients sont plus à risques de contracter de nouvelles infections pouvant ensuite causer la mort (Angus *et al.*, 2013, Biswas *et al.*, 2009). Afin de se protéger, l'organisme réduit ou inhibe sa production de cytokines pro-inflammatoires en régulant directement les voies de signalisation en réponse à une stimulation constante des TLR. Cela se traduit par une diminution de la production de TNF-α et d'IL-6 pour la voie NF-κB ou une diminution de la production d'IFN-I lorsque la voie des IRF est affectée (Albrecht *et al.*, 2008, Dalpke *et al.*, 2005, Hayashi *et al.*, 2009, Marshall *et al.*, 2007, Nahid *et al.*, 2016). Cette tolérance des TLR a été observée non seulement pour le TLR4 dans le cas de septicémie mais aussi pour plusieurs autres TLR et ce, en situation d'homo- et d'hétéro-tolérance (Tableau 1.2).

La tolérance des TLR serait majoritairement attribuée à la dégradation d'IRAK1 qui est impliqué dans les voies de signalisation dépendantes de la protéine adaptatrice MyD88. IRAK1 est une protéine kinase qui, avec IRAK4 et TRAF6, forme un complexe protéique permettant la transmission du signal suite au recrutement de MyD88 au récepteur (Kawai *et al.*, 2010). Plusieurs études ont démontré qu'IRAK1 est dégradé suite à une stimulation du TLR2 (Albrecht *et al.*, 2008, C. H. Li *et al.*, 2006, Liu *et al.*, 2012, S. Sato *et al.*, 2002, Siedlar *et al.*, 2004), du TLR4 (Albrecht *et al.*, 2008, Nahid *et al.*, 2016, Siedlar *et al.*, 2004), du TLR7 (Bourquin *et al.*, 2011, Koga-Yamakawa *et al.*, 2015, Nahid *et al.*, 2016, S. Sato *et al.*, 2002) ainsi que du TLR9 (Liu *et al.*, 2012). Cette dégradation serait due à l'ARN interférant miR146a, qui est exprimé suite à la stimulation du TLR4 (Nahid *et al.*, 2016, Nahid *et al.*, 2009), du TLR7 (Karrich *et al.*, 2013, Nahid *et al.*, 2016) et du TLR9 (Karrich *et al.*, 2013) bien que ces résultats soient controversés (Taganov *et al.*, 2006). La dégradation de l'ARN messager d'IRAK1 par l'ARN interférant miR146a inhiberait donc la production de nouvelles protéines d'IRAK1 (Park *et al.*, 2015, Taganov *et al.*, 2006), limitant ainsi la transmission des différents signaux via les cascades de signalisation des TLR.

Tableau 1.2 : Combinaisons de ligand de TLR induisant une diminution de production de cytokines suite à une tolérance des TLR

1ère stimulation	2e stimulation	Diminution du TNF-α	Diminution de l' IFN-I
TLR2	TLR2	(Albrecht *et al.*, 2008, Anstadt *et al.*, 2016, Dalpke *et al.*, 2005, Lehner *et al.*, 2001, C. H. Li *et al.*, 2006, S. Sato *et al.*, 2002, Siedlar *et al.*, 2004, J. H. Wang *et al.*, 2003)	
	TLR4	(Berglund *et al.*, 2008, Dalpke *et al.*, 2005, Lehner *et al.*, 2001, C. H. Li *et al.*, 2006, S. Sato *et al.*, 2002)	
	TLR7/8	(Anstadt *et al.*, 2016)	
	TLR9	(Anstadt *et al.*, 2016)	
TLR4	TLR2	(Berglund *et al.*, 2008, Dalpke *et al.*, 2005, Lehner *et al.*, 2001, Nahid *et al.*, 2016)	
	TLR4	(Albrecht *et al.*, 2008, Dalpke *et al.*, 2005, Lehner *et al.*, 2001, L. Li *et al.*, 2000, Nahid *et al.*, 2016, S. Sato *et al.*, 2002)	
	TLR7/8	(Nahid *et al.*, 2016)	
	TLR9		
TLR7/8	TLR2	(Hayashi *et al.*, 2009, Nahid *et al.*, 2016)	
	TLR4	(Nahid *et al.*, 2016, S. Sato *et al.*, 2002)	
	TLR7/8	(Hayashi *et al.*, 2009, Nahid *et al.*, 2016, Tsukada *et al.*, 2007)	(Koga-Yamakawa *et al.*, 2015, Marshall *et al.*, 2007)
	TLR9	(Hayashi *et al.*, 2009)	(Marshall *et al.*, 2007)
TLR9	TLR2		(Liu *et al.*, 2012)
	TLR4	(Dalpke *et al.*, 2005)	
	TLR7/8		
	TLR9	(Dalpke *et al.*, 2005)	(Jaehn *et al.*, 2008)

1.1.2 Les cellules dendritiques plasmacytoïdes (pDC)

Les pDC, qui représentent un type de cellules dendritiques (DC), n'ont qu'un faible potentiel de présentation antigénique, contrairement aux autres membres de la famille des DC. En effet, leur rôle principal réside dans la production d'IFN-I suite à une infection virale. En déplétant les pDC de la souris avant une infection virale, la production d'IFN-I diminue considérablement (Asselin-Paturel *et al.*, 2001, Dalod *et al.*, 2003, Jego *et al.*, 2003) et la protection de l'hôte contre l'infection est grandement affectée (Cervantes-Barragan *et al.*, 2012, Cervantes-Barragan *et al.*, 2007, Lund *et al.*, 2006, Smit *et al.*, 2006, Swiecki *et al.*, 2010b, Swiecki *et al.*, 2013, Yoneyama *et al.*, 2005). Les pDC sont aussi d'excellentes productrices d'IFN-I suite à la stimulation des TLR7 et TLR9, récepteurs majeurs des pDC qui reconnaissent le matériel génétique typiquement associé aux agents pathogènes viraux (Hornung *et al.*, 2002, Iwasaki *et al.*, 2004). Les pDC ont aussi la capacité de produire des cytokines pro-inflammatoires lorsque la voie de signalisation NF-κB est engagée (Gibson *et al.*, 2002, Swiecki *et al.*, 2010a, Swiecki *et al.*, 2015) bien que cette voie ne soit pas la principale sollicitée chez les pDC. La nature du récepteur engagé n'est pas le seul déterminant de la voie de signalisation qui sera empruntée. En effet, deux ligands du TLR9, soit le CpG-A et le CpG-B, engendrent une réponse différente chez les pDC. Les CpG-A, qui ont une forme particulaire, se retrouvent dans les endosomes des pDC et induisent la production d'IFN-I. Les CpG-B, qui ont une forme linéaire, se retrouvent dans des vésicules à caractère lysosomal et empruntent la voie de signalisation NF-kB pour induire la différentiation des pDC (Guiducci *et al.*, 2006, Honda *et al.*, 2005). Le choix de la voie de signalisation empruntée dépend donc de la nature et de la structure du ligand ainsi que de sa localisation dans la cellule une fois phagocyté. Bien qu'elles ne soient pas reconnues pour le faire, les pDC sont aussi capables d'effectuer la présentation antigénique sans toutefois le faire aussi bien que les cDC. L'activation des pDC par un ligand de TLR ou un agent infectieux induit l'expression du complexe majeur d'histocompatibilité (CMH) II ainsi que des molécules de costimulation (Takagi *et al.*, 2011, Villadangos *et al.*, 2008, Young *et al.*, 2008), tous les deux requis pour la présentation antigénique aux lymphocytes T $CD4^+$. Il a aussi été démontré que les pDC peuvent présenter des antigènes aux lymphocytes T $CD8^+$ par le biais du CMH I et ainsi participer au développement de la réponse adaptative cytotoxique (Salio *et al.*, 2004, Takagi *et al.*, 2011). Une grande source d'antigène doit être disponible afin que les pDC puissent effectuer la présentation antigénique (Villadangos *et al.*, 2008, Young *et al.*, 2008), leur conférant ainsi un rôle de pont entre la réponse immunitaire innée et adaptative.

Bien que les pDC soient reconnues pour leur production d'IFN-I suite à la stimulation de leurs TLR endosomaux, ce ne sont pas toutes les pDC qui détiennent ce potentiel. En effet, il a été démontré qu'il existe deux sous-populations de pDC qui répondent différemment à la stimulation par un ligand de TLR9 au niveau de leur production d'IFN-α (Bjorck *et al.*, 2011, Niederquell *et al.*, 2013, Omatsu *et al.*, 2005, Schwab *et al.*, 2010). Différents marqueurs d'expression ont été identifiés afin de différencier ces deux sous-populations dont Ly6C (Pelayo *et al.*, 2005), CD9 (Bjorck *et al.*, 2011), Ly49Q (Omatsu *et al.*, 2005), CD123 (Schwab *et al.*, 2010) ainsi que CD5 et CD81 (Zhang *et al.*, 2017) (Voir tableau 1.3). L'antigène de cellules souches 1 (Sca-1) a été identifié par l'équipe de Niederquell *et al.*, qui ont associé ce marqueur aux capacités de production d'IFN-I ainsi qu'au degré de maturation des pDC. Selon cette étude, les pDC n'exprimant pas Sca-1 (Sca-1$^-$) détiendraient la capacité de produire l'IFN-I alors que l'expression de Sca-1 (Sca-1$^+$) indiquerait un potentiel très faible de production d'IFN-I (Niederquell *et al.*, 2013). Sca-1, qui n'est pas connu pour être exprimé à la surface des pDC, est reconnu pour son expression à la surface des lymphocytes T activés suite à une stimulation du TLR4 (K. R. Kumar *et al.*, 2005) ou d'une infection par LCMV (Whitmire *et al.*, 2009). Le mécanisme derrière l'expression de Sca-1 à la surface des pDC reste encore aujourd'hui inconnu.

Tableau 1.3 : Phénotypes liés à la production d'IFN-α chez les cellules dendritiques plasmacytoïdes

Marqueur	Phénotype de production d'IFN-I	Phénotype produisant peu ou pas d'IFN-I	Références
Sca-1	Sca-1$^-$	Sca-1$^+$	(O'Neill *et al.*, 2013)
Ly6C	Ly6C$^+$	Ly6C$^-$	(Pelayo *et al.*, 2005)
Ly49Q	Ly49Q$^+$	Ly49Q$^-$	(Omatsu *et al.*, 2005)
CD123	CD123^{hi}	CD123^{lo}	(Schwab *et al.*, 2010)
CD9	CD9$^+$	CD9$^-$	(Bjorck *et al.*, 2011)
CD5 et CD81	CD5$^-$CD81$^-$	CD5$^+$CD81$^+$	(Zhang *et al.*, 2017)

1.2 La réponse immunitaire adaptative

La réponse immunitaire adaptative, tel que son nom l'indique, s'ajuste à l'environnement et aux situations auxquelles elle doit faire face. C'est grâce à cette branche de la réponse immunitaire que nous détenons une mémoire immunologique contre les agents pathogènes déjà rencontrés. Cette spécificité antigénique nous permet de nous défendre de façon plus rapide et plus efficace si le système immunitaire rencontre l'agent pathogène à nouveau. La réponse immunitaire adaptative est de plus longue durée que la réponse immunitaire innée mais nécessite plus de temps avant d'atteindre son efficacité maximum. La réponse immunitaire adaptative se divise en deux soit la réponse humorale et la réponse cellulaire cytotoxique.

1.2.1 La réponse immunitaire humorale

La réponse immunitaire humorale est largement menée par les anticorps, impliqués dans la reconnaissance, l'opsonisation et la destruction d'agents pathogènes. Ces protéines sont produites par les lymphocytes B, qui ont la capacité de produire différentes classes d'anticorps selon des cinétiques différentes. Les IgM sont les premiers anticorps sécrétés lors d'une infection et forment des pentamères peu spécifiques aux pathogènes (Schroeder *et al.*, 2010). L'opsonisation générale et peu spécifique d'agents pathogènes par les IgM facilite la reconnaissance et la destruction de l'agent pathogène par le système du complément (Schroeder *et al.*, 2010). Au cours de l'évolution de la réponse immunitaire, les IgM sont remplacés par des anticorps tel que les IgG, qui auront subi plusieurs mutations afin d'augmenter leur spécificité et leur affinité envers un épitope précis (Pieper *et al.*, 2013). Lorsque les anticorps reconnaissent l'épitope contre lequel ils sont spécifiques, des complexes anticorps-pathogènes sont formés et reconnus par les cellules phagocytaires ou par le complément, ce qui engendre la destruction de l'agent pathogène. Lorsque l'agent pathogène est éliminé, la majorité des lymphocytes B effecteurs seront éliminés car leur présence n'est pas nécessaire à l'état d'homéostasie. Par contre, certains lymphocytes B survivront en tant que cellules mémoires ou plasmocytes de longue durée. Les lymphocytes B mémoires produiront des anticorps rapidement lorsqu'ils rencontreront à nouveau l'agent pathogène contre lequel ils sont spécifiques alors que les plasmocytes de longue durée vont produire des anticorps de façon constitutive (Manz *et al.*, 1997, Pieper *et al.*, 2013, Purtha *et al.*, 2011, Slifka *et al.*, 1998), ce qui explique pourquoi les titres d'anticorps restent élevés à long terme suite à une immunisation ou une infection. La réponse immunitaire humorale permet de contrôler plusieurs agents infectieux tel que le virus de la poliomyélite, le virus de la rubéole ainsi que le virus des oreillons (Pantaleo *et al.*, 2004).

1.2.2 Les lymphocytes cytotoxiques CD8$^+$

Dans le cas d'infections impliquant des pathogènes extracellulaires, la production d'anticorps par les lymphocytes B suffit généralement à protéger l'hôte. Par contre, lorsque l'hôte est infecté par un pathogène intracellulaire, les anticorps sont incapables d'assurer la protection de l'hôte. Une réponse immunitaire plus robuste est nécessaire afin de contrôler et d'éradiquer l'infection. Pour ce faire, les lymphocytes T CD8$^+$ sont recrutés par le système immunitaire afin d'éliminer les pathogènes intracellulaires. Afin d'assembler une réponse immunitaire cellulaire cytotoxique protectrice, trois signaux sont nécessaires à l'activation des lymphocytes T CD8$^+$ (voir figure 1.2). Le premier signal est à la base de la spécificité antigénique des lymphocytes T. Lorsqu'un pathogène intracellulaire infecte une cellule du système immunitaire inné, cette dernière va présenter à sa surface des peptides provenant de l'agent pathogène. Cette présentation s'effectue par le biais du CMH I (Hewitt, 2003), qui est exprimé de façon ubiquitaire par toutes les cellules nucléées. Ces peptides ainsi que les CMH I seront reconnus par le récepteur des cellules T (TCR), qui n'est spécifique qu'à un seul antigène (Pennock *et al.*, 2013). Afin d'activer les lymphocytes T CD8$^+$ naïves, cette interaction entre le CMH I et le TCR doit être soutenue par deux autres signaux dont la costimulation. À la surface des lymphocytes T, le CD28 se lie aux molécules de costimulation des cellules immunitaires innées (Sharpe *et al.*, 2006) et permet de confirmer le nature de l'interaction entre les lymphocytes T et les cellules du système immunitaire inné. Au niveau des cellules du système immunitaire inné, le CD80 et le CD86, aussi connus sous B7-1 et B7-2, sont essentiels à l'activation des lymphocytes T cytotoxiques. En effet, ces deux molécules sont exprimées à la surface des cellules immunitaires innées lorsque ces dernières sont activées suite à une infection. Ces interactions permettent donc de confirmer aux lymphocytes T CD8$^+$ qu'il y a bel et bien une infection et qu'il ne s'agit pas d'une reconnaissance accidentelle d'un antigène (Sharpe *et al.*, 2006). Le troisième signal qui permet l'activation des lymphocytes T CD8$^+$ et l'élaboration d'une réponse immunitaire protectrice contre des pathogènes intracellulaires requiert certaines cytokines tel que l'IL-12 et l'IL-2. Ces cytokines vont permettre aux lymphocytes de proliférer et ensuite de devenir des cellules effectrices (Curtsinger *et al.*, 2003, Curtsinger *et al.*, 1999). En absence de ce troisième signal, les lymphocytes T CD8$^+$ sont activés mais ne pourront pas participer à la réponse immunitaire effectrice car ces cellules seront dans un état perpétuel de tolérance (Curtsinger *et al.*, 2003).

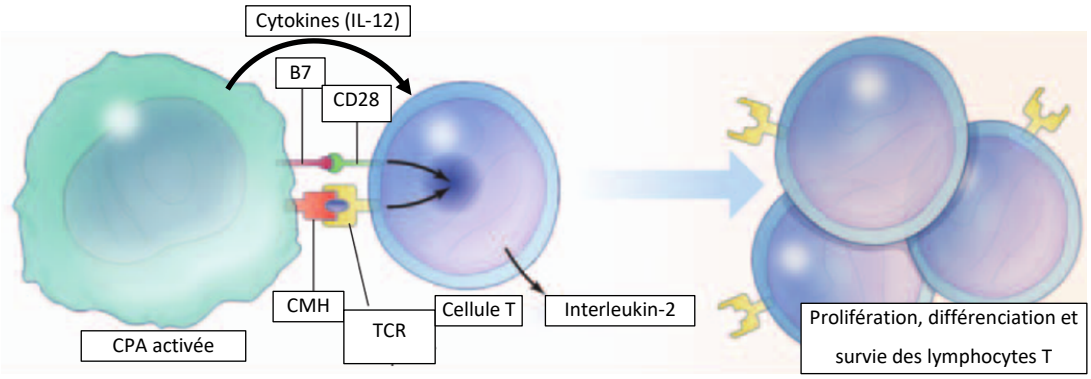

Figure 1.2 : Les trois signaux requis pour l'activation des lymphocytes T.
La cellule présentatrice d'antigènes (CPA) activée exprime le corécepteur B7, qui est reconnu par le CD28 à la surface du lymphocyte T. La CPA présente un antigène par le biais de son CMH qui sera reconnu par le TCR afin d'activer la cellule T. La production de cytokines de la CPA vers le lymphocyte T constitue le troisième signal requis pour l'activation des lymphocytes T, ce qui mène à la prolifération, la différenciation et la survie de ces derniers. Reproduit avec la permission de (Sharpe *et al.*, 2006), Copyright Massachussetts Medical Society.

Une fois les lymphocytes T $CD8^+$ devenus effecteurs, ces derniers visent à détruire les cellules infectées par l'agent pathogène. En utilisant le même processus que les cellules du système immunitaire inné infectées, toute cellule infectée va présenter des antigènes de l'agent infectieux par le biais du CMH I qui est exprimé par toutes les cellules nucléées. La reconnaissance de cet antigène par les TCR de lymphocytes T $CD8^+$ spécifiques, en plus d'autres signaux et interactions entre les cellules infectées et les lymphocytes T cytotoxiques, se termine par la mort de la cellule infectée (Pennock *et al.*, 2013). Cette fonction de cytotoxicité fait des lymphocytes T $CD8^+$ des cellules immunitaires importantes dans le contrôle d'infections tel que celles causées par le virus de l'hépatite A, B et C (VHA, VHB, VHC), le virus du papillome humain (VPH) ainsi que le virus de l'immunodéficience humaine (VIH). Cette réponse immunitaire cytotoxique protectrice est aussi importante dans le contrôle du cancer (Pantaleo *et al.*, 2004). Ces cellules peuvent donc servir d'indicateurs de l'évolution et de l'efficacité de la réponse immunitaire.

2 LA VACCINATION

C'est Edward Jenner qui, au 18e siècle, introduit pour la première fois le concept de la vaccination. Plusieurs développements tel que les travaux de Louis Pasteur nous permettent aujourd'hui d'être protégés contre une grande variété de maladies ou d'en réduire l'incidence. Depuis ses débuts, la vaccination a principalement été axée sur la prévention de maladies. La vaccination thérapeutique, qui applique la vaccination pour le traitement d'infections ou de maladies, gagne cependant de plus en plus d'intérêt dans le domaine médical.

2.1 La vaccination prophylactique

La majorité des vaccins en circulation sont de nature prophylactique. Cela signifie qu'ils sont administrés en prévention contre une infection. Il existe différents types de vaccins, chacun ayant ses avantages et ses inconvénients. Seulement trois types seront abordés ici soit les vaccins atténués, inactivés et sous-unitaires.

2.1.1 Les vaccins atténués

Les vaccins atténués ont été développés suite aux travaux effectués par Edward Jenner qui croyait que l'administration d'un virus animal chez l'humain engendrerait une protection car le virus animal y serait atténué (Minor, 2015, S. Plotkin, 2014). L'atténuation d'un agent pathogène permet d'induire une forte réponse immunitaire à long terme suite à une ou deux inoculations, réduisant ainsi la nécessité de doses de rappel. En effet, l'atténuation d'agents pathogènes permet de conserver la structure du pathogène, et donc les épitopes immunitaires, mais limite l'expression de caractéristiques infectieuses. Une des techniques d'atténuation consiste à effectuer plusieurs passages de l'agent infectieux chez une lignée cellulaire ou un hôte où la réplication du pathogène est plus difficile. Au fil des passages, le pathogène perd ses facteurs de virulence, réduisant ainsi sa pathogénicité (S. Plotkin, 2014) sans affecter sa structure. Bien que les vaccins vivants atténués soient très efficaces en prophylactie, ils doivent être utilisés avec précaution car les agents pathogènes atténués sont toujours vivants mais avec un potentiel de réplication moindre. Il est donc possible que ces derniers mutent et révertent à leur génome d'origine pouvant ainsi causer une infection (Mostow et al., 1979). Plusieurs vaccins sont aujourd'hui préparés à base d'agents pathogènes vivants atténués tel que le vaccin contre le virus de la poliomyélite, de la rougeole ou de l'influenza (Tableau 1.3).

Tableau 1.4 : Exemples de vaccins atténués, inactivés et sous-unitaires disponibles au Canada

(Compilation d'après l'Agence de la santé publique du Canada)

Type de vaccin	Pathogène (Maladie)	Nom commercial du vaccin	Composition du vaccin
Atténués	*Mycobacterium tuberculosis* (Tuberculose)	Bacille Calmette Guérin	Dérivé de *Mycobacterium bovis*
	Virus de l'influenza (Grippe)	FluMist®	Quadrivalent Vaporisation intranasale Souches adaptées au froid
	Poliovirus (Poliomyélite)	Imovax® Polio	Cultivé sur cellules Véro
	Virus de la rougeole (Rougeole)	Priorix®	Propagation sur des cellules d'embryons de poules
Inactivés	*Bordetella pertussis* *Bordetella parapertussis* *(*Coqueluche)	Adacel®	Vaccin acellulaire combiné aux antitoxines diphtériques et tétaniques
	Virus de l'influenza (Grippe)	Fluviral®	Virions propagés dans des œufs de poule embryonés
	Virus de l'hépatite A (Hépatite A)	Avaxim®	Cultivés sur des cellules diploïdes humaines Inactivation au formaldéhyde
	Virus de la rage (Rage)	Imovax®	Cultivé sur des cellules diploïdes humaines Inactivation au beta-propriolactone
Sous-unitaires	Virus du papillome humain (Verrues génitales, Cancers)	Cervarix®	Vaccin recombinant bivalent VPH 16 et 18 formant un VLP
	Virus de l'hépatite B (Hépatite B)	Recombivax® HB	Vaccin recombinant formant un VLP

2.1.2 Les vaccins inactivés

Introduits au 19e siècle, les vaccins inactivés sont fabriqués lorsque les agents pathogènes sont soumis à des traitements chimiques ou à de hautes températures (S. Plotkin, 2014). Les agents pathogènes ainsi inactivés ne détiennent plus de pouvoir de réplication mais la structure de l'agent pathogène doit être conservée afin de générer une réponse immunitaire efficace. Cela fait des vaccins inactivés une alternative plus sécuritaire aux vaccins vivants atténués bien que la réponse immunitaire élicitée soit plus faible. Afin d'obtenir une protection à long terme, une ou plusieurs doses de rappel sont donc nécessaires. Il faut aussi tenir compte du degré de protection généré par un vaccin inactivé, qui n'est parfois pas suffisant afin de protéger l'hôte. Afin d'améliorer la réponse immunitaire à un vaccin, des adjuvants sont souvent ajoutés à la formulation vaccinale. Les vaccins contre la coqueluche, le virus de l'influenza, le virus de l'hépatite A ainsi que le virus causant la rage sont tous des exemples de vaccins inactivés (Tableau 1.2).

2.1.3 Les vaccins sous-unitaires

Les vaccins sous-unitaires, qui constituent une technologie plus récente que les deux dernières classes de vaccins abordées, ne nécessitent pas l'utilisation de pathogènes entiers. En effet, seulement les antigènes ou les épitopes d'intérêts sont utilisés dans la formulation du vaccin et sont parfois couplés à du matériel génétique synthétique et sécuritaire. Les épitopes retrouvés à la surface des pseudoparticules virales (VLP) sont reconnus par le système immunitaire afin de monter une réponse protectrice contre l'agent pathogène exprimant ces épitopes. Une des caractéristiques importantes des VLP réside dans les possibilités de fusions en surface de la particule afin d'induire une réponse immunitaire contre plusieurs antigènes à la fois (Lebel *et al.*, 2015, Noad *et al.*, 2003). L'un des grands avantages de l'immunisation au moyen de VLP est la génération d'une réponse immunitaire humorale mais aussi une réponse immunitaire cellulaire protectrice (D. M. Smith *et al.*, 2013). Il existe présentement deux vaccins commerciaux composés de VLP soit le vaccin recombinant contre le virus de l'hépatite B ainsi que les vaccins bivalent, quadrivalent ou nonavalent contre le virus du papillome humain (VPH) (Tableau 1.2). Plusieurs autres sont présentement en phases cliniques.

2.2 **Article 1 : Plant viruses as nanoparticle-based vaccines and adjuvants**

Titre en français

Les vaccins et adjuvants à base de nanoparticules de virus de plantes

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Résumé en français

La vaccination est considérée comme un des plus grands développements dans la bataille contre les maladies infectieuses. Toutefois, le fait que plusieurs maladies, tel que l'hépatite C, le VIH/SIDA, la malaria, la tuberculose et le cancer, restent sans traitements pose problèmes car la vaccination traditionnelle s'est montrée inefficace dans leur prévention. Ces défis ont poussé l'émergence de nouvelles approches vaccinales, surtout dans l'élaboration de plateformes vaccinales et adjuvantes sécuritaires et efficaces. L'utilisation de nanoparticules de virus de plantes ainsi que de virus de plantes recombinants en tant que plateforme vaccinale a su gagner sa place en tant qu'outil dans le développement de nouveaux vaccins contre les maladies infectieuses ainsi que le cancer. Cette revue de littérature résume les avancées récentes dans l'utilisation de nanoparticules de virus de plantes ainsi que leurs mécanismes d'action. Exploiter les propriétés des nanoparticules de plantes permettra d'élaborer de nouvelles plateformes vaccinales qui sont sécuritaires et efficaces contre les maladies, que ce soit en contexte de vaccination prophylactique ou thérapeutique.

Abstract: Vaccines are considered one of the greatest medical achievements in the battle against infectious diseases. However, the intractability of various diseases such as hepatitis C, HIV/AIDS, malaria, tuberculosis, and cancer poses persistent hurdles given that traditional vaccine-development methods have proven to be ineffective; as such, these challenges have driven the emergence of novel vaccine design approaches. In this regard, much effort has been put into the development of new safe adjuvants and vaccine platforms. Of particular interest, the utilization of plant virus-like nanoparticles and recombinant plant viruses has gained increasing significance as an effective tool in the development of novel vaccines against infectious diseases and cancer. The present review summarizes recent advances in the use of plant viruses as nanoparticle-based vaccines and adjuvants and their mechanism of action. Harnessing plant-virus immunogenic properties will enable the design of novel, safe, and efficacious prophylactic and therapeutic vaccines against disease.

Keywords: recombinant plant virus; vaccine; adjuvant; immune response; production methods

2.2.1 Introduction

Although vaccines have allowed great achievements such as a significant reduction in incidence, mortality, and morbidity related to infectious diseases, the field of vaccinology has faced several challenges in recent years. Indeed, development of new vaccines has proven difficult especially against chronic infections or cancer. To overcome these obstacles, large research efforts are being devoted to better understand the key elements required to generate protective immunity. For example, while the majority of available vaccines mainly induce the generation of antibodies that neutralize targeted pathogens, it is now recognized that the cellular immune response is often necessary to protect against many infections. A balanced and complete immune response involving both humoral immunity, with high-affinity neutralizing antibodies limiting pathogen transmission and cellular immunity, with cytotoxic $CD8^+$ T cells killing infected cells, is in fact required to control malaria, hepatitis C, and HIV/AIDS, just to list a few (Barth *et al.*, 2011, de Souza, 2014, Holz *et al.*, 2015, Imai *et al.*, 2015, Pereyra *et al.*, 2014), highlighting the need for the development of new vaccine design approaches. Moreover, antigens and epitopes from several pathogens targeted by protective immune responses have recently been identified (Chakraborty *et al.*, 2014, Frosig *et al.*, 2015, Hafalla *et al.*, 2013, Linnemann *et al.*, 2015). This has led to the design of safer and more specific recombinant vaccines, although they are often less immunogenic than more traditional vaccines based on live/attenuated pathogens. Therefore, the choice of vaccine formulations and the addition of

suitable adjuvants will likely be required to achieve protective immunity with these novel vaccines. It is generally accepted that the best way to generate vaccines capable of generating a broad immune response with high levels of antibodies and cytotoxic T lymphocytes is to mimic a pathogenic infection while remaining as safe as possible. In this regard, virus-like nanoparticles (VLPs) have been increasingly studied in recent years with the aim of developing new effective vaccines.

2.2.2 Virus-Like Nanoparticles

VLPs mimic viruses by bearing similar protein composition while being non-infectious. In fact, most VLPs do not contain any viral genetic material while others contain nucleic acids that do not allow virus replication in mammals. For now, there are three vaccines composed of VLPs used in humans: the hepatitis B vaccine, the human papilloma vaccine, and the recently approved vaccine against hepatitis E in China (Riedmann, 2012). These generate robust humoral immune responses but still require the addition of an adjuvant to be fully effective. Other VLPs are currently in clinical trials (Clinicaltrials.gov, 2009, Clinicaltrials.gov, 2014a, Clinicaltrials.gov, 2014b) and many more are in development (J. H. Kim *et al.*, 2013, S. K. Kim *et al.*, 2015, Sun *et al.*, 2015). The increase in VLP use in the development of novel vaccines is related to their numerous qualities that meet new medical needs. For example, their highly ordered and repetitive structures facilitate recognition by the immune system and induce B-cell activation through B-cell receptor (BCR) cross-linking (Batista *et al.*, 2009). The size of VLPs is generally between 20 and 300 nm, which is ideal to be effectively recognized by dendritic cells (DCs) or other antigen-presenting cells (APC) (Bershteyn *et al.*, 2012, Fifis *et al.*, 2004, X. Li *et al.*, 2014, X. Li *et al.*, 2011). In addition, VLPs frequently display various Pathogen Associated Molecular Patterns (PAMPs) that are recognized by Pattern Recognition Receptors (PRRs), leading to the activation of the innate immune system and cytokine production such as type I interferon (IFN-I); notably, these cytokines were reported to increase the cross-presentation capacity of DCs and are beneficial towards cell-mediated, T-lymphocyte immune responses (Kolumam *et al.*, 2005, Le Bon *et al.*, 2006, Le Bon *et al.*, 2003). IFN-αβ can also increase the humoral response, induce isotype switching, and enhance the generation of T follicular helper cells (Cucak *et al.*, 2009, Le Bon *et al.*, 2001). Furthermore, many studies have shown the capacity of VLPs to induce a cellular immune response, therefore permitting the development of prophylactic and therapeutic vaccines targeting $CD8^+$ cytotoxic T lymphocyte (CTL) responses (Cubas *et al.*, 2009, Kammer *et al.*, 2007, Pinto *et al.*, 2006, Quan *et al.*, 2007). This strongly correlates with APC cross-presentation of antigens fused to VLPs to $CD8^+$ T cells through a Tap- and proteasome-independent pathway (Leclerc *et*

al., 2007, Ruedl *et al.*, 2002, Win *et al.*, 2011). This mechanism has also been shown to generate tumor-specific $CD8^+$ T-cell responses that efficiently slow down tumor growth and increase mice survival in various models (Cubas *et al.*, 2011, Peacey *et al.*, 2008, Speiser *et al.*, 2010). Importantly, non-infectious VLPs are safer than attenuated or inactivated viruses and could therefore potentially be administered to immunocompromised individuals. Finally, the use of VLPs could be advantageous for developing vaccines against viruses that are impossible or difficult to grow in culture such as the human norovirus, for example (Ma *et al.*, 2011). Altogether, these properties contribute to the effectiveness and usefulness of VLP vaccines.

2.2.3 Recombinant Plant Virus Particles (rPVPs)

Recombinant plant virus particles are increasingly studied as candidate vaccines. They can be classified as a subtype of VLP since they are viral particles that are non-infectious in mammals. However, most rPVPs retain their replication potential in plants, posing additional safety and environmental challenges. Nonetheless, rPVPs possess all of the advantages of VLPs and thus would allow the development of effective vaccines. Many plant viruses such as the tobacco mosaic virus (TMV), cowpea mosaic virus (CPMV), potato virus X (PVX), alfalfa mosaic virus (AlMV), and papaya mosaic virus (PapMV) (see Table 1 for more information) are currently used for the development of new vaccines. Typically, plant viruses are simple, rod shaped, or bearing icosahedral symmetry; they are composed of one or two repeated coat protein (CP) subunits and bear an RNA genome. These viruses are relatively easy to engineer, produced at low cost, and very stable, enabling storage at room temperature, which is desirable for vaccination in developing countries. CP modification of plant viruses allows dense expression of fused antigens, thereby contributing to the development of an effective immune response. Finally, while other virus platforms, such as adenovirus, suffer from platform-specific antibody development (Fausther-Bovendo *et al.*, 2014, Zaiss *et al.*, 2009), rPVPs seem to maintain their immunogenic properties in the presence of specific antibodies (Kemnade *et al.*, 2014, Mallajosyula *et al.*, 2014). In the following paragraphs, we will discuss the different methods of production of rPVPs for vaccination, the diverse approaches for antigen expression on their surface, and achievements made with rPVPs.

Table 1.5 Recombinant plant virus particles used in vaccine development.

Virus	Shape	Parameters Tested	Element of Response	References
CPMV	Icosahedral	Humoral response	Binding of neutralizing antibodies	(Brennan *et al.*, 1999a, Brennan *et al.*, 1999b, Brennan *et al.*, 1999d, Dalsgaard *et al.*, 1997, Durrani *et al.*, 1998, Kaltgrad *et al.*, 2007, Langeveld *et al.*, 2001, McInerney *et al.*, 1999, Miermont *et al.*, 2008, Nicholas *et al.*, 2003, Nicholas *et al.*, 2002, Rennermalm *et al.*, 2001, Taylor *et al.*, 2000)
			Protection against challenge	(Brennan *et al.*, 1999b, Dalsgaard *et al.*, 1997, Langeveld *et al.*, 2001, McInerney *et al.*, 1999)
		Cellular response	IFN- γ production	(Nicholas *et al.*, 2003, Nicholas *et al.*, 2002)
		Immunomodulation	APC activation	(Gonzalez *et al.*, 2009)
PVX	Rod	Humoral response	Binding of neutralizing antibodies	(Jobsri *et al.*, 2015, Lico *et al.*, 2009, Marconi *et al.*, 2006, Marusic *et al.*, 2001, Uhde-Holzem *et al.*, 2010)
			Protection against challenge	(Jobsri *et al.*, 2015)
		Cellular response	$CD8^+$ T cells activation	(Lico *et al.*, 2009, Massa *et al.*, 2008)
			IFN- γ production	(Lico *et al.*, 2009, Massa *et al.*, 2008)
			Protection against challenge	(Lico *et al.*, 2009, Massa *et al.*, 2008)

Table 1.5 Cont

Virus	Shape	Parameters Tested	Element of Response	References
		Immunomodulation	APC activation	(Jobsri *et al.*, 2015, Marusic *et al.*, 2001)
			Cytokine production	(Jobsri *et al.*, 2015)
TMV	Rod	Humoral response	Binding or neutralizing antibodies	(Jiang *et al.*, 2006, Mallajosyula *et al.*, 2014, Massa *et al.*, 2008, M. L. Smith *et al.*, 2006)
			Protection against challenge	(Jiang *et al.*, 2006, Koo *et al.*, 1999, Mallajosyula *et al.*, 2014, Palmer *et al.*, 2006, Staczek *et al.*, 2000)
		Cellular response	$CD8^+$ T cells activation	(Kemnade *et al.*, 2014, McCormick *et al.*, 2006a, McCormick *et al.*, 2006b)
			IFN-γ production	(McCormick *et al.*, 2006a)
			Protection against challenge	(McCormick *et al.*, 2006a)
		Immunomodulation	APC activation	(Kemnade *et al.*, 2014, McCormick *et al.*, 2006a)
CMV	Icosahedral	Humoral response	Binding or neutralizing antibodies	(Piazzolla *et al.*, 2005)
		Cellular response	$CD8^+$ T cells activation	(Piazzolla *et al.*, 2005)
			IFN-γ production	(Nuzzaci *et al.*, 2007, Piazzolla *et al.*, 2005)

Table 1.5 Cont

Virus	Shape	Parameters Tested	Element of Response	References
AIMV	Icosahedral	Humoral response	Binding or neutralizing antibodies	(Jones *et al.*, 2013, Yusibov *et al.*, 2005)
		Cellular response	IFN-γ production	(Yusibov *et al.*, 2005)
PapMV	Rod	Humoral response	Binding or neutralizing antibodies	(Babin *et al.*, 2013, Denis *et al.*, 2008, Denis *et al.*, 2007)
		Cellular response	$CD8^+$ T cells activation	(Hanafi *et al.*, 2010, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007)
			IFN-γ production	(Babin *et al.*, 2013, Hanafi *et al.*, 2010, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007)
			Protection against challenge	(Lacasse *et al.*, 2008)
		Immunomodulation	APC activation	(Mathieu *et al.*, 2013)
			Cytokine production	(Mathieu *et al.*, 2013)
			Protection against challenge	(Mathieu *et al.*, 2013)
		Adjuvant	APC activation	(Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014)
			Cytokine production	(Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014)
			Vaccine jointly administered	PapMV-M2e (Denis *et al.*, 2008), BMDC-OVA (Savard *et al.*, 2011), Outer Membrane Protein C (Acosta-Ramirez *et al.*, 2008), TIV (Savard *et al.*, 2011)

Table 1.5 Cont

Virus	Shape	Parameters Tested	Element of Response	References
			Vaccine jointly administered	PapMV-M2e (Denis *et al.*, 2008), BMDC-OVA (Savard *et al.*, 2011), Outer Membrane Protein C (Acosta-Ramirez *et al.*, 2008), TIV (Savard *et al.*, 2011),
BaMV	Rod	Humoral response	Binding of neutralizing antibodies	(T. H. Chen *et al.*, 2012, Yang *et al.*, 2007)
			Protection against challenge	(T. H. Chen *et al.*, 2012, Yang *et al.*, 2007)
		Cellular response	IFN- γ production	(Yang *et al.*, 2007)
			Protection against challenge	(Yang *et al.*, 2007)
TBSV	Icosahedral	Humoral response	Binding of neutralizing antibodies	(Joelson *et al.*, 1997)
Plum pox potyvirus	Rod	Humoral response	Binding of neutralizing antibodies	(Fernandez-Fernandez *et al.*, 1998, Fernandez-Fernandez *et al.*, 2002)

CMV: cucumber mosaic virus; BaMV: bamboo mosaic virus; TBSV: tomato bushy stunt virus.

2.2.4 Production Methods

rPVP production can be achieved by several methods. Production of rPVPs using plants, such as *Nicotiana benthamiana*, *Nicotiana tabacum*, or cowpea plants, is the most widely used and can be divided into two different strategies, both starting with the production of the desired cDNA. The cDNA can then be transcribed *in vitro* and used to inoculate plants depositing the RNA transcript on abraded leaves to induce a systemic infection (Palmer *et al.*, 2006). Another option is to transform *Agrobacterium tumefaciens* with a plasmid containing the cDNA coding for the modified viral genome and then agroinfiltrate plants to induce transient expression and generate recombinant viruses (Petukhova *et al.*, 2014). A week or two later, recombinant viruses are then purified by different techniques (Jones *et al.*, 2013, Koo *et al.*, 1999, M. L. Smith *et al.*, 2006). Remarkably, due to the possibility of CMV production in edible vegetables, e.g., celery, lettuce, cucumber, tomato, carrot, pepper, and banana (Natilla *et al.*, 2004), plant production also advances the use of oral delivery of vaccine via ingestible plants (Arakawa *et al.*, 1998, Thanavala *et al.*, 2005). This strategy would therefore reduce costs related to virus purification and eliminate the need for sterile needles and trained medical staff for vaccine administration. rPVP manufacturing *in planta* also allows for large-scale production devoid of contamination risks from human pathogens. However, a weakness of oral administration of rPVPs is related to the difficulty of controlling the amount of antigen taken by the patient and the potential development of tolerance to the antigen. In addition, genetic modifications that induce foreign antigen expression on plant virus proteins can sometimes affect viral replication, thus reducing production efficiency (Natilla *et al.*, 2004). A new method based on transgenic plant cell suspension cultures was recently developed. This process, based on the culture of calli derived from transgenic plant lines expressing viral cDNA, allows for continuous production of large amounts of rPVPs with high reproducibility (Muthamilselvan *et al.*, 2015). More conventional techniques are also used to produce rPVPs such as bacteria (Mathieu *et al.*, 2013, Plchova *et al.*, 2011), yeast (Brumfield *et al.*, 2004, Kadri *et al.*, 2013), and insect cell cultures (P. Kumar *et al.*, 2009a, Saunders *et al.*, 2009). Plants, bacteria, and yeast are all simple and low cost manufacturing methods. However, bacteria and yeast sometimes produce insoluble proteins, thus restricting particle self-assembly (Brumfield *et al.*, 2004, Mueller *et al.*, 2010). The less practical and more costly baculovirus expression system seems to avoid these problems (Brumfield *et al.*, 2004, P. Kumar *et al.*, 2009a, Saunders *et al.*, 2009). When capsid protein production does not induce particle formation, it is also possible to perform the assembly process *in vitro*, with or without the addition of nucleic acids (Arkhipenko *et al.*, 2011, Mathieu *et al.*, 2013, Mueller *et al.*, 2010, Tyulkina *et al.*, 2011). Although

this process is more laborious, it allows for better control of the RNA present inside the particle since diverse and uncontrolled sources of RNA can be incorporated in self-assembled viral particles *in vivo* (Brumfield *et al.*, 2004, Kadri *et al.*, 2013, P. Kumar *et al.*, 2009a). In addition, *in vitro* assembly allows for the packing of specific RNA transcripts in recombinant plant virus capsid proteins that will further induce *in vivo* gene expression (M. L. Smith *et al.*, 2007). Atabekov and colleagues generated spherical nanoparticles using thermal denaturation of the TMV CP protein (Atabekov *et al.*, 2011). These particles are devoid of RNA and can bind different proteins or peptides, making it a universal and immunogenic particle platform (Karpova *et al.*, 2012). Therefore, these different manufacturing processes generate rPVPs that either contain no RNA, host RNA, viral RNA, or inactive or replicative synthetic RNA. Plant virus particles are most probably safe enough for administration in humans, but many are still infectious in plants. Therefore, inactivation methods based on chemical treatment or UV irradiation, for example, were developed to ensure that rPVPs are innocuous (Langeveld *et al.*, 2001, Phelps *et al.*, 2007, Rae *et al.*, 2005). Finally, methods using eukaryotic cells have the advantage of allowing post-transcriptional modifications ensuring that the rPVP is more similar to the parental virus and more stable (Brumfield *et al.*, 2004, Kadri *et al.*, 2013). In summary several manufacturing processes have been developed to efficiently produce the desired rPVP, each with their advantages and drawbacks.

2.2.5 Antigen Expression on rPVPs

Several processes lead to the expression of foreign antigens on rPVPs. The most commonly used are molecular cloning techniques to fuse sequences coding for the antigen directly within the CP gene construct. In the case of icosahedral viruses such as CMV, CPMV, and AlMV, localization within sequences exposed on the viral surface as well as sites accepting peptide fusions have been well studied (Natilla *et al.*, 2004, Vitti *et al.*, 2010). In general, insertions of 10–50 amino acids are well tolerated and are structured in closed loops exposed on the surface of the virus. For example, most successful fusions with CPMV were achieved by inserting epitopes within loops between amino acids 22 and 23 of the S protein (Brennan *et al.*, 1999d, Langeveld *et al.*, 2001, Nicholas *et al.*, 2002). Nevertheless, others have managed to obtain stable particles using N-terminal or C-terminal fusions with AlMV and TBSV, respectively, even if the N-terminal region is known to be important for particle formation for some icosahedral viruses (Jones *et al.*, 2013, S. Kumar *et al.*, 2009b). However, in some cases, only 20%–30% of rPVP CPs express the fused antigen (Jones *et al.*, 2013). In the case of rod-shaped viruses, *N*-terminal and C-terminal fusions are the most commonly used (Babin *et al.*, 2013, Lacasse *et al.*, 2008, Lico *et al.*, 2009, Marusic *et al.*,

2001, Palmer *et al.*, 2006). This allows surface expression without causing destabilization of the structure. In addition, specific and well-defined sites in the CP sequence, other than the C- and N-termini, were also shown to accept fusion without destabilization while allowing surface expression of fused antigens and recognition by the immune system (Petukhova *et al.*, 2013, Rioux *et al.*, 2012b, Wu *et al.*, 2003). However, the tolerated size of peptides fused to rod-shaped viruses is usually more restricted (Bendahmane *et al.*, 1999, Uhde-Holzem *et al.*, 2010). Therefore, in order to fuse bigger peptides or complete proteins, fusion processes other than molecular cloning have been developed. One such technique consists in biotinylating the CP to attach streptavidin-linked proteins or peptides (Jobsri *et al.*, 2015, M. L. Smith *et al.*, 2006). Others also incorporate a reactive lysine in the sequence of the CP to chemically conjugate peptides using a heterobifunctional linker (McCormick *et al.*, 2006a, McCormick *et al.*, 2006b, M. L. Smith *et al.*, 2007) or perform copper catalyzed azide-alkine cycloaddition to covalently link antigens to the viral capsid (Kaltgrad *et al.*, 2007, Yin *et al.*, 2012). Spontaneous conjugation due to electrostatic and/or hydrophobic interactions between foreign antigens and viral CP can also happen and are further stabilized by formaldehyde treatment (Atabekov *et al.*, 2011, Karpova *et al.*, 2012). However, antigen insertion may potentially induce viral particle destabilization (Bendahmane *et al.*, 1999, Nuzzaci *et al.*, 2007, Porta *et al.*, 2003, Rioux *et al.*, 2012a). Apart from the size and the localization of the peptide, its charge and isoelectric point can also impact the particles' capacity to assemble (Bendahmane *et al.*, 1999, Porta *et al.*, 2003, Uhde-Holzem *et al.*, 2007). In some cases, the generation of mixed particles allows for the formation of stable rPVPs (Jobsri *et al.*, 2015). For example, in order to avoid interference with particle assembly, the insertion of the foot-and-mouth disease virus 2A peptide in the cDNA construct of PVX benefits from a ribosomal skipping process to produce mixed particles made of recombinant and wild-type CPs (Marconi *et al.*, 2006, Uhde-Holzem *et al.*, 2010). Finally, the localization of conjugated antigens may also affect the ability to elicit an immune response against fused antigens by influencing the accessibility and conformation of the antigen and thus the immunogenicity of the rPVP (Rioux *et al.*, 2012a, Taylor *et al.*, 2000, Yin *et al.*, 2012). In summary, with new techniques developed and a better comprehension of factors influencing the stability and immunogenicity of rPVPs, we are better equipped to generate effective vaccines and it is now possible to conjugate very large peptides (Marconi *et al.*, 2006, Werner *et al.*, 2006) or even complete protein antigens to such particles (M. L. Smith *et al.*, 2006).

2.2.6 rPVPs as Vaccines to Induce Humoral Immune Responses

Most of the currently used vaccines induce a humoral response upon administration, which will then protect individuals against infection or the appearance of disease (reviewed in (S. A. Plotkin, 2008)). As it is the case for current vaccines, plant viruses used as vaccine platforms are also able to trigger the production of antibodies. Not only are these platforms able to induce IgGs, mainly found in serum after sub-cutaneous, intra-peritoneal, and to some extent intra-nasal injections, IgAs are also found in mucosa after intra-nasal and oral administration (Brennan *et al.*, 1999a, Durrani *et al.*, 1998, Marusic *et al.*, 2001, Nicholas *et al.*, 2003, Nicholas *et al.*, 2002). Plant viruses used as carriers for foreign epitopes can therefore efficiently induce the production of both systemic and mucosal antibodies following administration by various routes, which broadens their potential targets since not all pathogens will require the same antibody response to be cleared from the host. The structure of epitopes presented on the surface of rPVPs will depend on their localization and size. When in an optimal localization, epitopes will adopt a structure similar to their native conformation, allowing antibodies to recognize the virus or bacteria against which they were mounted (Marconi *et al.*, 2006, Taylor *et al.*, 2000). As such, antibodies from HCV-infected patients were able to recognize a CMV engineered virus expressing the R9 peptide from HCV (Nuzzaci *et al.*, 2007), even though these patients were never exposed to the rPVP. rPVP-based experimental vaccines were also shown to be as effective or even more effective at inducing an antibody response compared to commercially available vaccines (T. H. Chen *et al.*, 2012), peptides alone (Babin *et al.*, 2013, Denis *et al.*, 2008, Denis *et al.*, 2007, Massa *et al.*, 2008, M. L. Smith *et al.*, 2006), or peptides conjugated to keyhole limpet haemocyanin (KLH) (Langeveld *et al.*, 2001, Nicholas *et al.*, 2002). Moreover, these vaccines are often administered in conjunction with less toxic adjuvants, compared to Freund's Complete Adjuvant (which is not approved for human use), like QS-21 and QuilA (saponin-based) or RIBI (McInerney *et al.*, 1999, Palmer *et al.*, 2006, Rennermalm *et al.*, 2001), or even without the use of any adjuvant (Brennan *et al.*, 1999a, Lico *et al.*, 2009, Marusic *et al.*, 2001). Plant viruses also often require less peptide to induce efficient antibody responses compared to commercially available vaccines or peptides conjugated to KLH (Brennan *et al.*, 1999d, Mallajosyula *et al.*, 2014).

Immunization in different experimental animal models not only generated specific antibodies but also protected against challenge with various viruses, bacteria, or tumor cell lines when epitopes were presented on CPMV (Brennan *et al.*, 1999b, Dalsgaard *et al.*, 1997, Khor *et al.*, 2002, Langeveld *et al.*, 2001, Rennermalm *et al.*, 2001), PVX (Jobsri *et al.*, 2015, Massa *et al.*, 2008), Bamboo Mosaic Virus (BaMV) (T. H. Chen *et al.*, 2012, Yang *et al.*, 2007), or TMV (Jiang *et al.*, 2006, Koo *et al.*, 1999,

Mallajosyula *et al.*, 2014, Palmer *et al.*, 2006, Staczek *et al.*, 2000). Protection against challenge (sometimes lethal) is thought to be partially achieved by neutralizing antibodies, generated following immunization with engineered CPMV (Fernandez-Fernandez *et al.*, 1998, Langeveld *et al.*, 2001, Marusic *et al.*, 2001, McInerney *et al.*, 1999, Nicholas *et al.*, 2002), PVX (Marusic *et al.*, 2001), and Plum Pox Potyvirus (Fernandez-Fernandez *et al.*, 1998). This protection can be broadened to various strains and species of the same pathogen using conserved epitopes, which are usually weakly immunogenic. When such epitopes are presented on the surface of rPVPs, their immunogenicity is increased, allowing for the production of a more effective antibody response and therefore a broader protection of the host (Mallajosyula *et al.*, 2014, Miermont *et al.*, 2008, Yin *et al.*, 2012). This has been demonstrated for peptides 10 and 18 of the outer membrane protein F of *Pseudomonas aeruginosa* presented at the surface of CPMV (Brennan *et al.*, 1999b, Brennan *et al.*, 1999c), as well as for the R9 peptide of HCV presented on PVX (Uhde-Holzem *et al.*, 2010). This broad recognition was also observed when mannose was engineered to be displayed on CPMV inducing antibodies interacting with various of its analogues and derivatives (Kaltgrad *et al.*, 2007).

Part of the mechanism by which plant virus carriers induce the production of antibodies is hypothesized to proceed through cross-linking of the BCR (Miermont *et al.*, 2008). The presentation of many epitopes in close proximity might favor such cross-linking, leading to the proliferation of B lymphocytes, presentation of epitopes to T cells, and differentiation of B lymphocytes into antibody-producing plasma cells(Batista *et al.*, 2009). In agreement with this, Nicholas *et al.* demonstrated that higher antigen expression on the surface of CPMV particles provided a better immune response (Nicholas *et al.*, 2002). It also explains why viral platforms seem more efficient at inducing antibodies than peptide-KLH conjugates, for example (Langeveld *et al.*, 2001, Nicholas *et al.*, 2002).

2.2.7 rPVPs as Vaccines to Induce Cellular Immune Responses

Even though antibodies generated following vaccination are often sufficient to protect against some diseases, elicitation of both cellular and humoral adaptive immune responses is sometimes necessary for protection (reviewed in (S. A. Plotkin, 2008)). The use of plant viruses as epitope carriers has proven effective in inducing cellular immune responses directed towards antigens presented on the particle. We have demonstrated that epitopes fused to the PapMV platform were able to induce the activation of $CD8^+$ T cells by a, cross-presentation pathway (Hanafi *et al.*, 2010, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007). Other rPVPs, such as TMV (Kemnade *et al.*, 2014,

McCormick *et al.*, 2006a, McCormick *et al.*, 2006b), CMV (Piazzolla *et al.*, 2005), CPMV (McInerney *et al.*, 1999) and PVX (Lico *et al.*, 2009, Massa *et al.*, 2008) were also shown to generate and enhance $CD8^+$ T cell-mediated immune responses against fused epitopes. The main correlate for induction of effective T-cell responses seems to be production of IFN-γ. IFN-γ production was observed when peripheral blood mononuclear cells (PBMCs) from healthy patients or patients infected with HCV were incubated with AlMV expressing a respiratory syncytial virus epitope (Yusibov *et al.*, 2005) or CMV expressing the R9 epitope from HCV (Nuzzaci *et al.*, 2007, Piazzolla *et al.*, 2005), both demonstrating that human PBMCs can be activated by rPVPs, while the latter also demonstrates that the epitope is efficiently processed and presented, leading to the potent activation of $CD8^+$ T cells (*i.e.*, IFN-γ production). Cellular immune responses also correlated with protection against challenge with tumor cell lines B16-OVA and Eg.7-OVA (McCormick *et al.*, 2006a), Lymphocytic Choriomeningitis Virus (Lacasse *et al.*, 2008), and Foot and Mouth Disease Virus (Joelson *et al.*, 1997).

2.2.8 rPVPs Used as Immunomodulators and Adjuvants

In order to generate an effective immune response, APCs must be activated and present antigens to cells of the adaptive immune system (Storni *et al.*, 2002). As previously mentioned, rPVPs possess many suitable characteristics for them to be taken up by APCs, processed, and presented to T cells. Indeed, DCs were shown to be activated following TMV (Kemnade *et al.*, 2014, McCormick *et al.*, 2006a), PVX (Jobsri *et al.*, 2015, Marusic *et al.*, 2001), CPMV (Gonzalez *et al.*, 2009), and PapMV (Acosta-Ramirez *et al.*, 2008, Lacasse *et al.*, 2008, Lebel *et al.*, 2014) immunization. Activated DCs upregulate various co-stimulatory molecules such as CD40, CD86, CD80, MHCII, MHCI, and CCR7 (Jobsri *et al.*, 2015, Lebel *et al.*, 2014) and also produce pro-inflammatory cytokines such as IL-12, TNF-α, IL-6, and IFN-α (Acosta-Ramirez *et al.*, 2008, Jobsri *et al.*, 2015, Lebel *et al.*, 2014, Mathieu *et al.*, 2013, Savard *et al.*, 2011). DCs are not the only cell population that was shown to be activated following immunization with rPVPs since B lymphocytes, macrophages, and NK cells can also upregulate activation markers following rPVP encounter (Acosta-Ramirez *et al.*, 2008, Gonzalez *et al.*, 2009).

Since rPVPs can be efficiently taken up by APCs and induce their activation, they can potentially be used as adjuvants to enhance the effectiveness of concomitantly administered vaccines. We have indeed demonstrated that PapMV can be used as an adjuvant in combination with various types of vaccines. When PapMV was administered jointly with bone marrow-derived DCs presenting OVA, cellular immune responses towards OVA were enhanced, leading to better

protection against a *Listeria monocytogenes*-OVA challenge in mice (Lebel *et al.*, 2014). Similar protection was observed when PapMV was administered in mice in concert with the seasonal trivalent influenza vaccine (TIV) (Savard *et al.*, 2011). When used as an adjuvant, PapMV was also shown to enhance the production of antibodies directed against TIV (Acosta-Ramirez *et al.*, 2008, Denis *et al.*, 2008, Savard *et al.*, 2011). PapMV has also been shown to be able to prime the immune system and protect mice from an influenza infection on its own, without being fused to an epitope or being administered with a separate vaccine (Mathieu *et al.*, 2013). Finally, it was shown to induce a broader immune response against TIV antigens, providing protection against strains of influenza not contained within the vaccine (Savard *et al.*, 2011). To our knowledge, PapMV is the first rPVP used as an adjuvant or an immunomodulator specifically to prime the immune system.

The mechanisms by which rPVPs activate APCs are slowly being uncovered. We have identified one such mechanism in the PapMV system as being the recognition of nucleic acid found within rPVPs that induces APC activation. Single-stranded RNA (ssRNA) within PapMV particles is in fact recognized by TLR7 in endosomes of APCs, mainly plasmacytoid DCs, leading to the production of IFN-α (Jobsri *et al.*, 2015, Lebel *et al.*, 2014). The lack of activation of murine splenocytes upon administration of PapMV monomers further illustrates the importance of ssRNA for its adjuvancy properties(Lebel *et al.*, 2014). Of note, despite RNA serving as the major immunomodulatory molecule following PapMV administration, the use of RNA alone cannot replace rPVPs since it would be rapidly degraded following administration due to the abundance of host RNAses found within the blood or other bodily fluids. The viral capsid therefore provides protection from degradation to the RNA molecule, allowing it to be efficiently delivered to endosomes. Plant virus particles thus carry their adjuvant properties inside a protective capsid (Acosta-Ramirez *et al.*, 2008), explaining their versatility both as vaccines and adjuvants.

2.2.9 Conclusions and Perspectives for rPVPs

Although there is still much research to be done before rPVPs are used as vaccines in humans, great achievements have been made in recent years in this field. Several production and antigen expression methods have been developed and improved, thus leading to the generation of many promising candidate vaccines. Beyond demonstrating that rPVPs are effective platforms to generate both humoral and cellular immune responses against fused antigens, we now know that rPVPs are efficiently recognized by the immune system of mammals, which efficiently activates the innate immune system. Therefore, rPVPs possess attractive intrinsic adjuvant properties that

can be used for immunomodulatory purposes. This has important implications for future vaccine design and opens the door for new applications. A strong testimony to the recent achievements made in using rPVPs as novel vaccines is the recent entry of two rPVPs in clinical trials, PapMV as an adjuvant for the influenza vaccine (Clinicaltrials.gov, 2014d) and AIMV as a vaccine against malaria (Clinicaltrials.gov, 2013). In light of this, it seems very likely that in the near future plant viruses will be used in humans to address unmet medical needs as prophylactic and therapeutic vaccines and immunomodulators against infection or cancer.

2.3 La vaccination thérapeutique

Dans les dernières années, la vaccination thérapeutique a pris de l'importance dans le traitement d'infections virales ainsi que dans le traitement du cancer. Même si les recherches sont de plus en plus nombreuses, très peu de traitements par la vaccination sont présentement approuvés. Plusieurs défis persistent dans l'élaboration de vaccins dirigés contre des agents pathogènes en constante évolution, notamment le choix d'épitopes et d'antigènes contre lesquels une réponse immunitaire doit être montée.

2.3.1 Traitement d'infections virales

L'administration d'IFN-α a longtemps été la thérapie de choix pour le traitement d'infections virales chroniques. Cependant, les signes de toxicité associés à cette plateforme de traitement limitent son utilisation (Sleijfer *et al.*, 2005). Ces signes d'inconfort varient, allant de la nausée aux maux de tête et ressemblent aux symptômes associés au virus de l'influenza (Sleijfer *et al.*, 2005). Ces inconforts démontrent bien l'équilibre précaire qui existe avec l'IFN-α. En effet, il a été démontré que l'IFN-α est bénéfique à la protection de l'hôte très tôt lors de l'infection (Cervantes-Barragan *et al.*, 2007, Sandler *et al.*, 2014, Smit *et al.*, 2006, Sullivan *et al.*, 2015, Swiecki *et al.*, 2015) alors que sa présence dans la phase plus tardive voire chronique de l'infection est dommageable à l'hôte (Sandler *et al.*, 2014, Teijaro *et al.*, 2013, Wilson *et al.*, 2013). Le traitement d'infections chroniques par l'administration d'IFN-α exogène est possible mais la dualité de l'IFN-α doit être bien exploitée. Dans un modèle murin d'infection chronique analogue au virus de l'hépatite C chez l'humain, soit le virus de la chorioméningite lymphocytaire (LCMV), le traitement de l'infection par des injections d'IFN-α est efficace dans la réduction des titres viraux et l'activation des cellules immunitaires (Y. Wang *et al.*, 2012) mais la fenêtre de traitement est très

étroite. Si cette fenêtre n'est pas respectée, l'IFN-α administré pourrait favoriser le pathogène au détriment de l'hôte.

L'utilisation de la vaccination thérapeutique pour le traitement d'infections chroniques nécessite encore beaucoup de travail. Certains traitements actuels contre des infections chroniques virales consistent à administrer des molécules synthétiques antivirales affectant le virus lui-même plutôt que d'activer le système immunitaire et ainsi l'aider à se défendre contre l'agent pathogène. Le traitement doit donc être répété à plusieurs reprises afin d'assurer l'élimination du pathogène lorsque cela est possible. Au contraire des traitements à base d'antiviraux, la vaccination thérapeutique est plus accessible car elle ne nécessite pas l'adhérence à un régime strict et quotidien de prise de médicaments. Plusieurs candidats sont présentement à l'étude pour le traitement de différentes infections chroniques. Le vaccin Dermavir, qui est le premier vaccin en phase clinique pour le traitement du VIH, est composé d'un plasmide d'ADN contenant 15 antigènes du VIH, permettant ainsi de monter une réponse immunitaire à large spectre (Rodriguez *et al.*, 2013). Ce vaccin est administré par voie topicale suite à une exfoliation de la peau afin de cibler les cellules de Langerhans, qui vont livrer les antigènes aux organes lymphoïdes (Lisziewicz *et al.*, 2005). Les résultats de la phase clinique ont démontré que la charge d'ARN viral demeure sous les limites de détection suite au traitement de patients avec 3 différentes doses du vaccin (Rodriguez *et al.*, 2013). Un autre vaccin, pour le traitement du VHS-2, est composé d'un plasmide d'ADN bivalent et est présentement en phase clinique II (Vical, 2017). La preuve de concept, effectuée chez le cobaye, a démontré que l'utilisation de ce vaccin en combinaison avec des liposomes comme adjuvant (Vaxfectin®) réduit les épisodes récurrents de réapparition et de libération du virus sans toutefois affecter la quantité d'ADN détectée à chaque épisode (Veselenak *et al.*, 2012).

2.3.2 Immunothérapies du cancer

La vaccination thérapeutique connaît un meilleur succès chez les immunothérapies du cancer. Les traitements conventionnels contre le cancer tel que la chimiothérapie et la radiothérapie visent à détruire les cellules cancéreuses directement mais peuvent aussi atteindre des cellules saines dans les environs. Ces traitements causent plusieurs inconforts chez le patient et sont donc difficiles à tolérer. Les immunothérapies cherchent plutôt à éduquer le système immunitaire du patient afin que ce dernier soit plus efficace dans son combat contre le cancer, limitant ainsi les effets secondaires. L'utilisation d'anticorps monoclonaux, d'inhibiteurs de points de contrôle, la thérapie cellulaire ainsi que l'utilisation de molécules immunomodulatrices sont toutes des

technologies présentement à l'étude ou approuvées dans le traitement du cancer chez l'humain (Illidge, 2015, Rini, 2014, Sharma *et al.*, 2015). Parmi les molécules immunomodulatrices approuvées, on retrouve deux ligands de TLR utilisés à titre de molécule active ou comme adjuvant ; le bacille Calmette-Guérin (BCG) est utilisé dans le traitement du cancer de la vessie et cible les TLR2, TLR3, TLR4 ainsi que le TLR9 alors que l'imiquimod, qui est un ligand du TLR7, est utilisé dans une crème topique contre le mélanome et les condylomes (Kaczanowska *et al.*, 2013). Parmi les traitements à l'étude, on note des ligands de TLR4 tel que le LPS (Stier *et al.*, 2013) et la HSP90 de *Mycobacterium tuberculosis* (Vo et al., 2015) ainsi que des ligands synthétiques du TLR7 tel que le R848 (Bourquin *et al.*, 2011, Stier *et al.*, 2013), le DSR 6434 (Koga-Yamakawa *et al.*, 2015) ainsi que le DSR 29133 (Dovedi *et al.*, 2016). Toutes ces molécules ont démontré la capacité de diminuer la croissance tumorale chez la souris lorsqu'utilisées en traitement principal. Les ligands de TLR peuvent aussi être utilisé comme adjuvant afin d'améliorer l'effet antitumoral de la plateforme vaccinale à laquelle ils sont couplés. Par exemple, la combinaison de ligands de TLR à des cellules dendritiques dérivées de la moelle osseuse (BMDC) (Lebel *et al.*, 2016a, Vo *et al.*, 2015), de la radiation fractionnée (Dovedi *et al.*, 2016) ou à d'autres ligands de TLR (Ayari *et al.*, 2016, Stier *et al.*, 2013) permet d'améliorer l'effet antitumoral de la plateforme. Une fois en phase clinique, les résultats sont moins prometteurs. Par exemple, l'utilisation d'un ligand de TLR7 dans le traitement du cancer engendre des effets secondaires marqués (Dudek *et al.*, 2007, Dummer *et al.*, 2008, Geller *et al.*, 2010, Weigel *et al.*, 2012) ainsi qu'un tolérance limitée aux traitements (Sauder *et al.*, 2003a). Une stabilisation de la maladie est observée suite au traitement (Dudek *et al.*, 2007, Dummer *et al.*, 2008, Geller *et al.*, 2010) bien que seulement une fraction des patients le démontre. Ces difficultés soulèvent donc l'importance de développer de nouveaux ligands de TLR ou tout simplement d'améliorer l'efficacité des ligands présentement disponibles.

3 LA NANOPARTICULE DU VIRUS DE LA MOSAIQUE DE LA PAPAYE (PAPMV)

Le développement de nouvelles technologies en vaccination amène de nouvelles plateformes de plus en plus polyvalentes provenant de sources diverses et non traditionnelles. Les nanoparticules du virus de la mosaïque de la papaye (PapMV) sous forme de VLP en sont un exemple. Notre équipe étudie présentement leur rôle potentiel comme adjuvant ou comme plateforme vaccinale dans différents modèles d'infections et de maladies.

3.1 **Caractéristiques**

Les nanoparticules de PapMV, qui miment le virus de la mosaïque de la papaye, ont fait l'objet de plusieurs études depuis les dernières années. Ces études ont permis de caractériser les propriétés physiques des nanoparticules du PapMV ainsi que ses propriétés immunomodulatrices, qui seront abordées dans cette section.

3.1.1 Caractéristiques physiques

Le virus de la mosaïque de la papaye est un virus de forme hélicoïdale au centre duquel on retrouve un ARN simple brin. Afin de produire des nanoparticules du virus de la mosaïque de la papaye (PapMV) qui sont sécuritaires pour l'humain et la souris, les protéines recombinantes de la capside sont produites *in vitro* chez *Escherichia coli* puis assemblées autour d'un brin d'ARN synthétique non codant et donc non infectieux (Mathieu *et al.*, 2013). La taille moyenne des nanoparticules du PapMV est de 90 µm en longueur et 15 µm en largeur (Mathieu *et al.*, 2013). Dû à sa structure en hélice, il est possible de fusionner des épitopes d'intérêt à la surface du PapMV sans affecter sa structure (Babin *et al.*, 2013, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007, Rioux *et al.*, 2012a). Les épitopes fusionnés en surface du PapMV permettent d'établir une réponse immunitaire à large spectre contre plus d'un épitope à la fois.

3.1.2 Capacités immunomodulatrices

Nos recherches ont démontré que le PapMV est bien reconnu par le système immunitaire. L'administration de PapMV, fusionné ou non à des épitopes d'intérêts, permet de générer une réponse immunitaire innée et adaptative chez la souris. Le PapMV est efficacement phagocyté par les cellules dendritiques (Lacasse *et al.*, 2008), résultant en l'expression de marqueurs d'activation à la surface des cellules du système immunitaire inné (Acosta-Ramirez *et al.*, 2008, Lacasse *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b). Cette activation provient de la reconnaissance de l'ARN par le TLR7 suivi de la production d'IFN-α principalement par les pDC (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b). D'autres cytokines sont aussi produites suite à une stimulation au PapMV tel que l'IL-6 et le TNF-α (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Mathieu *et al.*, 2013) ainsi que des chimiokines tel que KC et IP-10 (Mathieu *et al.*, 2013). Bien que les DC aient un rôle important à jouer dans la réponse immunitaire au PapMV, ces dernières ne sont pas les seules cellules du système immunitaire inné à être

activées. Que ce soit par contact direct avec le PapMV ou par activation indirecte via les cytokines sécrétées par les DC, les macrophages et les neutrophiles sont recrutés au site d'infection (Mathieu *et al.*, 2013) et les macrophages sont activés (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b). Nous avons aussi démontré que l'administration du PapMV induit l'activation des lymphocytes B (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b) et la production d'anticorps spécifiques au PapMV ainsi qu'aux épitopes fusionnés à sa surface (Acosta-Ramirez *et al.*, 2008, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lebel *et al.*, 2014, Rioux *et al.*, 2014, Savard *et al.*, 2011). L'administration de PapMV permet aussi d'induire l'activation des lymphocytes T $CD8^+$, ce qui active la réponse immunitaire adaptative cellulaire. Cette activation se traduit principalement par l'expression de marqueurs d'activation à la surface des lymphocytes T $CD8^+$ (Lebel *et al.*, 2014, Lebel *et al.*, 2016b) ainsi que la sécrétion de cytokines impliquées dans l'activité cytolytique des lymphocytes cytotoxiques.

De par sa forme hélicoïdale, le PapMV est une plateforme versatile pouvant supporter des fusions en surface sans que sa structure n'en soit affectée (Babin *et al.*, 2013, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007, Rioux *et al.*, 2012a). La présentation d'épitopes en surface du PapMV permet d'éliciter une réponse humorale contre ces épitopes tout en conservant la production d'anticorps dirigés contre le PapMV (Carignan *et al.*, 2015, Denis *et al.*, 2007, Rioux *et al.*, 2012a). Certaines fusions ont aussi démontré un fort potentiel d'activation des lymphocytes T, tel que démontré par la réactivation des lymphocytes T et à leur production d'IFN-γ suite à une restimulation *in vitro* avec le peptide d'intérêt (Babin *et al.*, 2013, Leclerc *et al.*, 2007). La fusion d'épitopes à la surface du PapMV permet aussi d'améliorer la phagocytose des particules par les DC, ce qui se traduit en une activation plus prononcée des DC (Lacasse *et al.*, 2008).

3.2 **Études des propriétés vaccinales du PapMV**

Le PapMV étant une bonne particule immunostimulatrice, son potentiel en tant qu'adjuvant ou plateforme vaccinale n'est pas surprenant. Son pouvoir d'activation du système immunitaire ainsi que la possibilité de fusionner des épitopes à sa surface sans affecter sa structure confèrent au PapMV un avantage face à d'autres plateformes présentement disponibles. La sécurité observée suite à l'administration du PapMV démontre aussi que le PapMV est une plateforme intéressante dans le développement de vaccins. Une phase clinique I est présentement en cours afin d'évaluer la sécurité de l'utilisation du PapMV comme adjuvant dans le vaccin contre la grippe chez l'humain (ClinicalTrials.gov, 2014c)

3.2.1 Rôle en tant qu'adjuvant

Dans une formulation vaccinale, le rôle d'un adjuvant est de renforcer la réponse immunitaire générée contre le vaccin. La majorité des antigènes que l'on retrouve dans les vaccins sont de très faibles immunogènes et ne sont pas capables à eux seuls d'éliciter une forte réponse immunitaire. Afin d'améliorer la réponse immunitaire contre ces faibles immunogènes et ainsi augmenter le niveau de protection engendré par la vaccination, certains adjuvants sont ajoutés à la formulation vaccinale. L'adjuvant que l'on retrouve en majorité dans les formulations vaccinales est l'alum. Bien qu'il soit efficace dans son rôle d'adjuvant, son mécanisme d'action est encore aujourd'hui controversé (Wen *et al.*, 2016). D'autres types de molécules peuvent agir à titre d'adjuvant tel que les VLP, qui favorisent la phagocytose des antigènes retrouvés dans le vaccin par les cellules présentatrices d'antigènes (D. M. Smith *et al.*, 2013), ce qui facilite le transport du vaccin aux ganglions lymphatiques et ainsi la génération d'une réponse immunitaire efficace. Des ligands de TLR peuvent aussi être utilisés à titre d'adjuvants dans un vaccin tel que le monophosphoryl lipid A (MPL) qui est retrouvé dans la formulation Cervarix contre le virus du papillome humain 16 et 18 (Administration, 2014, Kaczanowska *et al.*, 2013).

Afin de démontrer le pouvoir adjuvant du PapMV, ce dernier a été administré conjointement à différents types de vaccins afin d'évaluer la réponse immunitaire générée chez la souris, la charge virale suite à une infection ainsi que la survie des souris suite à une infection léthale. Lors de la vaccination de souris avec le vaccin trivalent contre l'influenza (TIV), la présence de PapMV en tant qu'adjuvant permet d'augmenter la production d'anticorps dirigés contre les antigènes retrouvés dans le vaccin (Rioux *et al.*, 2016, Rioux *et al.*, 2014, Savard *et al.*, 2011) ainsi que d'en accélérer la production (Rioux *et al.*, 2016). Cette caractéristique adjuvante du PapMV permet aux souris vaccinées d'être protégées contre une infection par une souche hétérologue d'influenza ne se retrouvant pas dans le vaccin (Rioux *et al.*, 2016, Rioux *et al.*, 2014, Savard *et al.*, 2011). D'autres antigènes peuvent être utilisés tel qu'OVA, HEL et OmpC. L'ajout du PapMV dans ces formulations vaccinales permet d'améliorer la réponse humorale et cellulaire générée contre ces antigènes (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014). L'utilisation de BMDC chargés du peptide OVA (Lebel *et al.*, 2014) ainsi que l'utilisation du PapMV fusionné à la protéine M2e d'influenza (Denis *et al.*, 2008) bénéficie de l'utilisation du PapMV nu comme adjuvant tel que démontré par l'amélioration de la réponse humorale et cellulaire. Lorsque le PapMV est utilisé comme adjuvant dans la vaccination contre *Listeria monocytogenes*-OVA (Lebel *et al.*, 2014), *Salmonella typhi* (Acosta-Ramirez et al., 2008), LCMV Cl13 (Lacasse *et al.*, 2008) ainsi que certaines souches d'influenza (Denis *et al.*, 2008, Rioux *et al.*, 2016, Rioux *et al.*, 2014, Savard

et al., 2011), la survie des souris est améliorée, ce qui démontre le potentiel du PapMV en tant que plateforme adjuvante.

3.2.2 Rôle en tant que plateforme vaccinale

L'utilisation de particules non-modifiées de PapMV ou dotées de fusions n'est pas restreinte au rôle d'adjuvant du PapMV. En effet, l'administration du PapMV en tant que plateforme vaccinale permet aussi d'engendrer une forte réponse immunitaire efficace qui protège l'hôte contre une infection subséquente. C'est le cas lors d'une infection LCMV où l'hôte a été immunisé à plusieurs reprises par du PapMV fusionné à l'épitope gp33 de LCMV (Lacasse *et al.*, 2008). Ces administrations multiples permettent de diminuer les titres viraux dans la rate sous la limite de détection suite à l'infection de souris immunisées (Lacasse *et al.*, 2008). Un autre régime d'administrations multiples, cette fois-ci au niveau du système respiratoire, permet de protéger les souris contre une infection par le virus de l'influenza (Mathieu *et al.*, 2013). Dans cette étude, plusieurs régimes d'administrations ont été testés et une fenêtre optimale de traitement a été déterminée. Lorsque les instillations intra-nasales sont effectuées dans un délai trop rapproché (moins de cinq jours) la protection de l'hôte n'est pas optimale ; la perte de poids est plus prononcée et la réponse immunitaire est moins importante (Mathieu *et al.*, 2013). Deux autres études ont aussi démontré que l'utilisation du PapMV en tant que plateforme vaccinale permet d'améliorer la condition des souris subséquemment infectée sans toutefois induire une protection complète (Denis *et al.*, 2008, Savard *et al.*, 2012).

4 PROBLEMATIQUE ET OBJECTIFS

Bien que la vaccination ait depuis longtemps fait ses preuves en prophylactie, son utilisation dans le traitement d'infections chroniques pose encore plusieurs défis. Les traitements d'infections et de certaines maladies chroniques ont longtemps été basés sur l'utilisation d'IFN-α. Ces traitements engendrent plusieurs effets secondaires importants (Sleijfer *et al.*, 2005), limitant ainsi leur utilisation à grande échelle. Plusieurs traitements sont présentement évalués afin de se départir de l'IFN-α mais les résultats restent peu concluants dans plusieurs cas. Un de ces traitements vise l'utilisation de ligands de TLR comme base vaccinale afin de déclencher la production d'IFN-α de façon endogène, limitant ainsi les effets reliés à l'administration d'IFN-α exogène. De cette façon, le système immunitaire serait amené à produire lui-même les médiateurs requis afin d'enrayer l'infection.

Il existe présentement seulement deux ligands de TLR approuvés pour l'utilisation dans le traitement du cancer en tant que plateforme vaccinale. Le bacille Calmette-Guérin, un ligand de plusieurs TLR, est couramment utilisé dans le traitement du cancer de la vessie alors que l'imiquimod, un ligand de TLR7, est utilisé dans le traitement du mélanome ainsi que des condylomes. Plusieurs ligands de TLR7 sont présentement à l'étude dans le traitement du cancer mais les résultats restent peu convaincants. Les effets secondaires sont très marqués et les régimes d'administrations ne sont pas bien adaptés aux patients (Dudek *et al.*, 2007, Dummer *et al.*, 2008, Geller *et al.*, 2010, Sauder *et al.*, 2003b, Weigel *et al.*, 2012). Afin de réduire ces effets secondaires, nous envisageons de remplacer ces régimes de vaccination par une molécule vaccinale moins toxique qui serait mieux tolérée chez les patients.

Notre équipe travaille depuis plusieurs années sur le PapMV, un ligand de TLR7, afin d'évaluer son potentiel en tant que plateforme vaccinale et adjuvant. Le PapMV peut être administré tel quel ou fusionné en surface à des épitopes d'intérêts. L'administration du PapMV, non-modifié ou fusionné, active la réponse immunitaire et permet de protéger l'hôte contre une infection léthale. Nous avons démontré que le PapMV peut être utilisé dans le traitement d'un mélanome murin (Lebel *et al.*, 2016a), faisant ainsi preuve du potentiel du PapMV dans le traitement d'infections et de maladies chroniques. De plus, le PapMV n'induit aucun signe de toxicité, faisant de cette plateforme une alternative sécuritaire à considérer.

Afin de démontrer le potentiel du PapMV comme traitement lors d'une infection virale chronique, trois objectifs ont guidés la poursuite de mon projet de maîtrise

1. Évaluer le potentiel du PapMV dans le traitement d'une infection chronique par le LCMV Cl13
2. Évaluer l'impact d'un prétraitement au PapMV sur la réponse immunitaire suite à une seconde immunisation au PapMV
3. Déterminer quels mécanismes sont responsables de la tolérance immunitaire engendrée suite à une pré-immunisation au PapMV

Afin de répondre au premier objectif et d'évaluer le potentiel du PapMV comme traitement, nous avons utilisé le LCMV Cl13 comme modèle d'infection chronique. Suite à l'infection de souris C57Bl/6, deux fortes doses de PapMV leur ont été administrées trois et cinq jours post-infection. Différents paramètres ont ensuite été évalués tel que la production d'IFN-α suite aux traitements, l'activation des lymphocytes T $CD8^+$ ainsi que la charge virale dans différents organes afin d'évaluer l'efficacité des traitements.

Pour le deuxième objectif, l'infection au LCMV Cl13 a été remplacée par un prétraitement au PapMV. Étant donné qu'une infection au LCMV Cl13 active plusieurs voies de signalisation, l'utilisation du PapMV comme prétraitement nous permet de nous concentrer uniquement sur la voie du TLR7 et sa régulation lors d'immunisations répétées du PapMV. Les différents régimes de vaccination nous ont permis d'évaluer l'impact d'un prétraitement au PapMV sur l'activation des cellules dendritiques plasmacytoïdes ainsi que leur production d'IFN-α.

Pour le dernier objectif, nous avons évalué trois mécanismes pouvant être à la source de la tolérance immunitaire observée. La régulation d'IRAK1 suite à une stimulation au PapMV a été évaluée *in vitro* chez les BMpDC. Le deuxième mécanisme évalué portait sur l'expression de Sca-1 suite à une immunisation au PapMV chez les souris C57Bl/6. Le troisième mécanisme a été évalué chez des souris J_HT déficientes en lymphocytes B. Nous avons aussi effectué des transferts de sérums immuns afin d'éliminer l'implication des lymphocytes B eux-mêmes et d'isoler le rôle des anticorps dans la tolérance immunitaire observée.

CHAPITRE 2 : RÉSULTATS

ARTICLE 2: EFFICACY OF A VIRUS-LIKE NANOPARTICLE AS TREATMENT FOR A CHRONIC VIRAL INFECTION IS HINDERED BY IRAK1 REGULATION AND ANTIBODY INTERFERENCE

Titre en français

L'efficacité d'une nanoparticule d'un virus de plante comme traitement contre une infection virale chronique est entravée par la régulation d'IRAK1 ainsi que par l'interférence causée par les anticorps

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Contributions des auteurs

KC : Conception et réalisation du projet, co-écriture du manuscrit

MEL : Conception et réalisation du projet
ET : Réalisation du projet

PS : Développement du processus de manufacture du PapMV

DL : Co-écriture du manuscrit

AL : Conception du projet, co-écriture du manuscrit

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Résumé en français

Bien que la vaccination ait été un des moyens les plus efficaces dans la prévention d'infections et ce, depuis le 18^{e} siècle, l'élaboration de vaccins thérapeutiques reste encore à être développée. Plusieurs défis affectent le développement de la vaccination thérapeutique tel que l'administration d'agents pathogènes entiers, atténués ou morts, suscitant ainsi des risques pour la santé de l'être humain. Une alternative sécuritaire à la vaccination classique réside dans l'utilisation de nanoparticules synthétiques ou dérivées. Nous avons récemment démontré que les nanoparticules du virus de la mosaïque de la papaye (PapMV) peuvent être administrées en tant qu'adjuvant dans un contexte d'immunothérapie où son administration ralentit la croissance locale d'un mélanome murin agressif. Le PapMV peut aussi être utilisé comme plateforme vaccinale en prophylactie et permet de protéger les souris contre une infection virale léthale et ce, de façon IFN-α dépendante. Nous nous sommes donc intéressés au potentiel du PapMV dans le traitement d'une infection virale chronique modèle tel que le virus de la chorioméningite lymphocytes chronique (LCMV Cl13). Le traitement de souris infectées par le LCMV Cl13 trois et cinq jours post-infection au moyen d'administrations systémiques de PapMV n'a pas été efficace. En effet, aucun changement n'a pu être observé dans les titres viraux ainsi que dans la réponse immunitaire au LCMV suite aux traitements. De plus, la production d'IFN-α suite au traitement des souris infectées au LCMV Cl13 par le PapMV est presque abolie. Afin d'isoler les mécanismes responsables, nous avons évalué l'influence d'un prétraitement au PapMV sur des administrations subséquentes de PapMV. Cela nous a permis d'éliminer les multiples facteurs associés à une infection au LCMV Cl13. Les prétraitements au PapMV ont eu un effet semblable à une infection au LCMV sur la production d'IFN-α, qui est abrogée pendant au moins 50 jours suivant la première immunisation. Nous avons démontré que deux mécanismes différents se chevauchant sont responsables de cette tolérance. L'inhibition à court terme semble être partiellement due à la dégradation d'IRAK1, une protéine cruciale dans la voie de signalisation du TLR7. L'inhibition à long terme pour sa part semble être principalement due à l'interférence des anticorps spécifiques au PapMV. Nous avons identifié quelques défis à l'utilisation de pseudoparticules virales dans le traitement systémique d'infections virales chroniques et discutons d'alternatives afin de contourner ces difficultés.

1.1 **Abstract**

Although vaccination has been an effective way of preventing infections ever since the 18th century, the generation of therapeutic vaccines and immunotherapies is still a work in progress. A number of challenges impede the development of these therapeutic approaches such as safety issues related to the administration of whole pathogens whether attenuated or inactivated. One safe alternative to classical vaccination methods gaining recognition is the use of nanoparticles, whether synthetic or naturally derived. We have recently demonstrated that the papaya mosaic virus-like (PapMV) nanoparticle can be used as a prophylactic vaccine against various viral and bacterial infections through the induction of protective humoral and cellular immune responses. Moreover, PapMV is also very efficient when used as an immune adjuvant in an immunotherapeutic setting at slowing down the growth of aggressive mouse melanoma tumors in a type I interferon (IFN-I)-dependent manner. In the present study we were interested in exploiting the capacity of PapMV of inducing robust IFN-I production as treatment for the chronic viral infection model lymphocytic choriomeningitis virus (LCMV) Clone 13 (Cl13). Treatment of LCMV Cl13-infected mice with two systemic administrations of PapMV was ineffective, as shown by the lack of changes in viral titers and immune response to LCMV following treatment. Moreover, IFN-α production following PapMV administration was almost completely abolished in LCMV infected mice. To better isolate the mechanisms at play, we determined the influence of a pre-treatment with PapMV on secondary PapMV administration, therefore eliminating potential variables emanating from the infection. Pre-treatment with PapMV led to the same outcome as an LCMV infection in that IFN-α production following secondary PapMV immunization was abrogated for up to 50 days while immune activation was also dramatically impaired. We showed that two distinct and overlapping mechanisms were responsible for this outcome. While short-term inhibition was partially the result of IRAK1 degradation, a crucial component of the TLR7 signaling pathway, long-term inhibition was mainly due to interference by PapMV-specific antibodies. Thus we identified a possible pitfall in the use of virus-like particles for the systemic treatment of chronic viral infections and discuss mitigating alternatives to circumvent these potential problems.

1.2 **Introduction**

Type I interferon (IFN-I), mainly IFN-α and IFN-β, are a family of cytokines with potent antiviral and immunomodulatory properties. The effects of these cytokines on their milieu are complex and affect multiple cells of the immune system by: (i) inducing activation of dendritic cells (DCs)

(Montoya *et al.*, 2002, Santini *et al.*, 2000); (ii) sustaining activation of CD8+ T cells (Kolumam *et al.*, 2005, Marrack *et al.*, 1999) and (iii) inducing differentiation of B cells into antibody secreting cells (Jego *et al.*, 2003, Le Bon *et al.*, 2001). Thus early IFN-I production is essential for the control of most viral infections such as mouse hepatitis virus (MHV) (Cervantes-Barragan *et al.*, 2007), lymphocytic choriomeningitis virus (LCMV) (Sullivan *et al.*, 2015, Y. Wang *et al.*, 2012) or simian immunodeficiency virus (SIV) (Sandler *et al.*, 2014). However, whereas early and transient expression of IFN-I controls the infection, prolonged exposure bears detrimental effects to the host's immune response (Sandler *et al.*, 2014, Teijaro *et al.*, 2013, Wilson *et al.*, 2013). The balancing act between the positive and negative effects of the IFN-I response is observed in the clinical setting as displayed by the well-documented adverse effects of IFN therapy (Aul *et al.*, 1997, Sleijfer *et al.*, 2005). Thus, despite being the standard-of-care against various diseases, emerging therapies now focus on IFN-free alternatives.

Upon viral infection, plasmacytoid DCs (pDCs) are characterized as the main producers of IFN-I (reviewed in (Gilliet *et al.*, 2008, Swiecki *et al.*, 2015)). This major characteristic is mainly due to their toll like receptor (TLR) expression profile. While other innate immune cells express a wide array of TLRs, pDCs mainly express the endosomal TLR7 and TLR9 (Swiecki *et al.*, 2015), which bind genetic material typically associated with viral pathogens, such as ssRNA and unmethylated DNA, respectively. These receptors are also capable of recognizing analogues of their natural ligands such as imidazoquinoline and CpG for TLR7 and TLR9, respectively (Lee *et al.*, 2003). Through their production of IFN-I, pDCs also serve as a bridge between innate and adaptive immune responses as illustrated by their ability to activate natural killer cells (NKs) (Swiecki *et al.*, 2010b), other DCs (Fonteneau *et al.*, 2004, Yoneyama *et al.*, 2005) as well as T cells (Fonteneau *et al.*, 2003, Swiecki *et al.*, 2010b). Consequently, upon pDC depletion, mice become highly susceptible to viral infection with murine hepatitis virus (MHV) (Cervantes-Barragan *et al.*, 2007), herpes simplex virus (HSV) (Lund *et al.*, 2006, Swiecki *et al.*, 2013), LCMV (Cervantes-Barragan *et al.*, 2012), vesicular stomatitis virus (VSV) (Swiecki *et al.*, 2010b) or the murine cytomegalovirus (MCMV) (Swiecki *et al.*, 2010b) among others.

By harnessing the central role of pDCs, we have demonstrated the potential for the use of a plant virus-like nanoparticle as a vaccine candidate as well as an adjuvant in various infectious models (Acosta-Ramirez *et al.*, 2008, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lacasse *et al.*, 2008, Lebel *et al.*, 2014, Mathieu *et al.*, 2013, Rioux *et al.*, 2016, Rioux *et al.*, 2014, Savard *et al.*, 2011). Our platform is based on the papaya mosaic virus (PapMV) nanoparticle that contains a non-replicative synthetic ssRNA rendering it safe for future human use. The synthetic

ssRNA found inside the capsid is recognized by the TLR7 of pDCs (Lebel *et al.*, 2014), leading to the production of IFN-α (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b), IL-6 (Lebel *et al.*, 2014, Mathieu *et al.*, 2013) along with other cytokines and chemokines. PapMV resultantly activates DCs, macrophages, T cells as well as B cells (Acosta-Ramirez *et al.*, 2008, Lacasse *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b), making this platform versatile in activating the immune system. Furthermore, we have shown that PapMV induces protective immune responses against pathogens when used as a vaccine platform or an adjuvant (Acosta-Ramirez *et al.*, 2008, Carignan *et al.*, 2015, Denis *et al.*, 2008, Lacasse *et al.*, 2008, Lebel *et al.*, 2014, Mathieu *et al.*, 2013, Rioux *et al.*, 2016, Rioux *et al.*, 2014, Savard *et al.*, 2011, Therien *et al.*, 2017) and slows melanoma development when used in an immunotherapeutic setting (Lebel *et al.*, 2016a).

Considering that PapMV induces strong IFN-I responses, we sought to evaluate its potential as an immune adjuvant for the treatment for chronic viral infections with the objective of replacing exogenous IFN-α administration with endogenous IFN-α secretion following systemic PapMV delivery. This approach would provide a universally applicable immune stimulatory molecule that could be used against all viral infections without requiring expression of defined viral antigens. We observed that treatment of mice chronically infected with the persistent strain of LCMV (LCMV-Cl13) with PapMV was unable to clear the infection. Moreover, multiple administrations of PapMV induced an immune tolerance as shown by the almost complete abrogation of IFN-α production following secondary PapMV administration. We show that this tolerization is the result of a combination of factors including IRAKI degradation and interference by PapMV-specific antibodies. This information will be crucial for further clinical development of the PapMV platform.

1.3 Material and methods

1.3.1 Ethics statement

This study was performed in accordance with the Canadian Council on Animal Care guidelines. All *in vivo* experiments were reviewed and approved by the Institut national de la recherche scientifique animal care committee.

1.3.2 Mice

Female 6- to 10-wk-old C57BL/6 mice were purchased from Charles River Laboratories. J_HT mice were kindly provided by Dr. Rolf Zinkernagel (Zurich University, Switzerland).

1.3.3 Cells and virus

LCMV Cl13 was kindly provided by Dr. Rolf Zinkernagel (Zurich University, Switzerland). MC57G fibroblast were cultured in minimal essential medium with Earle's salt (Wisent, St-Bruno, Canada) containing 5 % heat inactivated FBS (PAA laboratories, Mississauga, Canada).

1.3.4 PapMV nanoparticles

PapMV nanoparticles were provided by Folia Biotech (Quebec, Canada) and were produced as described before (Mathieu *et al.*, 2013). Briefly, coat proteins are self assembled *in vitro* around a non-coding ssRNA. LPS contamination was always < 50 endotoxin units/mg protein and considered as negligible.

1.3.5 LCMV Cl13 infection and treatment

Mice were infected with 2 x 10^6 pfu of LCMV Cl13 i.v. and treated with 400 µg of PapMV i.v. on days 3 and 5. Serum was collected 6 hours following each treatment to assess IFN-α production. Blood was collected 8 days post infection (dpi) and mononuclear cells were isolated by density gradient over Ficoll-Paque (GE Healthcare Life Sciences, Mississauga, Canada) and centrifuged at 1200 rpm for 20 min at room temperature. Cells were collected and washed with PBS then stained for 30 min at 37 °C with GP_{33-41} PE tetramers, which were synthesized as previously described (Altman *et al.*, 1996), to label $CD8^+$ T cells specific for the MHC-I gp33 epitope of LCMV. Extracellular staining was performed on unwashed cells for 20 min at 4 °C. Following a wash, cells were fixed with fixation buffer (Biolegend, San Diego, USA) for 20 min at room temperature then analyzed by flow cytometry on a BDLSR Fortessa (BD Biosciences, Mississauga, Canada). Spleens collected 30 dpi were disrupted between frosted microscope slides and cells were stained to assess $CD8^+$ T cells $GP_{33-41}{}^+$ cells as described above. Cells were also incubated with Brefeldin A (BFA) (Sigma, Oakville, Canada) for 5 hours at 37 °C to inhibit vesicular transport. Spleen cells were then stained for intracellular cytokine production (see below). Blood, spleen, kidney, liver and brain were also collected 30 dpi to assess viral burden. Organs were mechanically disrupted and supernatants were tittered on MC57G cells by focus forming assay to assess viral burden as previously described (Battegay *et al.*, 1991).

1.3.6 Immunizations

Mice were injected with 100 µg or 400 µg of PapMV i.v., 50 µg of imiquimod (R837) (InvivoGen, San Diego, USA), 25 µg of LPS (Sigma), 50 µg of Poly (I:C) (InvivoGen) or control.

1.3.7 Organ processing

Spleen and bones from hind legs were collected at various time points following immunization. Spleens were subjected to digestion with 1 mg/mL of Collagenase D (Roche, Mississauga, Canada) for 15 min at 37 °C. Femurs, tibias and iliac crests were flushed and single cell suspensions from both spleen and bone marrow were subjected to red blood cell lysis followed by flow cytometry staining.

1.3.8 Bone marrow derived plasmacytoid dendritic cells (BMpDCs)

Bone marrow derived plasmacytoid dendritic cells (BMpDC) were prepared by flushing the bone marrow of femurs, tibias and iliac crests, and subjected to red blood cell lysis. Cells were seeded at 2 x 10^6 cells/mL in RPMI 1640 (Wisent) containing 10 % FBS, 100 IU Penicillin, 100 µg/ml Streptomycin (Wisent), 55 µM β-mercaptoethanol, 1 mM sodium pyruvate, 1x MEM non-essential amino acids and 10 mM HEPES (Gibco) supplemented with 200 ng/ml of FLT3-L (BioXcell, Lebanon, USA). On days 7 to 9, cells were stimulated with 100 µg/ml of PapMV, 25 µg/ml of imiquimod (R837) (InvivoGen), 12,5 µg/ml of polyinosinic:polycyticylic acid (Poly I:C) (InvivoGen) or control. On days 8 to 10, supernatants were frozen at -20 °C for IFN-α detection and cells stained for flow cytometry analysis or cell sorting.

1.3.9 Serum transfer

Mice were immunized with 100 µg PapMV i.v. on day 0. On day 5 or 25, mice were euthanized and blood was collected by cardiac puncture. Blood was allowed to clot for 30 min at room temperature. Tubes were then centrifuged at 1500xg for 10 min at room temperature. Sera were pooled and injected i.p. to mice whereby the serum from two mice was used to inject one mouse. After 24 hours, mice were immunized with 100 µg of PapMV or control i.v. Serum was collected 6 hours later to quantify IFN-α and PapMV specific antibodies and activation of pDCs was assessed in spleen 24 hours post immunization.

1.3.10 Flow cytometry

Analysis of surface antigens were performed with the following antibodies and markers: CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), PD-1 (29F1A12), Zombie Aqua, CD11b (M1/70), CD11c (N418), CD45R/B220 (RA3-6B2), CD317 (927), CD86 (GL-1), CD69 (H1.2F3), Sca-1 (E13-161.7) (Biolegend). Fc receptors were blocked using a purified anti CD16/32 antibody (2.4G2) (BioXcell). Identification of pDCs was based on their viability (Zombie Aqua^{-}) and their surface antigen expression (CD11c^{int}, CD11b^{lo}, B220^{+} and CD317^{+}). For intracellular staining, IFN-ɣ (XMG1.2), TNF-α (MP6-XT22), IL-2 (JES6-5H4) (Biolegend), IFN-α (RMMA-1) (PBL assay science, Piscataway, USA), IRAK1 (D51G7) as well as isotype control antibodies were used (Cell Signaling Technologies, Beverly, USA) after permeabilization using the Intracellular Staining Permeabilization Wash Buffer 10 x and Fixation Buffer following instructions of manufacturer (Biolegend). Data was acquired using BDLSR Fortessa Flow Cytometer (BD Biosciences) and analyzed using the FlowJo software (FlowJo, LLC).

1.3.11 IFN-α intracellular staining

Mice were immunized with 400 µg of PapMV i.v. as described above. Spleens and bone marrows were collected 4 hours post immunization and processed as described above. Cells were then incubated with BFA for 4 hours at 37 °C followed by IFN-α intracellular staining as described above.

1.3.12 Cell sorting

BMpDCs were stimulated for 24 or 48 hours and stained for sorting. Fc receptors were blocked as previously described and pDCs were identified as CD11c^{+}B220^{+}PDCA1^{+}. Cells were collected in FBS then washed twice with cold PBS followed by protein extraction (see below). Sort was performed using a BD FACSJazz (BD Biosciences).

1.3.13 Immunoblotting

For immunoblotting, cells were harvested and lysed in Triton X-100 lysis buffer (20 mM Tris-HCl pH 8.0, 1 % Triton X-100, 10 % Glycerol, 150 mM NaCl, protease inhibitor cocktail (Roche)). Lysates were then loaded on a 10 % SDS-PAGE followed by transfer on a polyvinylidene difluoride membranes (BioRad, Mississauga, Canada). Membranes were blocked in 5 % dry milk

TBS-T (TBS, 0.1 % Tween-20) for 2 hours at room temperature. Primary antibodies against mouse IRAK1 and β-actin (Cell Signaling Technologies) were diluted in TBS-T and incubated with the membranes o/n at 4 °C. Anti-rabbit IgG HRP (Jackson ImmunoResearch, West Grove, USA) were used as secondary antibodies whereby they were diluted in TBS-T and incubated with the membranes for 1 hour at room temperature. Detection was performed with ECL chemiluminescence kit (BioRad).

1.3.14 ELISA and multiplex

IFN-α levels in mice serum or cell culture supernatants were quantified by ELISA according to the manufacturer's directions (Affymetrix eBiosciences). TNF-α, IL-6, IL-10, IL12p40, IL12p70, IL-9, IL-15, KC, G-CSF, M-CSF, RANTES, MIP-1α, MIP-1β, MIP-2, IP-10 and MCP-1 levels in mice serum were quantified using Milliplex Map Mouse Cytokine/Chemokine Premixed 32 Plex (Millipore, Etobicoke, Canada) according to manufacturer's directions. Measurement of Median Fluorescence Intensity (MFI) were performed using Bio-plex (Biorad). PapMV specific antibody titers were determined as described previously (Denis *et al.*, 2007). Detection of PapMV-specific IgM was performed with peroxidase-conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories).

1.3.15 Statistical analysis

Statistical analysis was performed with GraphPad Prism Software (GraphPad Software). Error bars represent SEM. 2-tailed Student *t* test was used and Welch's correction was applied when needed.

1.4 Results

1.4.1 Treatment of chronic LCMV Cl13 infection with PapMV does not improve viral clearance

The impact of IFN-α on viral infections in mice was shown to vary according to the kinetics and strength of production during the ongoing infection. Early IFN-α was shown to be essential to the control of the infection (Cervantes-Barragan *et al.*, 2007, Sandler *et al.*, 2014, Smit *et al.*, 2006, Sullivan *et al.*, 2015) while long term IFN-α was detrimental to the host and favored viral persistence (Sandler *et al.*, 2014, Teijaro *et al.*, 2013, Wilson *et al.*, 2013). In one such study, treatment of LCMV Cl13-infected mice with exogenous IFN-α on days 3 and 5 post-infection

resulted in the increase of GP_{33-41} specific $CD8^+$ T cells as well as a decrease in viral load 32 dpi (Y. Wang *et al.*, 2012). This led us to hypothesize that the treatment of a chronic infection such as LCMV Cl13 with PapMV could result in a similar clearance of the virus given the capacity of PapMV to induce potent IFN-α production following immunization (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b). We therefore infected mice with LCMV Cl13 and treated them 3 and 5 dpi with 400 µg of PapMV i.v. Blood was collected at various time points to assess viral load, which was not affected by the treatments with PapMV (Figure 2.1A). To assess the efficiency of the PapMV treatment, serum was collected 6 hours following the treatment on days 3 and 5 and IFN-α was quantified by ELISA. Although the administration of 400 µg of PapMV induced strong IFN-α production in naive mice, LCMV Cl13-infected mice barely produced IFN-α at all (Figure 2.1B) whether assessed after the first treatment on day 3 or the second treatment on day 5. These results indicate that the infection with LCMV Cl13 hinders IFN-α production following PapMV administration. Of note, PapMV not only induces IFN-α in immunized mice but also other cytokines and chemokines such as IL-6, IP-10 and MCP-1 (Lebel *et al.*, 2014, Lebel *et al.*, 2016b), which rely on different sets of signaling pathways than IFN-α for their production. It is therefore possible that immune cells were activated through these auxiliary cytokines following treatment with PapMV despite the absence of detectable IFN-α in the serum. To test this, we assessed activation of $CD8^+$ T cells as well as the proportion of GP_{33-41} specific $CD8^+$ T cells. The proportion of GP_{33-41} specific $CD8^+$ T cells in blood on day 8 post infection was similar in groups treated with the control or PapMV (Figure 2.1C) and a similar trend was noticed for the expression of CD44 on $CD8^+$ T cells (Figure 2.1D). We also assessed exhaustion of $CD8^+$ T cells by means of PD-1 expression, which was increased in mice treated with PapMV compared to mice treated with control (Figure 2.1D). Expression of CD62L on $CD8^+$ T cells was reduced in PapMV compared to mice treated with control (Figure 2.1D). Together, these data indicate that the treatment of LCMV Cl13 infected mice with PapMV on days 3 and 5 does not increase the activation of $CD8^+$ T cells. However, these treatments seem to increase the exhaustion of $CD8^+$ T cells as indicated by the increased expression of the inhibitory receptor PD-1 (Wherry *et al.*, 2007). PapMV treatment did not lead to homing of $CD8^+$ T cells in lymphoid organs, as shown by their decrease in CD62L expression, an adhesion molecule involved in the homing of lymphocytes to secondary lymphoid organs. The percentage of GP_{33-41} specific $CD8^+$ T cells was not different on day 30 post infection between control and PapMV treated group (Figure 2.1E). As well, the expression of PD-1, CD44 and CD62L was not significantly different between these two groups (Figure 2.1F). Furthermore, our results revealed that the functionality of GP_{33-41} specific $CD8^+$ T cells was not affected by the treatment of LCMV Cl13 infection with PapMV, as shown by the

similar percentage of IFN-ɣ, TNF-α or IL-2 positive $CD8^+$ T cells in the spleen (Figure 2.1G) between control and PapMV treated groups. To further confirm that the PapMV treatments had no effect on the clearance of LCMV Cl13, we collected lymphoid and non-lymphoid organs at 30 dpi and assessed the viral load of LCMV Cl13. In the spleen, kidney, liver and brain, both groups displayed similarly elevated LCMV Cl13 titers (Figure 2.1H), which, along with previous data, indicated that treatment with PapMV was ineffective in clearing an LCMV Cl13 infection. Notably, it has been shown that cells previously infected with chronic viruses such as HBV (Xie *et al.*, 2009, Xu *et al.*, 2012) or HCV (Rodrigue-Gervais *et al.*, 2007, Yonkers *et al.*, 2007) were unresponsive to further TLR ligand stimulation. Based on our findings, we surmise that a possible TLR tolerance mechanism might be at play after an LCMV Cl13 infection, therefore hindering further activation of immune cells by a subsequent TLR ligand stimulation such as PapMV.

1.4.2 Pre-treatment with PapMV inhibits further effects of secondary PapMV administration

LCMV Cl13 activates immune cells through the TLR7/MyD88 endosomal pathway (Borrow *et al.*, 2010) as well as the RIG-I/Mda5 cytosolic pathway (Zhou *et al.*, 2010). In order to exclude activation and cytokine production caused by engagement of the RIG-I/Mda5 pathway, we decided to move to a PapMV-only based model in which only TLR7 is stimulated. We therefore pre-treated mice with PapMV at different time intervals ranging from 1 to 50 days prior to a second immunization with PapMV. Sera were collected 6 hours following the second immunization to assess IFN-α production by ELISA. A single administration of PapMV induced strong IFN-α production, as detected in the serum 6 hours post immunization (Figure 2.2A). When mice were pre-treated with 100 µg of PapMV, the production of IFN-α was abolished following a secondary immunization for up to 50 days following pre-treatment (Figure 2.2A). A similar hindrance was observed in the production of TNF-α, IL-6, IL-12p40, IL-12p70 and IL-15 whereas production of IL-10 and IL-9 was enhanced and unaffected respectively (Supp. Figure 1). Similarly, production of various chemokines and growth factors was also suppressed following a second immunization as observed with the production of M-CSF, RANTES, MIP-1α, MIP-1β, IP-10 and MCP-1 whereas production of KC, G-CSF and MIP-2 was enhanced or unaffected (Supp. Figure 1). While there are other cytokines and chemokines that are differentially regulated upon PapMV administration, we focused on IFN-α production based on its wide-ranging use in therapeutic settings (Sleijfer *et al.*, 2005).

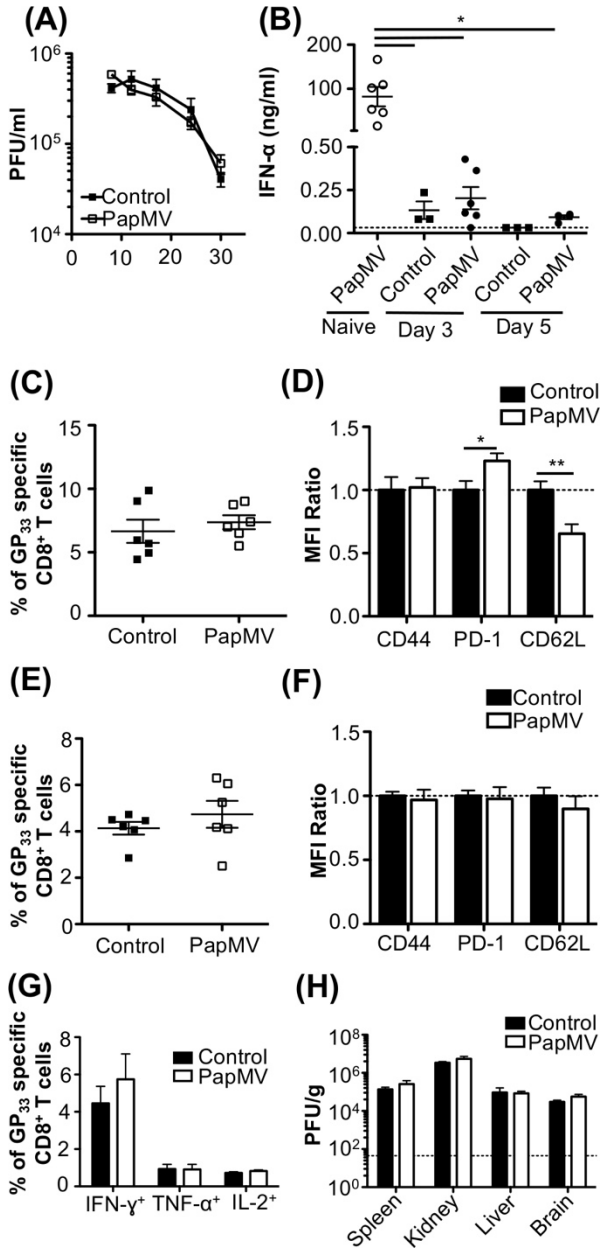


Figure 2.1: Treatment of an established LCMV Cl13 infection with PapMV does not improve viral clearance.

Mice were infected with LCMV Cl13 followed by treatments with PapMV on days 3 and 5 (A) Kinetics of viral burden expressed as LCMV Cl13 PFU/ml of blood (B) ELISA quantification of IFN-α in serum of mice 6 hours following treatment with control or PapMV. (C) Percentages of GP33-specific CD8+ T cells in the blood 8 dpi. (D) CD44, PD-1 and CD62L expression on CD8+ T cells in blood 8 dpi. (E) Percentages of GP33-specific CD8+ T cells in the spleen 30 dpi. (F) CD44, PD-1 and CD62L expression on CD8+ T cells in the spleen 30 dpi. (G) Percentages of CD8+ T cells producing IFN-ɣ, TNF-α or IL-2 in response to a stimulation with the GP33-41 peptide for 5 hours. (H) Viral loads of LCMV Cl13 in spleen, kidney, liver and brain 30 dpi. Results are expressed as PFU/g of each organ. For D and F, results are expressed as a ratio of the sample's mean fluorescence intensity (MFI) over the average MFI of control samples. (* $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$) (n = 2, three mice per group)

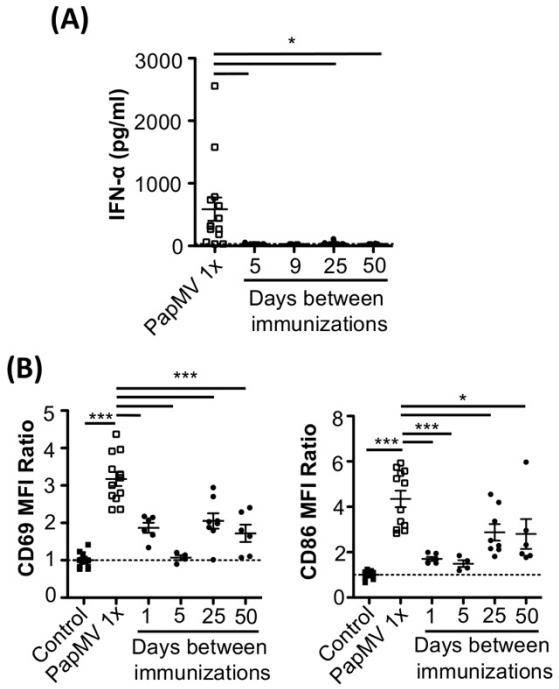


Figure 2.2: Pre-treatment with PapMV prevents pDCs from responding to a subsequent PapMV administration

(A) ELISA quantification of IFN-α in serum 6 hours following immunization with PapMV once (PapMV 1x) or preceded by pre-treatment with PapMV at various time intervals. (B) CD69 (left) and CD86 (right) expression on splenic pDCs 24 hours following immunization with PapMV once (PapMV 1x) or preceded by pre-treatment with PapMV at various time intervals. Results are expressed as a ratio of the sample's MFI over the average MFI of control samples. (* p <0.05; ** p <0.01 *** p < 0.001) (n = 2 to 8, two to four mice per group)

Since pDCs are major producers of IFN-α upon stimulation with a TLR7 ligand, we sought to determine whether the absence of IFN-α was due to a lack of activation of pDCs. Spleens of mice pretreated with 100 µg of PapMV were collected 24 hours following the second immunization and activation of pDCs was assessed by flow cytometry. As seen with IFN-α production, a single immunization with PapMV induced upregulation of CD69 and CD86 (Figure 2.2B) on pDCs. Conversely, when mice were pre-treated with PapMV 1 to 5 days prior to a second immunization, pDCs were unable to respond to the second immunization, as observed by the absence of CD69 or CD86 upregulation (Figure 2.2B). Here, we found that the expression of activation markers on pDCs on day 1 after the pretreatment was due to remnants of the initial immunization rather than the activation of pDCs following the second immunization (Supp. Fig 2). With a 25-day or more lag between the pre-treatment and the second immunization, pDCs were activated by the second

immunization although to a lesser intensity than mice treated only once with PapMV (Figure 2.2B). Altogether, these results demonstrate that the administration of PapMV induces a refractory state in pDCs rendering them unable to respond to further PapMV immunizations. In short intervals, this effect is completely inhibitory while for longer intervals the inhibition is only partial, indicating that distinct mechanisms are possibly concomitantly interfering with the response.

1.4.3 Refractory state induced by PapMV pre-treatment affects the response to other TLR7 and TLR4 ligands but not TLR3

To assess if the refractory state induced by a pre-treatment with PapMV affected only further administrations of PapMV, we pre-treated mice with PapMV and then challenged them 5 days later with LPS, R837 or Poly I:C, which are ligands for TLR4, TLR7 and TLR3, respectively. We then assessed the expression of CD69 on pDCs (Figure 2.3A). As expected, mice treated with LPS, R837 or Poly I:C alone showed an upregulation of CD69 on pDCs, indicating activation. When mice were pre-treated with PapMV, subsequent immunizations with LPS or R837 were not as efficient in inducing the activation of pDCs as immunizations in naive animals but still showed some degree of CD69 upregulation. These results indicate that the inhibition induced by pre-treatment with PapMV not only impacts subsequent immunizations with PapMV but also other TLR7 ligands as well at other TLR pathways, such as TLR4, although the inhibition is less pronounced. Strikingly, unlike the inhibition observed with LPS and R837, administration of PapMV prior to Poly I:C resulted in the expression of CD69, indicative of pDC activation, which was similar in the control and treated groups (Figure 2.3A). Altogether, these results point towards an inhibitory mechanism induced by PapMV pre-treatment that affects TLR7 and TLR4 pathways while the TLR3 pathway remains unaffected.

1.4.4 Stimulation with PapMV induces degradation of IRAK1 in pDCs

TLR pathways are not specific to each receptor. Indeed, most of the complexes implicated in the signaling cascades are shared across pathways (reviewed in (Blasius *et al.*, 2010, Lester *et al.*, 2014)). If PapMV were to affect the TLR7 signaling pathway, other TLR signaling pathways would also be affected, resulting in the cross-inhibition observed when cells are pre-treated with PapMV. Indeed, such cross-inhibition was observed in response to stimulations with various TLR ligands such as TLR4 ligands (Dalpke *et al.*, 2005, Lehner *et al.*, 2001, Nahid *et al.*, 2016) TLR7 ligands (Hayashi *et al.*, 2009, Nahid *et al.*, 2016) and TLR9 ligands (Dalpke *et al.*, 2005, Hayashi *et al.*, 2009), establishing the presence of homo and hetero tolerance in the TLR signaling pathways. In

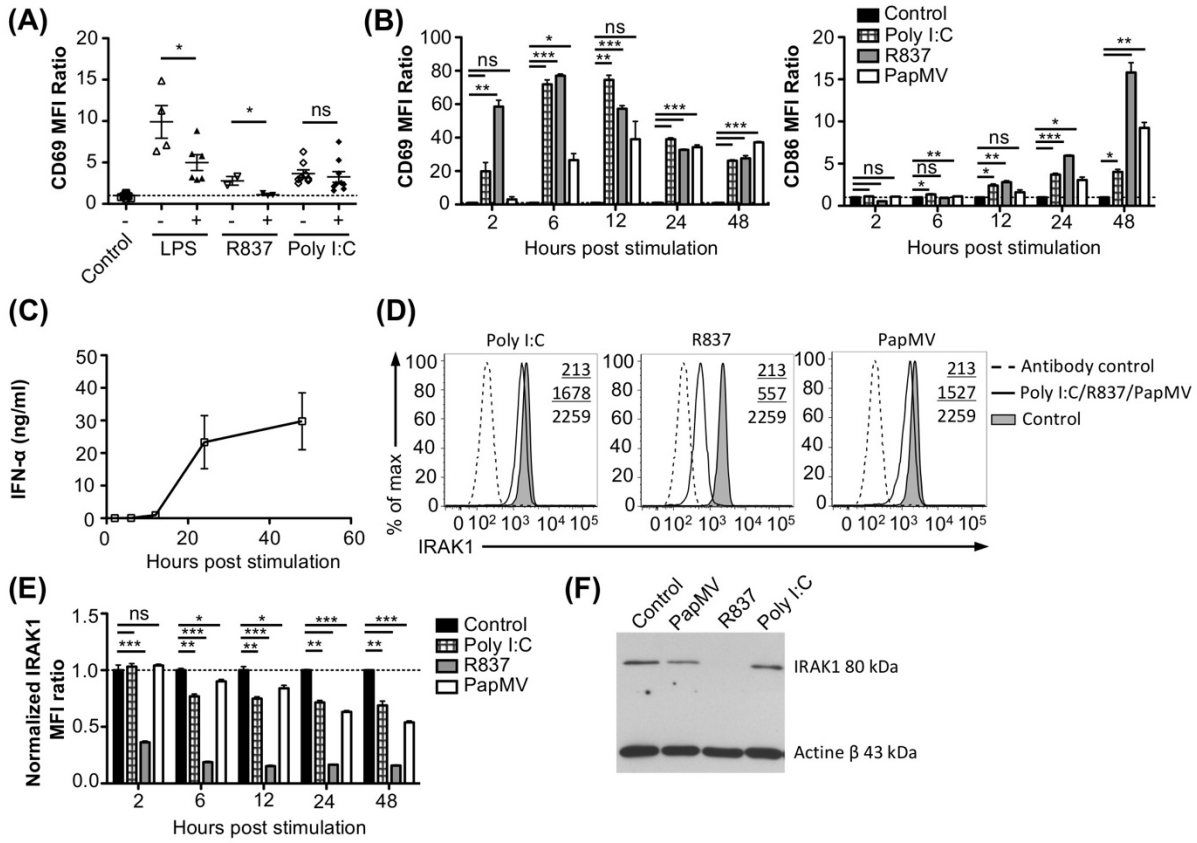


Figure 2.3: IRAK1 is degraded by PapMV in bone marrow derived plasmacytoid dendritic cells.

(A) CD69 expression on splenic pDCs 6 hours following the last immunization with the control and R837 and 24 hours following immunization with the control, LPS or Poly I:C once (-) or preceded with a pre-treatment with PapMV (+) 5 days prior. (*n* = 2 to 3, two to three mice per group, one representative experiment is shown for R837 samples) (B) CD69 (left) and CD86 (right) expression on bone marrow derived BMpDCs 2 to 48 hours post stimulation with control (black bars), Poly I:C (checkered bars), R837 (grey bars) or PapMV (white bars). (C) ELISA quantification of IFN-α in culture supernatant from BMpDCs stimulated 2 to 48 hours with PapMV. (D) Representative overlay histograms of IRAK1 expression in BMpDCs following a stimulation of 24 hours with Control (filled graph), Poly I:C, R837 or PapMV (full line) or secondary antibody staining control (dashed line) (E) IRAK1 expression in BMpDCs 2 to 48 hours following stimulation with the control (black bars), Poly I:C (checkered bars), R837 (grey bars) or PapMV (white bars) (F) Immunoblot of IRAK1 (top) and actin-β (bottom) of BMpDCs 24 hours following stimulation with control, PapMV, R837 or Poly I:C. BMpDCs were sorted to isolate pDCs prior to protein extraction and immunoblotting. (A, B, E) Results are expressed as a ratio of the MFI of the sample on the average MFI of control samples. (A)(*n*=2, two to three mice per group) (B, D, E) (representative experiment of two to ten experiments, two to three replicates per group) (C) (*n* = 2, 2 replicates per time point) (* p <0.05; ** p <0.01; *** p < 0.001)

these studies, one common mechanism reported to be involved in the observed cross tolerance was through the degradation of IRAK1 (Bourquin *et al.*, 2011, Nahid *et al.*, 2016, Siedlar *et al.*, 2004), a kinase implicated in all MyD88-dependant TLR signaling pathways, therefore excluding TLR3, which signals through a MyD88-independent pathway (Kawai *et al.*, 2010). Therefore, we

assessed IRAK1 expression in pDCs by flow cytometry and immunoblotting to analyze the regulation of IRAK1 following PapMV stimulation. Given that pDCs account for only 0.2 % of all splenocytes, we evaluated the response of pDCs to PapMV *in vitro* using BMpDCs. After differentiation of bone marrow cells with Flt3L for 8 days, cells were stimulated with PapMV. Similar to our observations from *in vivo* splenic pDCs, BMpDCs were readily activated by various TLR ligands including PapMV as shown by the upregulation of CD69, CD86 (Figure 2.3B) and the accumulation of IFN-α in culture supernatants following stimulation (Figure 2.3C). Since PapMV activates BMpDCs, we investigated the regulation of IRAK1 following stimulation with various TLR ligands. We first assessed IRAK1 expression by flow cytometry following various incubation periods of BMpDCs with TLR7 ligands, PapMV and R837, or TLR3 ligand, Poly I:C. As expected, R837 induced a strong down-regulation of IRAK1 after 24 hours in BMpDCs *in vitro* (Figure 2.3D, 2.3E). Surprisingly, stimulation of BMpDCs with Poly I:C induced a small down-regulation of IRAK1 starting at 6 hours post-stimulation. Stimulation of BMpDCs with PapMV induced the degradation of IRAK1 albeit to a lesser extent than R837 and with slower kinetics (Figure 2.3D, Figure 2.3E). To confirm the modulation of IRAK1 expression, we sorted pDCs 24 hours post-stimulation with PapMV, R837 and Poly I:C and extracted total cellular proteins to evaluate the expression of IRAK1 by immunoblotting. We found that 24 hours post R837-stimulation, the expression of IRAK1 was undetectable (Figure 2.3F) while stimulation with Poly I:C did not induce any degradation of IRAK1 (Figure 2.3F). IRAK1 was also degraded following stimulation of BMpDCs with PapMV although the extent of degradation was lower in comparison to that observed with R837 (Figure 2.3F). When comparing IRAK1 expression ratios obtained by western blot and flow cytometry (Table 1, Figure 2.3E and 2.3F), we noticed similar ratios between the two assays for R837 and PapMV stimulated BMpDCs while the ratios vary for Poly I:C stimulated BMpDCs. Taken together, these results indicate that PapMV induces the degradation of IRAK1 in pDCs, which could in part explain the tolerance observed when mice are pre-treated with PapMV.

1.4.5 PapMV induces the upregulation of Sca-1 on pDCs despite its expression not being associated to IFN-α production

Heterogeneity in the pDC population has been described (Bjorck *et al.*, 2011, Omatsu *et al.*, 2005, Pelayo *et al.*, 2005, Zhang *et al.*, 2017) although the biological significance of this phenomenon is still largely unknown. Various reports have, however, indicated that two subsets of pDCs expressing different sets of markers were differentially associated with IFN-α production following TLR stimulation (Bjorck *et al.*, 2011, Niederquell *et al.*, 2013, Omatsu *et al.*, 2005, Pelayo *et al.*,

Table 2.1: Comparison of IRAK1 regulation ratios by flow cytometry and western blot.

Sample	Flow cytometry ratio	Immunoblot band intensity ratio
Control	1.000	1.000
PapMV	0.631	0.587
R837	0.166	0.026
Poly I:C	0.716	1.011

2005). Among these studies, it has been suggested that expression of Sca-1 could discriminate between IFN-α producing pDCs and those that do not: Sca-1$^+$ pDCs were weak producers of IFN-α while Sca-1$^-$ pDCs were strong producers (Niederquell *et al.*, 2013). Thus, by evaluating the expression of Sca-1 in pDCs, we sought to determine whether it could explain the inhibition in IFN-α production observed with longer periods between PapMV treatments. As previously described (Niederquell *et al.*, 2013), pDCs from the spleen are mostly Sca-1$^+$ (88.45 % Figure 2.4A; Spleen; Naive) while pDCs from the bone marrow, although still in majority Sca-1$^+$ (69.90 % Figure 2.4A; Bone marrow; Naive), display of a larger population of Sca-1$^-$ than in the spleen. After an immunization with PapMV, the proportion of pDCs expressing Sca-1 increased to close to 100 % in both the spleen and the bone marrow (Figure 2.4A) and remained elevated for at least 5 days in both organs. The distribution of Sca-1 expression among pDCs returned to naive and control levels by 25 days post PapMV immunization. To determine which of the Sca-1 expression profile was associated with IFN-α production, we performed IFN-α intracellular staining on pDCs from spleen and bone marrow of mice immunized with 400 µg of PapMV 4 hours prior. In the spleen, most IFN-α$^+$ pDCs were Sca-1$^+$ (Figure 2.4B). However, in the bone marrow, both Sca-1$^-$ and Sca-1$^+$ pDCs were expressing IFN-α after a PapMV immunization (Figure 2.4B). No significant difference was denoted between the two groups in the bone marrow. Niederquell *et al.* (Niederquell *et al.*, 2013) proposed that Sca-1$^-$ pDCs could be precursors of Sca-1$^+$ pDCs. In this regard, it is possible that the proportion of Sca-1$^+$ IFN-α$^+$ pDCs are in fact Sca-1$^-$ cells that are upregulating Sca-1 in response to stimulation. When intracellular staining for IFN-α was performed 2 and 6 hours post immunization to assess the progression of the IFN-α$^+$ population with respect to Sca-1 expression in both spleen and bone marrow, no difference was observed between the three time points (data not shown). Altogether, these results indicate that although

PapMV induces expression of Sca-1, IFN-α production does not seem to be associated with Sca-1 expression in this experimental setting.

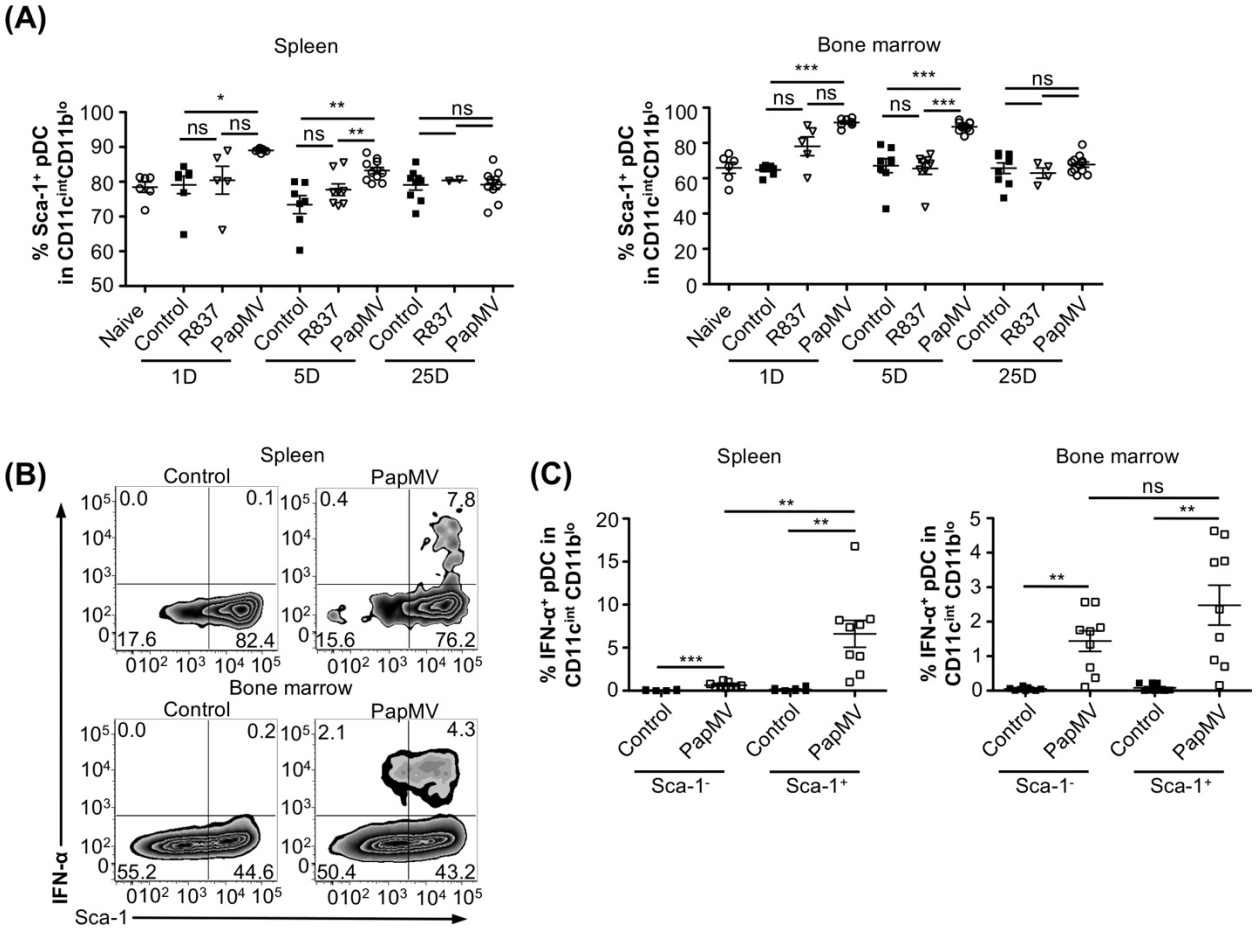


Figure 2.4: PapMV induces Sca-1 on splenic and bone marrow pDCs despite its expression not being associated to IFN-α production.

(A) Percentages of pDCs from spleen (left) and bone marrow (right) positive for Sca-1 expression 1, 5 and 25 days following an immunization with control (black squares), R837 (open inverted triangles) and PapMV (open circles) (n = 2 to 4, 1 to 3 mice per group) (B) Representative flow cytometry plots of IFN-α production by pDCs according to their Sca-1 expression profile 4 hours post immunization followed by a 4 hour incubation with BFA. Spleen (top) and bone marrow (bottom) samples are represented. (C) Percentages of intracellular IFN-α$^+$ pDCs found in the spleen (left) and the bone marrow (right) 4 hours following an immunization with control or PapMV followed by 4 hour incubation with BFA before intracellular staining (* p <0.05; ** p <0.01; *** p < 0.001)

1.4.6 Antibodies are responsible for long-term attenuation of the response of pDCs to PapMV immunization

Our results suggest that inhibition of the response to multiple administrations of PapMV is induced through shared mechanisms between TLR-associated pathways for short periods (Figure 2.3A) and PapMV-specific components that affect IFN-α production (Figure 2.2A) as well as pDC

activation (Figure 2.2B) for longer periods. We were therefore interested in assessing the role played by antibodies in the refractory state induced by PapMV pre-treatment knowing that they were shown to affect responses to prime-boost vaccine regimens in other systems (Barouch *et al.*, 2004, Sumida *et al.*, 2004). We determined the antibody-mediated impact of PapMV pre-treatment on further PapMV immunizations using J_HT mice, which lack functional B cells and consequently also lack antibodies (J. Chen *et al.*, 1993). As observed in C57Bl/6 mice (Figure 2.2A), a single immunization with PapMV in J_HT mice induced the production of IFN-α (Figure 2.5A) although in a slightly more pronounced fashion. When a PapMV pre-treatment was administered 5, 9 or 25 days prior to secondary PapMV immunizations, the production of IFN-α following the second immunization was equivalent to the response of naive animals, which differed significantly from results obtained in C57Bl/6 mice (Figure 2.5A). Since IFN-α production was not affected by the pre-treatment, we examined the expression of CD69 and CD86 on pDCs after multi-treatments with PapMV. Similarly to what was observed with IFN-α production, CD69 and CD86 (Figure 2.5B) expression on pDCs was not significantly different between naive mice and PapMV pre-treated mice receiving a PapMV immunization. To confirm that the IFN-α production and pDC activation in J_HT mice was due to the absence of antibodies and not of B cells, we performed transfer experiments with immune serum. Since two different profiles are observed at day 5 and day 25 in WT mice with respect to the inhibition generated by a PapMV pre-treatment, we assessed the kinetics of IgM and IgG production in the serum of mice after a PapMV immunization (Figure 2.5C). As expected, both IgM and IgG specific for PapMV were found in the serum of WT mice 5 days post immunization while 25 days post immunization, only a high IgG titer was detected. Transfers were carried out with serum collected either 5 or 25 days after PapMV administration pooled from matched groups. The serum from the equivalent of 2 mice was injected into one recipient mouse, which was immunized with PapMV 24 hours later (Figure 2.5D). IFN-α production was assessed in the serum 6 hours post immunization while pDC activation was assessed 24 hours post immunization. Injection of naive serum did not affect the production of IFN-α (Figure 2.5E compared to Figure 2.2A) nor did it affect the activation of pDCs as shown by the expression of CD69 and CD86 (Figure 2.5F compared to Figure 2.2B). However, when PapMV-immune serum taken at 5 or 25 days was administered to mice, the following immunization with PapMV showed no production of IFN-α (Figure 2.5E) and a significant decrease in the expression of CD69 and CD86 on pDCs (Figure 2.5F) although not as much as observed in WT mice. Altogether, these results indicate that PapMV-specific antibodies play a significant role in the long-term inhibition and attenuation of the immune response following a pre-treatment with PapMV.

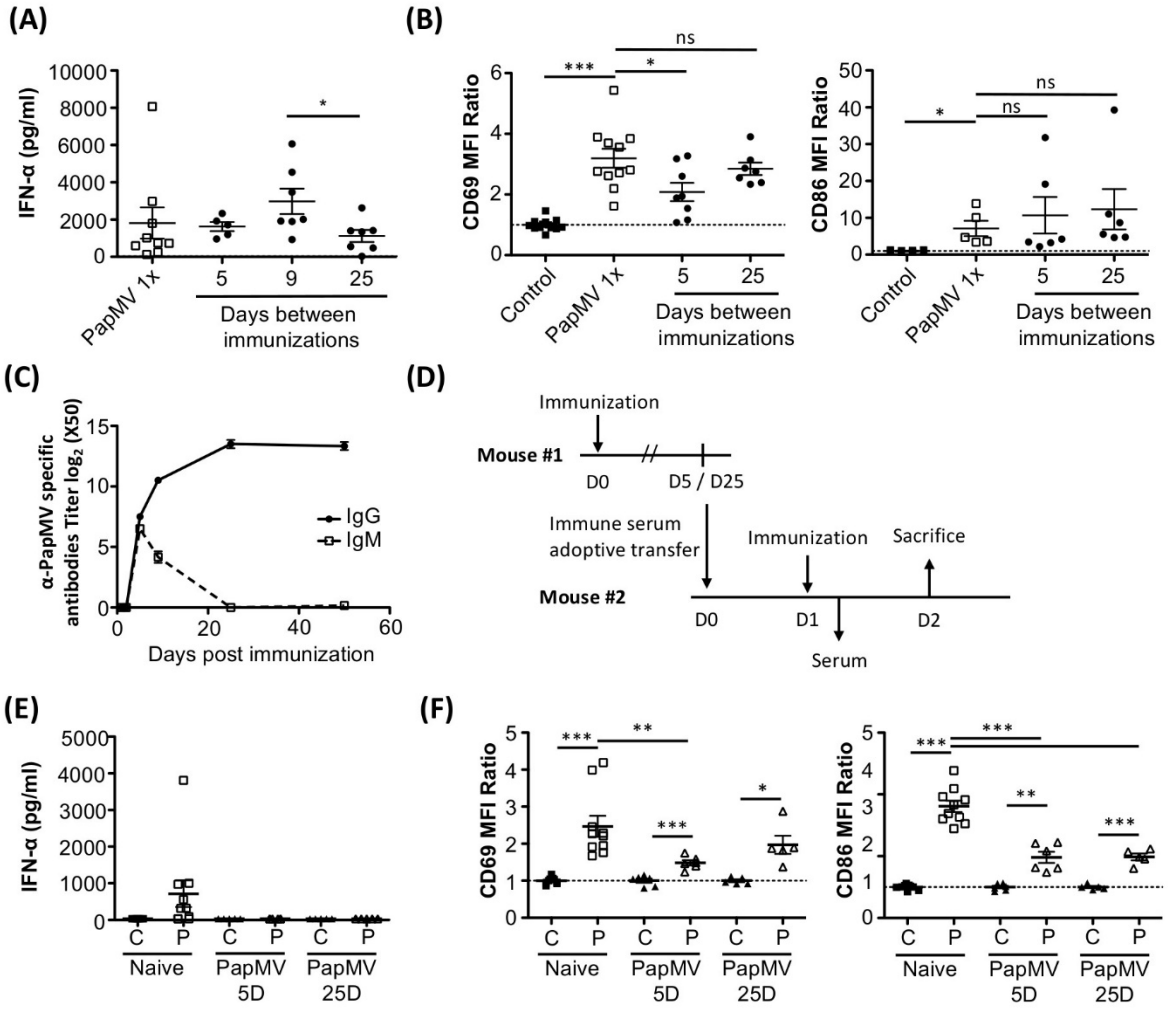


Figure 2.5: PapMV-specific antibodies are responsible for the long-term attenuation of pDC activation in response to a secondary immunization with PapMV.

(A) ELISA quantification of IFN-α in serum of J_HT mice 6 hours following immunization with PapMV once (PapMV 1x) or preceded by a PapMV pre-treatment at various intervals. (*n* = 2 to 5, two to four mice per group). (B) CD69 (left) and CD86 (right) expression on splenic pDCs of J_HT mice 24 hours following immunization with control, PapMV once (PapMV 1x) or preceded with a pre-treatment with PapMV at various time intervals. (C) Kinetics of PapMV-specific IgG and IgM development in C57Bl/6 mouse serum after immunization with PapMV as determined by ELISA. (D) Schematic representation of the serum transfer experiments. (E) ELISA quantification of IFN-α in serum of C57Bl/6 mice transferred with immune serum 6 hours following an immunization with control (C) or PapMV (P). (F) CD69 (left) and CD86 (right) expression on splenic pDCs of serum transferred C57Bl/6 mice immunized with control (C) or PapMV (P). (B, E) Results are expressed as a ratio of the MFI of the sample of the average MFI of controls. (*n* = 3 to 6, one to three mice per group) (* p <0.05; ** p <0.01; *** p < 0.001)

1.5 **Discussion**

IFN-α has long been a treatment of choice for chronic viral infections, whether used alone or in combination with other treatments such as ribavirin in the treatment of HCV. Due to its toxicity, the medical community is moving away from IFN-α-based treatments towards alternatives bearing better adverse event profiles. One alternative towards this end would be to induce endogenous IFN-α production by the host instead of administering high doses of exogenous IFN-α. It is with this aim that we used PapMV nanoparticles, which contain a non-coding ssRNA molecule rendering it non-replicative. PapMV induces the production of IFN-α by pDCs without causing any adverse effects when administered systemically and could therefore potentially accelerate the clearance of a persistent LCMV Cl13 infection. This approach would also be potentially applicable to other viral infections, as it would not require expression of virus-specific antigens. We observed that sequential application of PapMV treatment has limitations attributed to regulation of the TLR7 pathway as well as the presence of PapMV specific antibodies upon the first immunization.

The treatment of an LCMV Cl13 infection with PapMV proved to be inefficient as no changes in viral loads or in LCMV-specific immune responses were observed following treatment (Figure 2.1). Like PapMV, LCMV Cl13 is also a TLR7 ligand (reviewed in (Gilliet *et al.*, 2008, Swiecki *et al.*, 2015)). It is therefore possible that the stimulation of TLR7 by LCMV Cl13 induces TLR tolerance similar to that observed when other TLR ligands are used as stimulators (Dalpke *et al.*, 2005, Hayashi *et al.*, 2009, Lehner *et al.*, 2001, Nahid *et al.*, 2016). Further stimulation of the TLR pathways would therefore be inefficient in LCMV-infected mice. Similarly, previous research has shown that stimulation of HBV- (Xie *et al.*, 2009, Xu *et al.*, 2012) or HCV- (Rodrigue-Gervais *et al.*, 2007, Villacres *et al.*, 2008, Yonkers *et al.*, 2007) infected human cells with TLR ligands was unable to induce the production of cytokines and activate infected DCs.

Whereas we were unable to clear an LCMV Cl13 infection using PapMV administration, treatment of viral infections with exogenous IFN-α early in the course of the infection has been shown to be efficient in the control of LCMV (Y. Wang *et al.*, 2012), SIV (Sandler *et al.*, 2014) or RSV (Smit *et al.*, 2006). Of note, in the successful treatment of an LCMV Cl13 infection with IFN-α, Wang *et al.* administered the IFN-α5 subtype. However, the IFN-α subtype profile elicited by PapMV has yet to be determined. Thus, the discrepancy observed could be due to a difference in the IFN-α subtype given the disparity in the immunomodulatory effects and antiviral capacities borne by different subtypes (Gerlach *et al.*, 2009, van Pesch *et al.*, 2004, Yeow *et al.*, 1998). Furthermore, contrary to direct IFN-α injection, treatment with PapMV requires uptake of the nanoparticle, release and degradation of the ssRNA inside the endosome before IFN-α can be produced

following activation of the TLR7 signaling cascade (Acosta-Ramirez *et al.*, 2008, Lacasse *et al.*, 2008, Lebel *et al.*, 2014). Although this sequence of events ensures specificity and safety, it is likely more susceptible to various regulatory mechanisms.

LCMV Cl13 stimulates immune cells not only through the TLR7/MyD88 pathway (Gilliet *et al.*, 2008, Swiecki *et al.*, 2015) but also through the RIG-I/Mda5 pathway (Zhou *et al.*, 2010). In order to further study the mechanisms at play in this setting and isolate the TLR7 pathway from other variables of the LCMV infection, we pre-treated mice with PapMV followed by a second immunization at various time points. This approach recapitulated the results observed in LCMV-infected mice with almost complete abrogation of IFN-α production and pDC activation following the secondary PapMV immunization for short time intervals between immunizations and significant impairment for longer intervals (Figure 2.2). A similar outcome was observed for the production of various cytokines and chemokines such as TNF-α, IL-6, IL-12p40, IL-12p70, IL-15, M-CSF, RANTES and IP-10 while others were either not affected or enhanced by the secondary immunization (Supp. Figure 1). This suggests that PapMV stimulates other pathways in addition to TLR7 leading to a broad activation of the immune system and that these pathways might be differently affected by multiple PapMV administrations. Nonetheless, the main outcome of multiple systemic administrations of PapMV, at least for the TLR7 pathway, is the suppression of the secondary response. This outcome was also observed in previous studies and is indicative of TLR tolerance (discussed below) (Hayashi *et al.*, 2009, Lehner *et al.*, 2001, Nahid *et al.*, 2016, Siedlar *et al.*, 2004). We posit that the inability of subsequent PapMV immunizations to drive a robust response may also be dependent on the route of administration. This conclusion comes from our previous findings showing that sequential intranasal instillations could potentiate PapMV treatments (Mathieu *et al.*, 2013). In this previous study, immunizations were separated by seven days and the last immunization led to a higher production of various cytokines in bronchoalveolar lavages. In an intratumoral injection model, we also observed that multiple administrations of PapMV led to decreased tumor growth when administered alone or in combination with other immunotherapies and sustained IFN-α following multiple administrations (Lebel *et al.*, 2016a). Pre-treatment with PapMV is therefore able to potentiate further PapMV administrations when delivered locally. Limitations are however observed when PapMV is administered systemically, as shown in this study. The development of immunization regimens alternating between various administration routes could therefore be an interesting alternative to mitigate the pitfall of sequential systemic treatments.

With regard to the tolerization of TLRs, we found that degradation of IRAK1 played a central role. This is in agreement with other studies illustrating that this kinase, which is shared across most of the TLR pathways (Kawai *et al.*, 2010), is degraded following TLR2, TLR4, TLR7 and TLR9 stimulation (Albrecht *et al.*, 2008, Bourquin *et al.*, 2011, Liu *et al.*, 2012, Nahid *et al.*, 2016). Although this degradation has been shown to last at least 48 hours post stimulation (Bourquin *et al.*, 2011, Siedlar *et al.*, 2004) both *in vivo* and *in vitro,* the length of this refractory period has yet to be determined. Here, we show that the stimulation of BMpDCs with PapMV induces a partial degradation of IRAK1, which was observed by flow cytometry and later confirmed by immunoblotting (Figure 2.3). Of note, we observed a stronger IRAK1 degradation with R837 than with PapMV, which might be due to the different nature of both TLR7 agonists. Indeed, R837 is a small synthetic molecule that does not require uptake to reach the endosome of cells. It is therefore easier and faster for this molecule to reach more cells and induce the degradation of IRAK1 in a more robust fashion. On the other hand, PapMV is a particulate molecule that has to be taken up by immune cells to reach the endosome thus elongating the interval between the stimulation and the apparent degradation of IRAK1 (D. M. Smith *et al.*, 2013). It would also be of particular interest to verify the regulation of IRAK1 in pDCs *in vivo.* However, due to the low proportion of pDCs in the spleen, we were limited to conducting our analyses *in vitro* to determine the regulation of IRAK1 in BMpDCs. It is important to note that our results revealed only a partial role played by IRAK1 in the tolerance observed following multiple administrations of PapMV. Indeed, stimulation of BMpDCs with PapMV did not induce complete degradation of IRAK1, suggesting that there could be residual proteins left in the cells capable of proceeding through the signaling cascade when further encountering PapMV. This led us to investigate other potential inhibitory mechanisms.

Niederquell *et al.* suggested that expression of Sca-1 could discriminate between subsets of pDCs able or not to produce IFN-α in response to TLR stimulation (53). We therefore hypothesized that sequential PapMV administrations could preferentially stimulate or expand pDC subsets unable to produce IFN-α, which might explain the abrogation of IFN-α production upon secondary immunizations. However, in our system, Sca-1 expression on pDCs was not associated with the capacity to produce IFN-α in response to PapMV. While we used a particulate molecule, Niederquell *et al.* used CpG ODN as a TLR9 ligand. Given that TLR7 and TLR9 are not stimulated by the same ligands (RNA vs DNA) and similar to R837, CpG ODN is a small synthetic molecule, the kinetics of activation are therefore different in both models. In this regard, other markers such as Ly49Q (Omatsu *et al.*, 2005), CD123 (Schwab *et al.*, 2010) and CD9 (Bjorck *et al.*, 2011) that have also been associated with IFN-α production might be more informative.

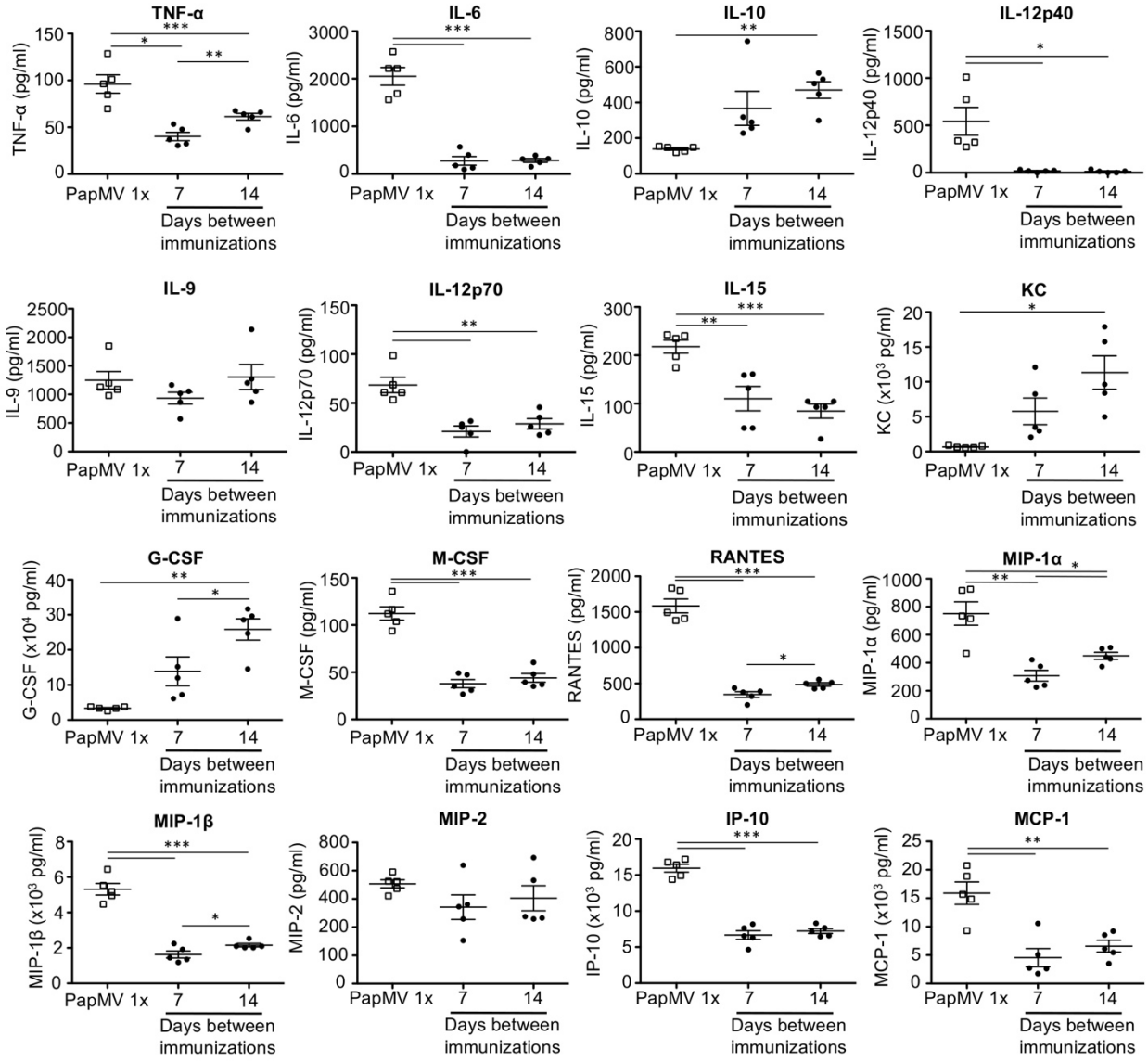
Administration of plant virus-like particles in mice leads to the rapid production of specific antibodies (reviewed in (Lebel *et al.*, 2015)). Since antibodies are generated following PapMV injection, we were interested in assessing their impact on multiple administration regimens. We showed by immunizing J_HT mice, which are devoid of B cells and antibodies, as well as performing serum transfer, that PapMV-specific antibodies generated after the first administration were largely responsible for the tolerance to a second PapMV injection. In the short-term immunization regimen (5 days), there was retained inhibition of pDC activation in J_HT mice as shown by the slightly diminished expression of CD69 following a second immunization relative to the naive group (Figure 2.5B). When transferring PapMV immune serum from day 5 post immunization into naive C57Bl/6 mice followed by PapMV immunization, we observed lower expression levels of CD69 and CD86 compared to mice receiving a single PapMV administration (Figure 2.5F compared to Figure 2.2B). This is either due to an underlying mechanism independent of antibodies or due to the titer of antibody transferred. Although the titer of PapMV specific antibodies found in mice receiving serum transfers is lower than what is found in PapMV immunized mice (Supp Fig. 3), this was enough to interfere with the subsequent PapMV immunization by inhibiting the production of IFN-α both on days 5 and 25. To overcome this limitation, one could administer immunogenically distinct plant virus-like particles to circumvent the effect of antibodies. It would also be interesting to explore the use of different injection routes and whether or not antibodies can also interfere with the response to subsequent injections.

Other directions are currently being assessed to potentiate systemically administered PapMV. We have previously shown that our platform could be modified in order to display various epitopes on the surface of the nanoparticle (Babin *et al.*, 2013, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007, Rioux *et al.*, 2012b). These engineered particles showed immunostimulatory properties similar and sometimes better than the original platform following immunization (Babin *et al.*, 2013, Carignan *et al.*, 2015, Denis *et al.*, 2008, Denis *et al.*, 2007, Lacasse *et al.*, 2008, Leclerc *et al.*, 2007). A new strategy that we are currently investigating is the use of a sortase-mediated antigen coupling technique, which permits the fusion of epitopes directly on the surface of PapMV without the need to genetically modify the sequence of the coat protein (Therien *et al.*, 2017). Immunizations with such PapMV-fused platforms lead to the development of protective humoral responses (Therien *et al.*, 2017). Different immunization regimen as well as different chronic viral infection models could here be tested to evaluate the potential of PapMV in treating other diseases. The results obtained during this study open the way to study other potential uses for PapMV such as in the treatment of autoimmune diseases. It was previously shown that in the absence of IFN-α, whether in IFNAR-

deficient mice or through the use of IFNAR antibody blockade, autoimmune symptoms of lupus prone mice were improved (Baccala *et al.*, 2012, Santiago-Raber *et al.*, 2003). Multiple systemic administrations of PapMV induced an inhibition of IFN-α production providing a potential therapeutic approach for such an application.

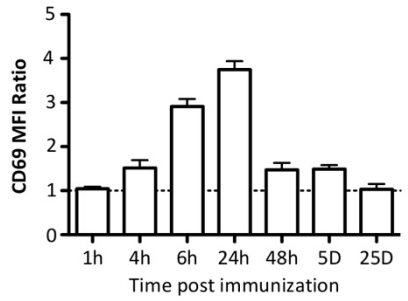
In this study, we showed that treatment of a chronic virus infection with PapMV has limitations and still needs to be improved. Although a single administration of PapMV induces strong immune responses, recall systemic immunizations are much less potent partly due to IRAK1 degradation but mainly to interference by PapMV-specific antibodies. Our results also demonstrate that the PapMV platform is able to induce an immune response following a pre-treatment although not yet to a degree that would be able to clear an ongoing viral infection. Although this outcome is not favorable in the context of chronic viral infections, it could be of interest for other diseases such as autoimmunity. Further improvements will therefore be needed for this promising therapeutic approach to be used in the treatment of chronic viral infections or other IFN-dependent chronic diseases.

1.6 **Suplementary figures**



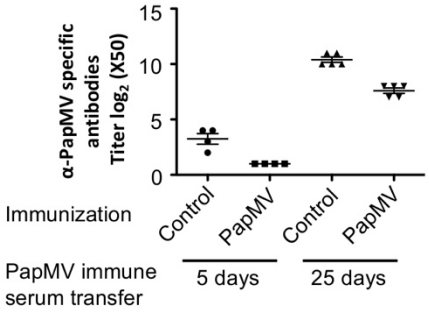
Supplementary Figure 1: Multiplex quantification of cytokines and chemokines in serum 6 hours following the last immunization with PapMV.

Immunizations were performed 7 or 14 days following a first immunization with PapMV. (*n* = 1, five mice per group)



Supplementary Figure 2: CD69 expression kinetics on pDCs after PapMV immunization.

Results are presented as a ratio of the MFI of the sample over the average MFI of controls. (*n* = 1 to 7, one to three mice per group)



Supplementary Figure 3: ELISA quantification of PapMV-specific IgG in serum of mice transferred with immune sera 6 hours following an immunization with control or PapMV.

Immune sera were collected 5 and 25 days following PapMV immunization (*n* = 2, two to three mice per group)

CHAPITRE 3 : DISCUSSION

Un des meilleurs moyens de protection contre les infections virales et bactériennes reste encore aujourd'hui la vaccination prophylactique. Bien qu'elle ait été implantée au 18^{e} siècle, la vaccination n'est que très peu utilisée en traitement d'infections et de maladies. L'IFN-α a longtemps été un traitement de choix pour les infections virales chroniques mais les effets adverses reliés à ce type de traitement posent problème (Sleijfer *et al.*, 2005). Le remplacement de l'IFN-α exogène par des molécules capables d'induire la production endogène d'IFN-α en stimulant le système immunitaire permettrait de contourner ces effets adverses. Plusieurs approches sont à l'étude dont les ligands de TLR, qui activent le système immunitaire inné et induisent la production de cytokines pro-inflammatoires, nécessaires à l'induction d'une réponse immunitaire efficace.

Nous avons précédemment démontré que les nanoparticules du virus de la mosaïque de la papaye (PapMV) ralentissent la croissance tumorale d'un mélanome murin local (Lebel *et al.*, 2016a) et ce, qu'elles soient utilisées seules en tant que plateforme vaccinale ou comme adjuvant combiné à un vaccin à base de BMDC. Cette étude a permis de démontrer le potentiel du PapMV en tant que plateforme de traitement local. Afin de traiter des infections et maladies chroniques, il est important de démontrer l'efficacité du traitement dans un modèle systémique. Nous avons ici évalué le potentiel du PapMV comme base thérapeutique dans le traitement d'une infection virale chronique modèle tel que LCMV Cl13. Ce régime de traitement s'est par contre avéré inefficace tel que démontré par l'absence de production d'IFN-α suite aux traitements ainsi que l'absence d'effet sur les titres viraux. Nous avons choisi l'IFN-α comme signe d'efficacité du traitement car le PapMV induit une forte production d'IFN-α suite à son administration systémique (Acosta-Ramirez *et al.*, 2008, Lebel *et al.*, 2014, Lebel *et al.*, 2016b) bien que cette production semble complètement inhibée lors du traitement de l'infection par LCMV Cl13. Le PapMV étant une particule versatile, il est possible que d'autres voies de signalisation aient été empruntées, ce qui pourrait se traduire en une production d'autres cytokines pro-inflammatoires tel que de l'IL-6. Une étude plus approfondie du profil de cytokines et de chimiokines produites suite au traitement de LCMV Cl13 avec PapMV devra être effectuée afin de compléter les résultats obtenus et mieux caractériser l'inefficacité du traitement au PapMV. Il a déjà été démontré qu'une infection LCMV Cl13 pouvait être traitée au moyen d'IFN-α (Y. Wang *et al.*, 2012). Dans cette étude, la plateforme de traitement consistait uniquement d'IFN-α alors que nous avons utilisé un ligand de TLR7 induisant la production d'IFN-α par les pDC (Lebel *et al.*, 2014). Dans l'étude de Wang *et al.*, les traitements ont été effectués avec de l'IFN-α5. La famille des interférons alpha comprend différents sous-types ayant tous des affinités différentes pour le récepteur IFNAR et

donc des rôles antiviraux différents (Gerlach *et al.*, 2009, van Pesch *et al.*, 2004). La production des différents sous-types d'interféron alpha est aussi régie par une cinétique qui est propre à chacun (Marie *et al.*, 1998). Il est donc possible que l'IFN-α produit suite à une immunisation au PapMV ne détienne pas l'activité antivirale optimale dans le traitement d'une infection chronique tel que LCMV Cl13 ou que sa production soit retardée en comparaison à l'interféron produit suite à l'administration d'IFN-α5. L'IFN-α, comme plusieurs cytokines sécrétées, exerce son potentiel antiviral lors de sa liaison avec son récepteur, l'IFNAR. Ce dernier se retrouve à la surface cellulaire, ce qui le rend facilement accessible en plus d'être un récepteur ubiquitaire. Lors de l'administration directe d'IFN-α, la réponse immunitaire est rapidement générée car l'IFN-α atteint rapidement son récepteur. Au contraire, l'expression du TLR7 est restreinte principalement aux endosomes des pDC ainsi que plus faiblement aux lymphocytes B (Edwards *et al.*, 2003, Hornung *et al.*, 2002). Les cellules cibles du PapMV sont donc moins nombreuses et les récepteurs cibles nécessitent plus d'efforts et de travail avant d'être atteints. En effet, l'ARN du PapMV doit être reconnu par le TLR7, qui se retrouve dans les vésicules endosomales (Kawai *et al.*, 2010, Nishiya *et al.*, 2004). Cela implique une phagocytose ainsi que la destruction de la capside protéique afin de libérer l'ARN de PapMV et ainsi permettre sa liaison au TLR7. La réponse immunitaire sera donc plus lente à organiser en comparaison à la réponse immunitaire générée suite à une administration systémique d'IFN-α. Bien que l'administration systémique d'IFN-α ait été efficace dans le traitement d'une infection au LCMV Cl13 par l'équipe de Wang *et al.*, il faut tout de même être prudent car une présence prolongée d'IFN-α favorise la chronicité de l'infection et non son traitement (Sandler *et al.*, 2014, Teijaro *et al.*, 2013, Wilson *et al.*, 2013). Suite à notre étude, il nous est impossible de déterminer si les traitements de PapMV pourraient favoriser la chronicité de l'infection aux dépends de l'hôte. Il serait par contre intéressant de comparer plusieurs régimes de traitement afin d'évaluer si des administrations plus hâtives de PapMV seraient bénéfiques dans le traitement du LCMV Cl13 bien qu'une fenêtre si courte de traitements pourrait être trop contraignante pour un possible traitement chez l'humain.

Le virus de la chorioméningite lymphocytaire (LCMV) est un virus à ARN simple brin qui est reconnu entre autres par le TLR7 (Borrow *et al.*, 2010, Jung *et al.*, 2008) mais peut aussi être reconnu par d'autres voies cytosoliques tel que Mda-5 et RIG-I (Zhou *et al.*, 2010). Afin de limiter les effets observés à la voie de signalisation du TLR7 et d'évaluer le potentiel du PapMV en tant que traitement systémique, nous avons remplacé l'infection au LCMV Cl13 par une immunisation au PapMV. Le prétraitement au PapMV semble avoir le même effet qu'une infection LCMV Cl13 sur la production de cytokines tel que l'IFN-α, TNF-α, l'IL-6, l'IL-12p40 et l'IL12p70 suite à une

seconde administration. Le prétraitement de PapMV semble par contre augmenter la production d'IL-10, ce qui a auparavant été démontré suite à l'administration répétée d'un ligand de TLR7 lors du traitement d'un modèle de cancer (Bourquin *et al.*, 2011). Il a été démontré que l'IL-10 est produit lors d'une infection LCMV et que le blocage du récepteur IL-10R permet de limiter l'infection (Ejrnaes *et al.*, 2006). Cette caractéristique devra donc être étudiée afin d'évaluer si la production d'IL-10 suite aux immunisations répétées de PapMV induit une immunopathologie qui ne serait pas observée suite à une immunisation simple.

Nous avons par la suite évalué si les pDC, principales productrices d'IFN-α lors d'une infection virale, seraient affectées par les immunisations répétées de PapMV, ce qui pourrait expliquer l'absence de production d'IFN-α et d'autres cytokines. Dans un régime d'administrations court, les pDC sont incapables de répondre à une seconde immunisation de PapMV. Cela semble indiquer un mécanisme inhérent aux pDC, qui est affecté suite à un prétraitement de PapMV. Un tel mécanisme permettrait de protéger l'hôte lors d'une infection afin d'éviter de générer une réponse immunitaire en continu ce qui peut engendrer des effets indésirables importants (Foster *et al.*, 2009). L'organisme paralyse alors certaines voies de signalisation afin d'empêcher la transmission d'un signal suite aux stimulations subséquentes du récepteur et ainsi limiter la production de médiateurs inflammatoires. Ce mécanisme de protection a tout d'abord été observé lors de chocs septiques, où les bactéries se retrouvent dans la circulation sanguine, induisant ainsi un état de stimulation continu. Les TLR4 des cellules immunitaires sont alors grandement sollicités. Afin de limiter la production continue de médiateurs inflammatoires, l'hôte paralyse son système immunitaire en dégradant certaines protéines retrouvées dans la cascade de signalisation des TLR. Une de ces protéines est IRAK1, kinase que l'on retrouve dans un complexe protéique permettant le relai du signal du récepteur au facteur de transcription (Figure 1.1). Lorsqu'il y a déficience en IRAK1, les souris ou les cellules étudiées ne parviennent pas à répondre à une stimulation des TLR (Gottipati *et al.*, 2008, Swantek *et al.*, 2000), dénoté entre autres par l'absence d'IFN-α produit suite à une stimulation du TLR9 (Uematsu *et al.*, 2005). La dégradation d'IRAK1 suite à la stimulation de certains TLR serait expliquée par l'expression du micro ARN miR146a. Ce micro ARN cible la séquence d'ARN d'IRAK1 ainsi que de TRAF6 et limite leur expression protéique. La dégradation de protéines de signalisation empêche donc le relai du signal entre le récepteur et les facteurs de transcription, inhibant ainsi la réponse immunitaire suite aux stimulations subséquentes des TLR. En induisant la dégradation d'IRAK1 *in vivo* grâce au miR146a, cela nous permettrait de déterminer si cette dégradation est à l'origine de la tolérance induite par le PapMV. Il ne faut pas non plus exclure tous les autres mécanismes

possibles. En effet, la cascade de signalisation des TLR est complexe et requiert plusieurs acteurs. Il est donc possible que d'autres protéines soient affectées suite à une immunisation au PapMV. Il serait donc important de caractériser plus en détails la cascade de signalisation des TLR afin de déterminer si IRAK1 est la seule protéine affectée par une immunisation au PapMV.

Le modèle *in vitro* de cellules dendritiques plasmacytoïdes dérivées de la moelle osseuse (BMpDC) nous a permis d'établir que le PapMV induit une dégradation partielle d'IRAK1. Un des facteurs pouvant influencer la régulation d'IRAK1 est la quantité de ligand administré. En effet, même si 100 µg de PapMV ont été utilisés pour la stimulation de BMpDC, seulement l'ARN de PapMV pourra lier le TLR7 (Lebel *et al.*, 2014), ce qui représente environ 5 % de la masse totale de PapMV (Erickson *et al.*, 1976). En présence de ligands synthétiques, tel que le R837, l'intégralité des molécules administrées détiennent le potentiel de lier un récepteur. Avec une plus grande quantité de molécules stimulatrices disponibles, plus de cellules peuvent être atteintes, ce qui pourrait se traduire en une dégradation plus prononcée d'IRAK1. Le PapMV étant une molécule particulaire, cette dernière doit être phagocytée afin d'atteindre les endosomes, où se retrouvent les TLR7. Au contraire, le R837, qui est une molécule chimique de plus petite taille, ne requiert pas de processus complexe afin d'atteindre le TLR7. La réponse immunitaire induite suite à une stimulation au R837 est donc plus rapide qu'avec le PapMV, ce qui explique la différence de cinétique mais aussi la différence dans le niveau de régulation d'IRAK1. Si la régulation d'IRAK1 se confirme *in vivo,* cela pourrait expliquer l'hétérotolérance partielle observée avec le LPS et le R837 suite à un prétraitement au PapMV. L'hétérotolérance entre les différents TLR dû à la régulation d'IRAK1 a auparavant été démontrée et engendre la diminution de la production d'IFN-α (Koga-Yamakawa *et al.*, 2015, Liu *et al.*, 2012) et de TNF-α (C. H. Li *et al.*, 2006, Nahid *et al.*, 2016, S. Sato *et al.*, 2002). Bien qu'une hétérotolérance soit observée lorsqu'une immunisation au LPS ou au R837 est précédée d'un prétraitement au PapMV, l'inhibition n'est pas totale. Un mécanisme spécifique au PapMV serait donc impliqué dans l'homotolérance au PapMV.

Bien que les pDC soient les principales productrices d'IFN-α lors d'une infection virale, il a été démontré que ce ne sont pas toutes les pDC qui ont un potentiel de production d'IFN-α identique (Bjorck *et al.*, 2011, Niederquell *et al.*, 2013, Omatsu *et al.*, 2005, Pelayo *et al.*, 2005, Schwab *et al.*, 2010, Zhang *et al.*, 2017). Cette dichotomie a été étudiée par l'équipe de Niederquell *et al.* qui se sont servis de Sca-1 comme marqueur de dichotomie chez les pDC. Ce marqueur est peu connu comme étant exprimé à la surface des pDC mais plutôt comme étant exprimé à la surface des lymphocytes suite à leur activation via un ligand ou une infection virale (H. C. Chen *et al.*,

2003, K. R. Kumar *et al.*, 2005, van de Rijn *et al.*, 1989, Whitmire *et al.*, 2009). Dans cette étude, l'équipe de Niederquell *et al.* a démontré que les pDC exprimant Sca-1 (Sca-1$^+$) sont de faibles productrices d'IFN-α alors que les pDC n'exprimant peu ou pas Sca-1 (Sca-1$^-$) seraient responsables de la forte production d'IFN-α suite à une stimulation du TLR9. Cette association entre l'expression de Sca-1 et la production d'IFN-α concorde avec des observations faites chez la souris *lpr,* un modèle classique du lupus systémique érythémateux. Lorsqu'elles sont vieilles, ces souris expriment un haut niveau d'ARN de *Sca1* mais sont incapables de produire de l'IFN-α suite à une stimulation du TLR9. Au contraire, les souris *lpr* plus jeunes ont des niveaux d'expression de l'ARNm de *Sca1* beaucoup plus faibles mais démontrent une forte production d'IFN-α suite à une stimulation du TLR9 (Liao *et al.*, 2015). Nous avons par contre démontré que l'expression de Sca-1 à la surface des pDC n'est pas un indicateur des capacités de production de l'IFN-α suite à une stimulation au PapMV. Bien que plusieurs de nos résultats concordent avec les résultats de Niederquell *et al.*, certaines différences peuvent expliquer notre incapacité à corréler l'expression de Sca-1 à la production d'IFN-α par les pDC suite à une stimulation au PapMV. Tel que mentionné précédemment, la différence dans les ligands utilisés pourrait expliquer certaines différences. En effet, dans cette étude, des CpG ont été utilisés, qui réagissent de façon similaire au R837 étant donné leur nature synthétique et leur petite taille. Il est aussi possible que d'autres marqueurs soient plus appropriés pour l'étude de la dichotomie des pDC en réponse au PapMV. Par exemple, les pDC Ly49Q$^+$ produisent des cytokines pro-inflammatoires suite à une infection au moyen de virus de l'influenza PR8 alors que les Ly49Q$^-$ n'y parviennent pas (Kamogawa-Schifter *et al.*, 2005). Le PapMV étant à la base un virus, le marqueur Ly49Q serait potentiellement plus approprié vu son implication dans la dichotomie face à une infection par le virus de l'influenza. Une autre étude a aussi démontré que la population de pDC responsable de la production d'IFN-α exprime un plus haut taux d'ARNm d'IRF7 en comparaison avec la population de pDC ne produisant pas d'IFN-α suite à une stimulation de TLR (Zhang *et al.*, 2017). Une combinaison de facteurs pourrait donc expliquer les divergences en production d'IFN-α. Une meilleure caractérisation de l'effet à long terme d'une immunisation au PapMV serait ici très importante.

Lors d'un régime d'administrations à plus long terme, le prétraitement de PapMV semble affecter les pDC sans toutefois inhiber complètement leur réponse à une immunisation subséquente. La durée de vie des pDC a été évaluée à 15 jours dans la rate (O'Keeffe *et al.*, 2002). Lorsque l'on administre le PapMV plus de 15 jours suivant le prétraitement, les pDC qui vont interagir avec le PapMV seront naïves ; ces dernières n'auront jamais rencontré le PapMV. La réponse

immunitaire suite à une immunisation répétée devrait donc être aussi forte qu'une immunisation simple. Les résultats ici présentés indiquent par contre qu'un mécanisme externe, qui n'est pas inhérent aux pDC, serait responsable de la tolérance observée. Ce mécanisme serait plutôt spécifique au PapMV et aurait une influence moyenne à plus long terme. Suite à une première immunisation au PapMV, une grande quantité d'anticorps spécifiques sont produits. Lors d'une seconde immunisation au PapMV, un état de compétition s'installe entre les anticorps spécifiques au PapMV et les cellules immunocompétentes (Birnbaum *et al.*, 1975, Siskind *et al.*, 1968). Cette compétition pour le PapMV limiterait le nombre de particules disponibles pour monter une réponse immunitaire ce qui signifie que moins de cellules immunitaires seront stimulées par PapMV.

Notre équipe a précédemment démontré que la présence d'anticorps anti-PapMV générés suite à une immunisation sous-cutanée n'affecte pas la production subséquente d'anticorps contre un antigène spécifique lors d'immunisations doubles (Denis *et al.*, 2008, Denis *et al.*, 2007, Rioux *et al.*, 2012a, Savard *et al.*, 2011) ni la protection contre une infection léthale (Rioux *et al.*, 2012a, Savard *et al.*, 2011). Les résultats présentés dans ce mémoire indiquent que les immunisations systémiques sont régies par un mécanisme plus contraignant, se traduisant en l'incapacité de potentialiser les immunisations subséquentes. Lors d'une immunisation sous-cutanée, les molécules composant le vaccin sont retenues plus longtemps dans le système lymphatique et au site d'injection, limitant ainsi leur disponibilité dans la circulation sanguine (Beaulieu *et al.*, 2010, Richter *et al.*, 2012). Cette rétention d'antigènes vaccinaux au site d'injection ralentit leur reconnaissance et interaction avec le système immunitaire, ce qui permettrait d'allonger la durée de la réponse immunitaire. Lors d'immunisations systémiques via la voie intra-veineuse, la disponibilité du vaccin dans la circulation sanguine est immédiate, ce qui se traduit en une élimination rapide des molécules vaccinales (Beaulieu *et al.*, 2010, Estcourt *et al.*, 2005). Lors d'immunisations systémiques répétées, le vaccin est rapidement traité par le système immunitaire qui a déjà été en contact avec ces molécules. Cette interaction rapide entre le système immunitaire et le vaccin pourrait être trop courte pour l'élaboration d'une réponse immunitaire efficace. Il a aussi été démontré qu'une immunité préexistante, par exemple due à une vaccination antérieure, inhibe la génération d'une immunité cellulaire. Les anticorps dirigés contre les nanoparticules du VPH limitent la liaison et la phagocytose des VLP par les CPA (Da Silva *et al.*, 2001). Dans notre modèle, les anticorps générés suite au prétraitement de PapMV pourraient être responsables de l'inhibition observée suite aux immunisations subséquentes et empêcheraient la génération d'une réponse immunitaire efficace.

Bien que l'emploi du PapMV dans le traitement d'infections virales chroniques semble apporter son lot de défis, ce projet de maîtrise semble indiquer que le PapMV pourrait être utilisé dans le traitement d'une maladie auto-immune où l'IFN-α joue un rôle important dans l'immunopathologie. En effet, le lupus systémique érythémateux est une maladie auto-immune où la production d'IFN-α est très élevée et cela contribue à la progression et l'immunopathologie de la maladie. Il a été démontré que la déplétion hâtive des pDC améliore les symptômes cliniques de souris prônent au lupus (Rowland *et al.*, 2014). Dans un contexte de lupus, les pDC seraient responsables de l'activation aberrante de la réponse immunitaire adaptative ainsi que de la progression de la maladie. Leur déplétion tôt dans l'établissement de la maladie permet donc de contrôler les symptômes. Lorsque la déplétion est effectuée dans les développements plus tardifs de la maladie, certains symptômes sont améliorés mais la majorité restent inchangés (Rowland *et al.*, 2014). D'autres études ont aussi démontré que l'absence de l'activité de l'IFN-α, que ce soit via le blocage de l'IFNAR (Baccala *et al.*, 2012) ou la déplétion du gène du récepteur de l'IFN-α (Santiago-Raber *et al.*, 2003), améliore l'immunopathologie du lupus chez les souris. Suite à ce que nous avons démontré dans cette étude, il semblerait que le PapMV pourrait être étudié dans le traitement du lupus. La tolérance induite suite à un traitement systémique de PapMV pourrait aider dans l'amélioration de l'immunopathologie reliée au lupus. Plusieurs régimes de traitements devront être évalués afin de déterminer la fenêtre de traitement optimale.

En résumé, cette étude a permis de démontrer que deux mécanismes seraient responsables de l'incapacité du PapMV à traiter une infection virale chronique chez la souris soit un mécanisme général, partagé par les TLR ainsi qu'un mécanisme spécifique au PapMV. Ces deux mécanismes méritent d'être approfondis afin de mieux comprendre le mode d'action du PapMV et ainsi d'en potentialiser son utilisation. De plus, il s'agit ici d'une exploration des différents mécanismes potentiels. Il nous est donc impossible d'exclure tous les mécanismes possibles, qui devront être étudiés afin de dresser un portrait plus adéquat de la tolérance au PapMV.

CHAPITRE 4 : CONCLUSION

Ce travail de maîtrise visait à évaluer le potentiel du PapMV comme plateforme vaccinale dans le traitement d'infections virales chroniques. Nous avons démontré que le traitement d'une telle infection modèle au moyen du PapMV est limité par deux mécanismes inhibiteurs. Le premier mécanisme, qui consiste en la dégradation rapide d'IRAK1 suite à une immunisation au PapMV, serait responsable de la tolérance immunitaire à court terme. Il serait intéressant de confirmer ce mécanisme *in vivo* ainsi que d'établir la durée de la dégradation d'IRAK1. Cette fenêtre indiquerait les limites d'un régime d'administrations thérapeutiques de PapMV. Le deuxième mécanisme potentiellement responsable de la tolérance immunitaire suite à une immunisation au PapMV serait médiée par les anticorps spécifiques au PapMV, qui inhibent partiellement la réponse immunitaire au PapMV. L'utilisation de différentes plateformes vaccinales hétérologues ou couplées à des protéines d'intérêts pourrait permettre de contourner cette inhibition à long terme. Il serait aussi intéressant d'évaluer la tolérance immunitaire induite suite à l'utilisation du PapMV exprimant différents antigènes à sa surface. Cela nous permettrait de prévoir l'utilisation de telles plateformes dans le traitement de maladies. Afin de potentialiser les immunisations de PapMV, il serait aussi important de vérifier si l'effet est le même lorsque l'on administre le PapMV conjointement avec un autre vaccin déjà utilisé sur le marché. Même si la réponse au PapMV n'est pas optimale, son utilisation en tant qu'adjuvant pourrait permettre de diminuer les doses de vaccin utilisées et ainsi pouvoir traiter plus de patients.

Suite à ces travaux, nous proposons le modèle suivant afin d'expliquer la tolérance immunitaire suite aux immunisations répétées du PapMV (Figure 8). Lors d'une première immunisation, les cellules naïves tel que les pDC vont phagocyter le PapMV, qui va se retrouver dans les endosomes. À ce moment, le PapMV libère son ARN simple brin afin qu'il soit reconnu par le TLR7. Ceci engendre une cascade de signalisation recrutant le complexe protéique MyD88-IRAK1-IRAK4-TRAF6. Suite au recrutement de ce complexe protéique, IRF7 est phosphorylé et dimérise, ce qui lui permet de transloquer au noyau et ainsi induire la production d'IFN-α (Figure 8). L'activation de la cascade de signalisation du TLR7 mène aussi à la dégradation d'IRAK1, ce qui va causer un déséquilibre dans la voie de signalisation. Suite à une première immunisation, les cellules immunitaires innées et adaptatives sont activées, menant à la production de plusieurs médiateurs pro-inflammatoires ainsi que des anticorps anti-PapMV. Lors d'une deuxième immunisation dans un court intervalle de temps, les anticorps anti-PapMV présents, bien que la production n'ait pas encore atteint le seuil maximal, peuvent limiter la disponibilité de quelques particules. Ceci s'ajoute à la dégradation d'IRAK1 suivant la première immunisation. L'absence d'IRAK1 dans la cascade de signalisation empêche donc la transmission du signal. Aucune cytokine n'est alors produite et les cellules immunitaires ne sont pas activées de nouveau (Figure

8). Lors d'une seconde immunisation séparée d'un plus long intervalle de temps du prétraitement, les anticorps anti-PapMV sont présents à capacité maximale et une compétition entre les cellules immunitaires et les anticorps prend place. Plusieurs particules de PapMV ne sont donc pas disponibles pour la phagocytose par les cellules immunitaires. Trois options sont ici possibles : soit la production d'IFN-α est si faible qu'elle ne peut pas être détectée, l'IFN-α produit en faible quantité est trop rapidement utilisé par les cellules environnantes pour être détecté ou les pDC sont incapables de produire de l'IFN-α (Figure 8).

Plusieurs paramètres restent encore à être évalués afin de mieux caractériser la tolérance immunitaire induite par le PapMV. Nous avons par exemple démontré une tolérance par la voie intra-veineuse mais est-ce que cette tolérance est aussi observée lors de l'utilisation d'autres voies d'immunisation ? Si ce n'est pas le cas, serait-il possible de varier les voies d'administration afin d'optimiser la réponse immunitaire au PapMV ? Une caractérisation plus approfondie des différentes conditions de traitement avec le PapMV est donc nécessaire afin d'optimiser son utilisation future. L'utilisation en parallèle de plateformes de traitement déjà établies pourrait aussi être une alternative à prendre en considération afin de diminuer les doses de traitement ainsi que les effets indésirables.

En conclusion, l'utilisation du PapMV en tant que plateforme pour le traitement d'infections et de maladies chroniques nécessite encore de l'optimisation. Nous avons démontré deux mécanismes pouvant être responsables de la tolérance immunitaire observée. Ces pistes de solutions pourront nous aider dans l'élaboration d'un traitement éventuel à base du PapMV.

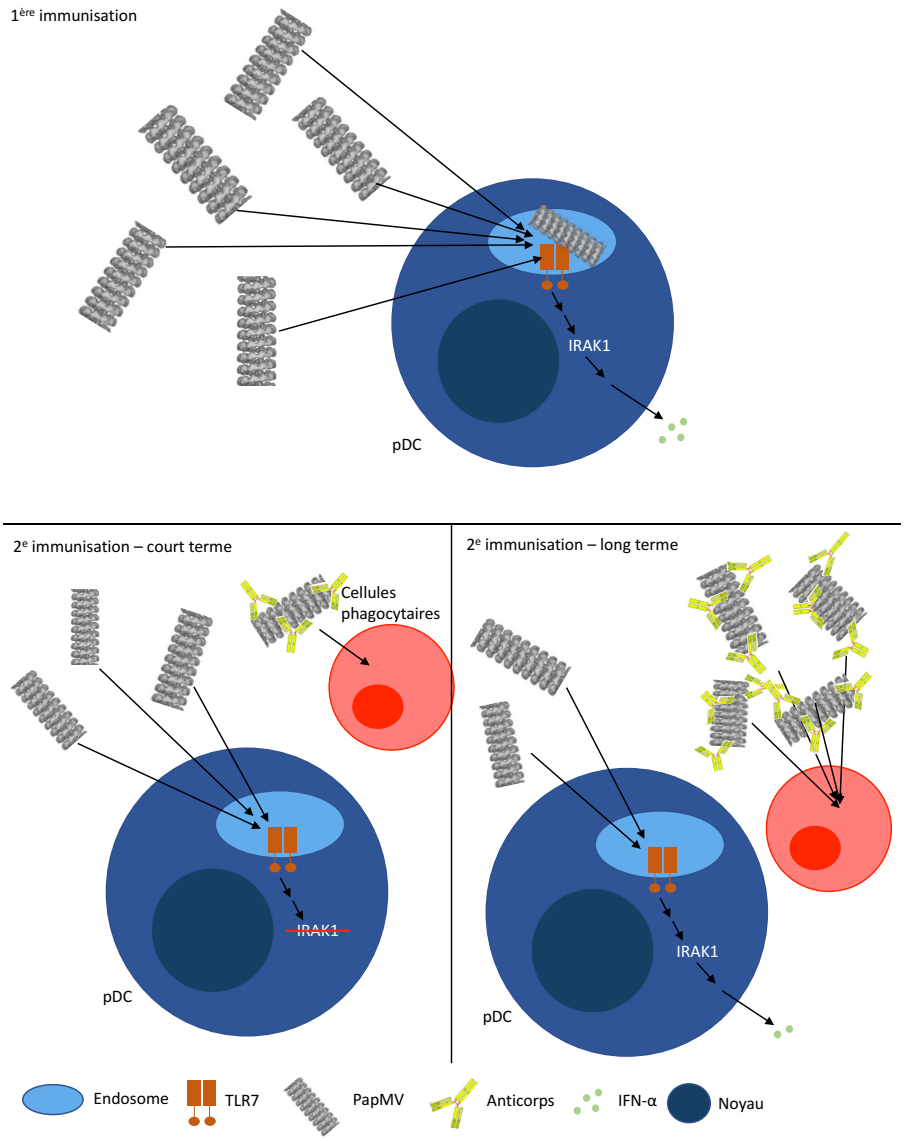


Figure 4.1 : Mécanismes de tolérance induit suite aux immunisations au PapMV

Lorsque le PapMV est administré chez une souris naïve, l'ARN de PapMV est reconnu par le TLR7, ce qui engendre une cascade de signalisation passant par IRAK1 et induisant la production d'IFN-α. Lors d'une seconde immunisation dans un court délai, la cascade de signalisation du TLR7 ne peut s'effectuer car IRAK1 a été dégradée suite au prétraitement de PapMV. Les anticorps anti-PapMV présents en faible nombre peuvent aussi affecter la disponibilité du PapMV. Lors d'immunisations répétées dans des délais plus long, la présence massive d'anticorps anti-PapMV limite de beaucoup le nombre de particules disponibles afin d'engendre une réponse immunitaire. Bien que la cascade de signalisation du TLR7 soit fonctionnelle, la faible quantité de PapMV présent limite la production d'IFN-α.

CHAPITRE 5 : RÉFÉRENCES

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CHAPITRE 6 : ANNEXES

ARTICLE 3: NANOPARTICLE ADJUVANT SENSING BY TLR7 ENHANCES CD8$^+$ T CELL-MEDIATED PROTECTION FROM *LISTERIA MONOCYTOGENES* INFECTION

Titre en français

La reconnaissance d'une nanoparticule adjuvant par le TLR7 augmente la protection contre *Listeria monocytogenes* médiée par les lymphocytes T CD8$^+$

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UK : Conception du projet

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Résumé en français

Le développement de plateformes vaccinales et adjuvantes est primordial dans la lutte contre plusieurs maladies infectieuses et le cancer. Très peu d'adjuvants sont présentement approuvés pour l'usage chez l'humain et la majorité d'entre eux activent principalement la réponse immunitaire humorale. Toutefois, les anticorps spécifiques ne sont pas suffisants pour conférer une protection contre les infections persistantes ainsi que le cancer. Le développement d'adjuvants et d'immunomodulateurs pouvant améliorer la réponse immunitaire cellulaire représente donc un besoin médical majeur. Nous avons récemment démontré que les nanoparticules du virus de la mosaïque de la papaye (PapMV), qui s'auto-assemblent à partir des protéines de la capside (CP) autour d'un brin d'ARN synthétique non codant, sont hautement immunogéniques chez la souris. Le PapMV peut être utilisé autant à titre de plateforme vaccinale par la fusion d'épitopes variés aux CP ainsi qu'à titre d'adjuvant pour améliorer la réponse immunitaire humorale générée contre des antigènes ou des vaccins conjointement administrés. Les mécanismes conférant ces propriétés immunomodulatrices au PapMV ainsi que son habileté à améliorer la réponse au vaccins T restent encore inconnus. Grâce aux immunisations en souris, nous démontrons que le PapMV est un nouvel agoniste du TLR7 détenant de fortes propriétés immunomodulatrices. De plus, le prétraitement avec du PapMV augmente de façon significative la réponse effectrice et mémoire des lymphocytes T $CD8^+$ générées lors de la vaccination au moyen de cellules dendritiques, améliorant ainsi la protection contre une infection avec *Listeria monocytogenes.*

Introduction

Vaccination is considered to be the most effective method of protection against infectious diseases. Indeed, vaccines have not only helped to reduce the incidence of several infectious diseases such as measles or diphtheria, they also contributed to the decrease

in mortality and morbidity related to infectious diseases and in the eradication of smallpox (1, 2). However, the generation of safe vaccines with the capacity to generate protective cellular immunity, which is essential to protect against most chronic infections (3, 4) and cancers (5, 6), is still a challenge. Cell-mediated immune responses are often induced following the detection of pathogen associated molecular patterns (PAMPs) by APCs leading to their activation, which increases their stimulatory capabilities towards pathogen-specific T lymphocytes. This has lead to the development of numerous vaccination trials that include various PAMPs in the formulation of candidate vaccines (7-9) instead of traditional adjuvants such as alum, which mostly induces a humoral response (10). As such, TLR7/8 ligands that induce the production of IL-12 and interferon-alpha (IFN-α), which are important for the generation of an appropriate cellular immune response, are actively being evaluated for this purpose (11). Unfortunately, the natural ligand of TLR7/8, ssRNA, when used alone, is rapidly degraded *in vivo* (12, 13). To solve this problem and allow optimal presentation of vaccine antigens, researchers have developed various delivery systems to increase the bioavailability of such molecules. These tools include molecules that induce the formation of aggregates such as alum, liposomes, oil in water emulsions and nanoparticles (14). These formulations of vaccine antigens increase their phagocytosis by APCs, the first step required to generate a potent immune response. Another way to efficiently trigger a T cell response is to mimic a viral infection through the use of virus-like particles (VLPs). VLPs do not contain infectious genetic material providing a safer alternative to attenuated or inactivated viruses (15). In addition, the shape of such particles and the display of repeated arrays of epitopes on their surface renders them easily recognizable by APCs which are then activated by intrinsic PAMPs present on the VLPs (16). However, many VLPs in development still require the coadministration of adjuvants to be fully effective (17, 18). VLPs can easily be engineered to express foreign epitopes using genetic fusion or chemical conjugation (19-21). At present, there are two vaccines used in humans derived from VLPs: The hepatitis B virus vaccine and the human papillomavirus vaccine.

We have previously shown that papaya mosaic virus-like nanoparticles (PapMV) are efficiently recognized and taken-up by immune cells leading to their activation (22). In addition, we have shown that various foreign antigens can be fused to the surface of the

PapMV without affecting its ability to self-assemble. Vaccination with such nanoparticles generates a specific cellular and humoral immune response against displayed antigens and provides protection against various viral infection models (19, 22-25). Moreover, we have demonstrated that the fused antigens are efficiently cross-presented on MHC-I of human APCs and cause the expansion of human antigen-specific T cells (26). Finally, we recently showed that PapMV used as an immunomodulator leads to the development of protective immune responses against influenza or *Streptococcus pneumoniae* challenges (27). Thus, PapMV represents a promising candidate adjuvant for the development of novel vaccines or treatments. However, the mechanisms by which PapMV activates the immune system are unknown.

We demonstrate here that PapMV induces immune activation through TLR7 ligation and type I interferon production. In addition, PapMV enhances effector and memory $CD8^+$ T cell responses induced through bone marrow-derived dendritic cell (BMDC) vaccination increasing protection against a *Listeria monocytogenes* challenge. These results suggest that PapMV could be useful for the development of T-cell vaccines against infectious diseases.

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 INRS-IAF institutional animal care committee.

Mice. Female 6- to 10-week-old C57BL/6 mice were purchased from Charles River. *Tlr7* KO and *Myd88* KO mice were purchased from The Jackson Laboratory. Type I Interferon Receptor knockout mice (*Ifnar* KO) on a C57BL/6 genetic background were kindly provided by Ulrich Kalinke (Institute for Experimental Infection Research, Germany) and *Irf5/7* KO mice by Paula Pitha-Rowe (Johns Hopkins University, USA).

PapMV nanoparticles. PapMV nanoparticles used in this study were kindly provided by Folia Biotech and were produced as described in our previous study (27). LPS contamination was always below 50 endotoxin units (EU)/mg of protein and considered as negligible.

Generation of BMDC. BMDC were differentiated as described before (28). On day 6, LPS (Sigma-Aldrich) (1 μg/ml) was added to cultures to induce maturation and the $OVA_{257–264}$ peptide (SIINFEKL) (2 μg/ml) (Midwest Bio-Tech) was added (BMDC-OVA) or not (unloaded BMDC) overnight. BMDCs were harvested on day 7.

Immunization. PapMV (Folia Biotech) injections were done i.v. or s.c. with 100 μg. For BMDC-OVA immunization experiments, 100 μg of PapMV or 100 μl of PBS were injected i.v. or s.c. 6 h before i.v. or s.c. immunization with 1.25×10^6 mature BMDC-OVA or unloaded BMDC. OVA-specific $CD8^+$ T cell responses were analyzed at days 7 and 45 post-immunization in the spleen or blood. To follow BMDC phenotype following immunization, BMDC were labeled with 5 μM CFSE (Life Technologies) for 10 min at 37°C. WT mice were injected i.v. with 100 μg PapMV or PBS and 6 h later with 10×10^6 BMDC-CFSE. The expression of CD86 and CD70 were then analyzed on the $CD11c^+CFSE^+$ population, 6 h after BMDC immunization.

LM-OVA infection. Mice were infected with $2\text{-}5 \times 10^3$ *Listeria monocytogenes* expressing OVA (LM-OVA) i.v. at least 45 days post BMDC immunization. Five days post-infection (p.i.) spleen and liver were harvested, homogenized in distilled water plus 0.5% NP-40 (Sigma) and fold serial dilutions were plated onto brain heart infusion (BHI, BD Biosciences) agar plates containing 200 μg/ml streptomycin (Bio Basic). Plates were incubated at 37°C for 24 h and colonies were enumerated.

Plasmacytoid dendritic cell depletion. The hybridoma cell line producing monoclonal antibody 927, specific for the mouse bone marrow stromal antigen 2 (BST2), used for the depletion, was kindly provided by Dr Marco Colonna (Washington University school of Medicine, USA). Depletion was done as described previously (29). Briefly, mice were injected (i.p.) with 500 μg of purified antibody 927 or an isotype control 24 and 48 hours before PapMV immunization. Depletion leads to a reduction of at least 60% of pDC numbers in the spleen as measured by flow cytometry.

Flow cytometry analysis and antibodies. Flow cytometry analysis of mouse surface antigens was performed with the following antibodies: anti-CD69 (H1.2F3), -CD86 (GL1), -CD11c (N418), -CD8a (53-6.7), -CD45R/B220 (RA3-6B2), -CD19 (6D5), -CD317

(PDCA-1) (927), and -CD44 (IM7) (Biolegend) and H-2Kb (AF6-88.5.5.3) (eBioscience). Staining was performed for 20 min at 4°C. H-2Kb-OVA monomers were purchased from CANVAC tetramer core facility and tetramers were generated using extravidin-PE (Sigma). PE-coupled Kb-OVA tetramer staining was done at 37°C for 15 min. For intracellular cytokine staining, splenocytes were cultured with OVA (2 μg/ml) in the presence of brefeldin A (10 μg/ml) for 5 h at 37°C. Following staining for surface antigens as described above, cells were stained for intracellular cytokines using fixation/permeabilization buffer (Biolegend) according to the manufacturer's instructions. Antibodies used for intracellular cytokine detection were anti-IFN-γ (XMG1.2), -TNF-α (MP6-XT22), –IL-2 (JES6-5H4) (Biolegend) and -granzyme B (NGZB) (eBioscience). Flow cytometry analyses were performed on a BDLSRII or BDLSR fortessa flow cytometer (BD) and data analyzed using the FlowJo software (Tree Star).

ELISA. PapMV-specific antibody titers were determined as described previously (30). Results are expressed as an antibody endpoint titer, determined when the OD value is 3-fold greater than the background value obtained with a 1:50 dilution of serum from PBS-injected mice. IFN-α and IL-6 levels in sera and spleen homogenates from immunized mice were determined following the manufacturer's instructions (PBL InterferonSource and Biolegend).

Statistical analysis.

Data were analyzed for statistical significance using Student t test. Statistical significance was determined as $p < 0.05$.

Results

PapMV induces the activation of a broad range of immune cells through TLR7. To evaluate the extent of the immunomodulatory properties of PapMV, we first measured the activation of various immune cells following i.v. immunization of C57BL/6 wild type (WT) mice. We analyzed the expression of co-stimulatory molecule CD86, early activation marker CD69 and MHC molecule H-2Kb by flow cytometry in the spleen, peripheral lymph nodes and blood. We observed that every subtype of immune cells analyzed expressed

higher levels of CD86, CD69 and H-2Kb 24 h following administration of PapMV (Fig. 1A and S1). For example, a 5-fold increase in the expression level of CD69 on splenic T cells was measured in the PapMV-treated group in comparison with PBS-treated controls. To determine which component of the PapMV was responsible for its immunomodulatory properties, mice received either the coat protein (CP) subunit alone (containing monomers, dimers and trimers of the CP) or the complete nanoparticle. In contrast to intact PapMV nanoparticles, immunization with the CP alone did not lead to an increased expression of CD86, CD69 or H-2Kb on splenic DCs, B cells or $CD8^+$ T cells when compared to negative controls (Fig. 1B and S1B). This suggests that the ssRNA molecule contained within the VLP could be responsible for its immunomodulatory properties. To confirm this hypothesis, we immunized mice deficient in the ssRNA sensor TLR7 or molecules involved in its signaling pathway and measured immune cell activation. In contrast to WT mice, the expression levels of CD86, CD69 or H-2Kb on splenic DCs, B cells or $CD8^+$ T cells in *Tlr7*, *Myd88* or *Irf5/7* KO mice remained unchanged upon treatment with PapMV (Fig. 1B and S1B). Since activation through TLR7 signaling can lead to type I interferon production, we tested whether PapMV induced the production of IFN-α in immunized mice. Indeed, immunization with PapMV lead to the production of IFN-α in the serum and spleen of WT mice with a peak at 6 h post-immunization whereas it was completely undetectable in *Tlr7* or *Myd88* KO mice (Fig 1C and S2A). We next sought to determine the contribution of plasmacytoid dendritic cells (pDCs), which strongly express TLR7 and secrete high amounts of IFN-α, to this response. We depleted pDCs by injection of a neutralizing antibody directed against BST2, which has been shown in steady-state conditions to be mostly expressed on pDCs (31). *In vivo* depletion of $BST2^+$ cells reduced IFN-α production in serum below the detection limit (Fig. 1C) and significantly decreased the expression levels of activation markers on immune cells following PapMV immunization (Fig. 1B and S1B). Since depletion of $BST2^+$ cells caused such a dramatic decrease in the activation of immune cells with no detectable IFN-α production, we next assessed the importance of this cytokine in the immunomodulatory effect of PapMV. While IFN-α production remained high following PapMV immunization in *Ifnar*-deficient mice (Fig. 1C), expression levels of CD86, CD69 and H-2Kb in splenic DCs, B lymphocytes and $CD8^+$ T cells were not increased demonstrating a crucial role

played by type-I interferon signaling in PapMV-dependent immune activation (Fig. 1B and S1B). Finally, we observed that immunization with PapMV leads to IL-6 production in serum and spleen of WT mice but not in *Ifnar* KO and *Tlr7* KO mice (Fig. 1D and S2B). Taken together, these results demonstrate that injection of PapMV in mice induces the activation of a broad range of immune cells through IFN-α production by pDCs in a TLR7-dependent manner.

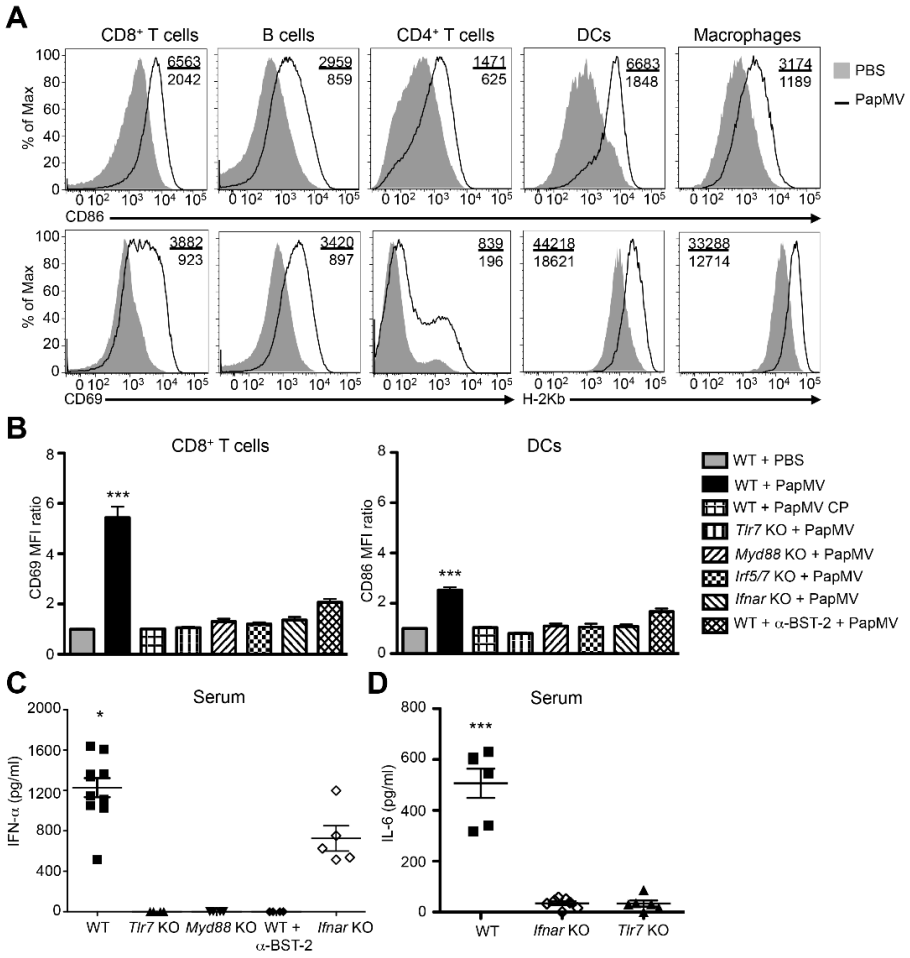

Figure 1. TLR7, MYD88, IRF5/7 and IFNAR are essential for the activation of murine splenocytes by PapMV. (**A**) Representative overlay histograms showing CD86, CD69 and H-2Kb expression on CD8+ T cells, B cells (CD19+), CD4+ T cells, macrophages (F4/80+) and DCs (CD11c+) of WT mice 24 h after i.v. immunization with PapMV (bold lines) or PBS (filled histograms). (**B**) Compilation of CD69 and CD86 expression on CD8+ T cells and DCs 24 h post-immunization. Results are presented as a ratio of the Mean Fluorescence Intensity (MFI) of the analyzed sample on the MFI of the PBS sample. Results are shown as the mean ± SEM. (**C**) ELISA quantification of serum IFN-α or IL-6 (**D**) 6 h post-immunization with PapMV. (n = 3; 2-5 mice per group). **p* < 0.05,****p* < 0.001.

Humoral immune response generated following administration of PapMV depends on TLR7 and MYD88. To determine whether ligation of TLR7 and strong IFN-α production induced upon injection of PapMV affects the generation of the adaptive immune response we first analyzed the development of PapMV-specific antibodies. We observed that *Tlr7* and *Myd88* KO mice produced significantly reduced amounts of PapMV-specific IgG and IgM in serum compared with WT mice (Fig. 2). In contrast to early immune activation, production of PapMV-specific antibodies was not greatly affected by the absence of IFNAR since production of PapMV-specific IgG (Fig. 2A) was only slightly delayed in *Ifnar* KO mice while IgM levels were not affected at day 7 post-immunization (Fig. 2B). In addition, we observed that immunization with PapMV induced the production of high titers of IgG2c and IgG3 isotypes and low titers of IgG1 isotype (Fig. 2C). These results demonstrate that activation through TLR7 and MYD88 is important to induce the development of PapMV-specific antibody responses while absence of type I interferon signaling only slightly delays IgG production.

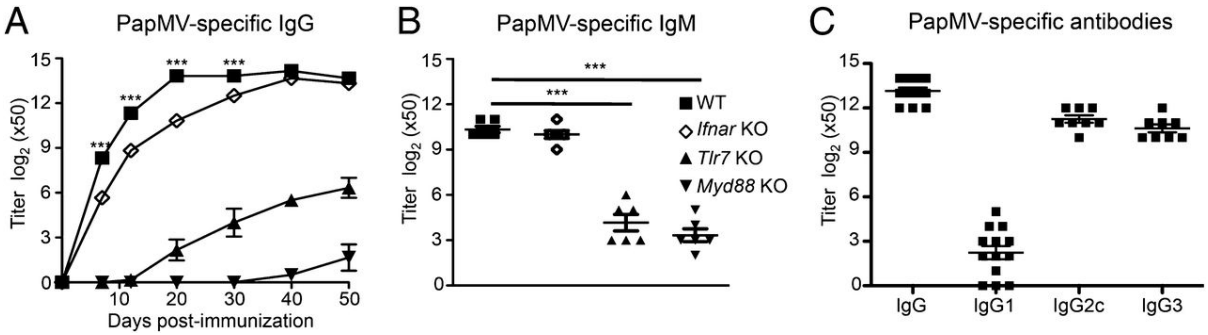

Figure 2. TLR7 and MYD88 are important for the humoral response generated following PapMV nanoparticle immunization. (**A**) Kinetics of anti-PapMV IgG production in serum of WT, *Ifnar* KO, *Tlr7* KO and *Myd88* KO mice following immunization with PapMV measured by ELISA. (**B**) Anti-PapMV IgM production at day 7 post-immunization in serum of WT and KO mice. **(C)** IgG isotypes (IgG1, IgG2c and IgG3) titer in the serum of WT mice 20 days post-immunization. (n = 3; 2-5 mice per group). ***$p < 0.001$.

Pretreatment of mice with PapMV prior to BMDC vaccination increases CD8⁺ T cell responses. Several studies have shown that TLR7 ligands and IFN-I can act as adjuvants for the induction of cellular immune responses (9, 11). Moreover, BMDC immunization is a promising type of vaccination to induce $CD8^+$ T cell responses against loaded antigens (32). We therefore investigated the adjuvant potential of PapMV for the

induction of $CD8^+$ T cell responses generated following vaccination with BMDC-OVA. BMDC-OVA were injected at the peak of IFN-α production following PapMV administration (6 h) and the generation of an effector $CD8^+$ T cell response was analyzed. At day 7 post-immunization (peak of the response) PapMV-treated mice immunized with BMDC-OVA showed significantly higher proportions (Fig. 3A) and numbers (Fig. 3B) of OVA-specific $CD8^+$ T cells in comparison with mice receiving only BMDC-OVA. Moreover, PapMV pre-treatment increased the functionality of OVA-specific $CD8^+$ T cells since they produced more IFN-γ, TNF-α and IL-2, following *in vitro* restimulation (Fig. 3A and 3C). Mice deficient in TLR7 treated with PapMV did not show any increase in OVA-specific $CD8^+$ T cell numbers while *Ifnar* KO mice showed only a slight increase (Fig. 3D). Moreover, pretreatment of mice with PapMV increases the expression of co-stimulation markers such as CD86 and CD70 (Fig. 3E) and MHC molecule H-2Kb (not shown) on BMDC. Finally, administration of PapMV significantly increases the proportion of OVA-specific memory $CD8^+$ T cells at day 45 post-immunization (Fig. 3F). In addition, this pretreatment slightly increased the proportion of cells that produce IFN-γ, TNF-α and IL-2, although this did not reach statistical significance (Fig 3G). Similar results were obtained when PapMV was injected by the subcutaneous (s.c.) route 6 h before s.c. BMDC immunization (Fig. S3).

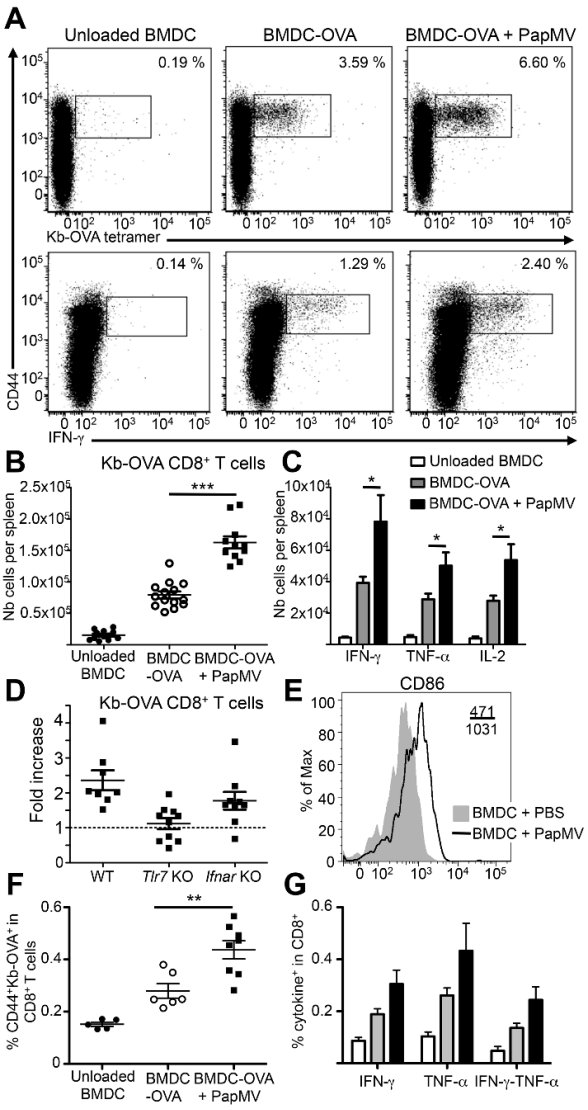

Figure 3. PapMV administration increases the number of OVA-specific effector and memory CD8$^+$ T cells generated by BMDC-OVA immunization. (**A**) Representative results of the percentage of Kb-OVA CD8$^+$ T cells (upper panel) or that produce IFN-γ following restimulation with OVA (lower panel). (**B**) Compilation of the numbers of OVA-specific CD8$^+$ T cells in the spleen or (**C**) CD8$^+$ T cells producing IFN-γ, TNF-α or IL-2 after OVA restimulation. (**D**) Measurement of the increase in the numbers of OVA-specific CD8$^+$ T cells in the spleen detected following pretreatment with PapMV in WT, *Tlr7* KO and *Ifnar* KO mice. The fold increase was calculated by dividing the number of OVA-specific CD8$^+$ T cells in the BMDC-OVA + PapMV group by that of the BMDC-OVA group. (**E**) Representative overlay histogram of CD86 and CD70 expression on BMDCs (CD11c$^+$CFSE$^+$) 6 h post-immunization in the spleen of WT mice pretreated with PBS (filled histogram) or PapMV (bold line) (**F**) Proportions of Kb-OVA CD8$^+$ T cells and (**G**) cytokine producing CD8$^+$ T cells detected following splenocyte restimulation 45 days post-immunization. (n = 3; 3 mice per group) *p < 0.05, **p < 0.01,***p < 0.001.

PapMV administration increases protection against LM-OVA. We next investigated whether administration of PapMV prior to BMDC-OVA immunization could provide greater protection against a bacterial challenge at the memory stage. To address this question,

we infected mice at day 45 post-immunization with LM-OVA. As shown in figure 4A, mice immunized with BMDC-OVA showed a greater proportion of OVA-specific $CD8^+$ T cells in the spleen compared to the unloaded BMDC group at day 5 post-infection indicating that OVA-specific memory $CD8^+$ T cells expanded following infection with LM-OVA. More interestingly, PapMV treatment increases the secondary $CD8^+$ T cell response when compared to BMDC-OVA treatment alone group since there are a greater proportion of OVA-specific secondary effector $CD8^+$ T cells (Fig. 4A) and more $CD8^+$ T cells producing TNF-α or IFN-γ in the PapMV-treated group (Fig. 4B). In addition, more polyfunctional $CD8^+$ T cells producing both, IFN-γ and TNF-α or IFN-γ and granzyme B were induced in the PapMV-treated group (Fig. 4B). This led to a decreased number of LM-OVA in the spleen and liver of the PapMV-treated group compared with the group vaccinated with BMDC-OVA alone (Fig. 4C). In summary, administration of PapMV 6 h before BMDC-OVA immunization results in an increased $CD8^+$ T cell memory response and better protection against a bacterial challenge.

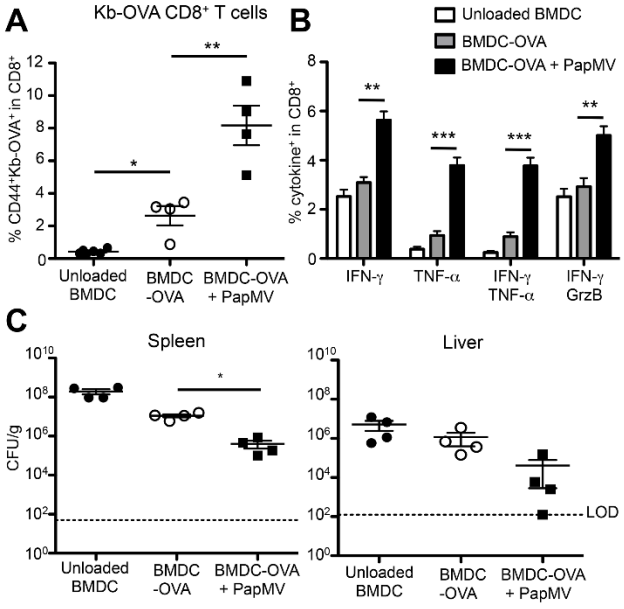

Figure 4. PapMV increases the quality of the immune response generated by BMDC-OVA vaccination and protection against LM-OVA. (**A**) Compilation of the percentage of $CD44^+Kb\text{-}OVA^+$ $CD8^+$ T cells and (**B**) $CD8^+$ T cells that produce cytokines following OVA restimulation. (**C**) Numbers of LM-OVA CFU per gram (g) of spleen and liver enumerated 5 days post-infection. LOD : Limit of detection. (n = 3; 4 mice per group, a representative experiment is shown) $*p < 0.05$, $**p < 0.01$,$***p < 0.001$.

Discussion

The efficiency of PapMV nanoparticles when used as adjuvant or as a vaccine platform has been largely demonstrated (19, 22-24, 30, 33, 34). In this study we have elucidated the mechanism by which PapMV induces immune activation and demonstrated its potential as an adjuvant for T-cell vaccination. In previous studies, we showed that following i.v. immunization, splenic DCs take up PapMV *in vivo* and acquire a mature phenotype (22). In addition, we recently showed that subcutaneous (34) and intranasal (27) administration of PapMV leads to the production of pro-inflammatory cytokines and chemokines. In the present paper, we explored more deeply the immunogenic properties of PapMV upon i.v. immunization. Twenty-four hours after injection of PapMV in mice, we observed that $CD8^+$ and $CD4^+$ T cells, B lymphocytes, macrophages, monocytes and dendritic cells isolated from the spleen, the blood and the peripheral lymph nodes showed increased expression of activation markers demonstrating the induction of a generalized state of activation of the immune system. This was observed only with the complete nanoparticle and not with the CP. PapMV nanoparticles, are produced *in vitro* from monomers of the capsid protein and a synthetic ssRNA by self-assembly into a filamentous rod-shape nanoparticle. Thus, the composition of the complete nanoparticle and of the CP differs only by the presence of the nucleic acid. This strongly suggested that the ssRNA was responsible for the immunogenicity of the PapMV. This hypothesis was further supported by the fact that PapMV failed to induce the activation of immune cells in mice deficient in TLR7, MYD88 or IRF5/7. Furthermore, IFN-α, a cytokine produced upon TLR7 activation, was rapidly detected in serum and spleen of immunized mice. We next sought to determine whether the generalized immune activation observed following PapMV administration was resulting from this strong IFN-α production. Indeed, absence of the type I interferon receptor completely abolished the immunomodulatory effect of PapMV nanoparticles on immune cells *in vivo*. Moreover, similar results were obtained following depletion of BST2 positive cells, which in naïve mice, mainly consists of pDCs, the main source of IFN-α. We thus suggest that following immunization with PapMV, pDCs and most certainly other APCs as we have shown previously (22, 30, 35), take-up the particles and degrade them in the endosome allowing recognition of the ssRNA by TLR7, which leads to a massive production of IFN-α in a MYD88 and IRF5/7-

dependent fashion. Subsequently, IFN-α is detected by various immune cells through IFNAR leading to their activation as observed by an increased expression of activation markers such as CD69, co-stimulatory molecules like CD86 and MHC proteins, and the production of other immune mediators such as the proinflammatory cytokine IL-6.

Having uncovered a major role played by TLR7 in the activation of the innate immune response following i.v. immunization with PapMV, we next analyzed its impact on the humoral immune response. We observed that in the absence of TLR7 and even more in the absence of MYD88, production of PapMV-specific IgG and IgM was greatly reduced. Since absence MYD88 had a greater impact on PapMV-specific IgG production than TLR7 deficiency, it is possible that PapMV activates another MYD88-dependent receptor such as TLR8. In fact, TLR7 and TLR8 are known to sense similar ligands such as ssRNA and small synthetic molecules like imidazoquinolines and nucleoside analogs. In addition, many ligands (CL097, CL075, R848, Poly(dT)) are known to activated both. Therefore we cannot exclude the possible implication of TLR8 in IgG production following PapMV immunization. However, the involvement of TLR8 in the activation of immune cells and IFN-α production seems negligible since absence of TLR7 completely abrogates the effect of PapMV on these processes. In addition, absence of type I interferon receptor only transiently decreased the amount of IgG in the serum of immunized mice. This result suggests that detection of PapMV by TLR7 on B lymphocytes is required for specific antibody production, while B lymphocyte activation by IFN-α is not necessary. Consistently with this hypothesis, it was demonstrated that, in an immunization context involving a PRR ligand, direct PRR stimulation of B cells was required to obtained a robust antibody response (36, 37) especially when immunization was performed with VLPs (38). Moreover, Le Bon *et al*. showed that type I interferon enhances humoral immunity and promotes isotype switching by stimulating dendritic cells (39). We showed that IgG isotypes produced following PapMV administration were mainly composed of IgG2c and IgG3 with low IgG1 titers arguing for the skewing of the $CD4^+$ T cell response towards a Th1 phenotype. In addition, IgG3 production is indicative of a T-independent antibody response in the presence of IFN-γ (40). This is likely the result of the direct cross-linking of specific B cell receptors by repeated antigenic patterns found on the surface of PapMV nanoparticles, which could induce antibody production without help from $CD4^+$ T cells.

Although some studies have shown that antibodies directed towards a vaccine platform can be detrimental to the effectiveness of a recall injection (41), the presence of high levels of antibodies against PapMV does not affect the efficacy of a booster vaccination (19, 27, 34).

The cellular immune response is critical for the control of viral infections, intracellular microbial infections and cancer (4, 5, 42-44). To determine the capacity of PapMV to enhance T cell-mediated immune responses we used PapMV as adjuvant in a BMDC-OVA vaccination regimen. We observed an increased proportion of OVA-specific effector $CD8^+$ T cells producing cytokines such as TNF-α, IFN-γ and IL-2, when PapMV was administered before BMDC-OVA vaccination. It is noteworthy that this increased number of OVA-specific effector $CD8^+$ T cells was not observed in *Tlr7* KO mice highlighting once again the importance of this receptor in the immunogenic effect of PapMV. Interestingly, absence of IFNAR did not abrogate totally the effect of PapMV on the number of OVA-specific $CD8^+$ T cells. In past years, several studies have shown that direct stimulation of T cells by IFN-I was required for optimal $CD8^+$ T cell responses (45, 46). In our case, it seems that IFN-I produced following PapMV immunization acts not only on $CD8^+$ T cell but also directly on injected BMDC since IFN-I produced in *Ifnar* KO mice can only act on these cells as they are the only one expressing the IFNAR. Additionally, it was shown that IFN-α facilitates terminal differentiation of DCs *in vitro* (47). Accordingly, we observed that pretreatment of mice with PapMV increases the expression of co-stimulatory molecules on injected BMDC, which could explain the adjuvant effect observed with PapMV. CD4 help is very important for the functionality of the memory CTL response induced following BMDC immunization [48]. Since we have shown that PapMV immunization induces $CD4^+$ T cell activation, it is possible that CD4 help plays a role in PapMVs enhancement of CTL responses. In addition, it has recently been shown that the CD4 help signal is transmitted from antigen presenting cells to $CD8^+$ T cells via CD27–CD70 interactions [49]. In agreement with these results, we observed that PapMV induced an increased expression of CD70 on BMDCs. More importantly, PapMV also increased long-term memory $CD8^+$ T cell response, the ultimate goal of any vaccine. Since $CD8^+$ T cells are important to control infection with LM (50, 51), an enhanced memory response should result in better control against a recall infection. Fittingly, we showed that the use of PapMV as adjuvant for a

BMDC-OVA vaccine increases the quality of the secondary effector T cell response and protection of mice against a LM-OVA challenge. Thus, we demonstrated that PapMV is a suitable adjuvant for the generation of protective $CD8^+$ T cell responses.

The type of immune response generated by a vaccine and therefore the choice of the adjuvant used is crucial to ensure an effective protection or treatment. Thus, increasing efforts are being made to determine the modes of action of adjuvants and to understand the type of immune responses necessary to protect against various infections or disease. The findings of this study are very important to further develop novel effective adjuvants for T-cell vaccines. We have established that PapMV is a potent TLR7 agonist that induces strong IFN-α production and broad immune cell activation. TLR7 agonists such as imidazoquinolines (like R837) have been shown to increase the efficiency of vaccines that trigger cellular immune responses against pathogens or cancer (11, 52-55). However, use of imidazoquinolines is limited to specific applications such as topical creams because of there side effects and short half-life when administrated systemically (56). PapMV have not shown any signs of toxicity *in vivo* in pre-clinical studies and have a stable repetitive and crystalline structure that protects the ssRNA from degradation and efficiently targets APCs. Other VLPs displaying various TLR ligands have provided encouraging results in various pathological settings. In particular, VLPs loaded with CpG oligodeoxynucleotides (TLR9 ligands) have shown a very promising therapeutic potential against cancer (57, 58), but also in other context such as allergen-specific immunotherapy (59-61). Cancer immunotherapy with DC-based vaccines has also provided great promises in the last few years, but requires optimization to become fully effective (62, 63). Our results demonstrate that PapMV is a suitable adjuvant for BMDC-based vaccines that could be applicable to the development of improved therapeutic DC vaccination strategies against cancer and chronic infections.

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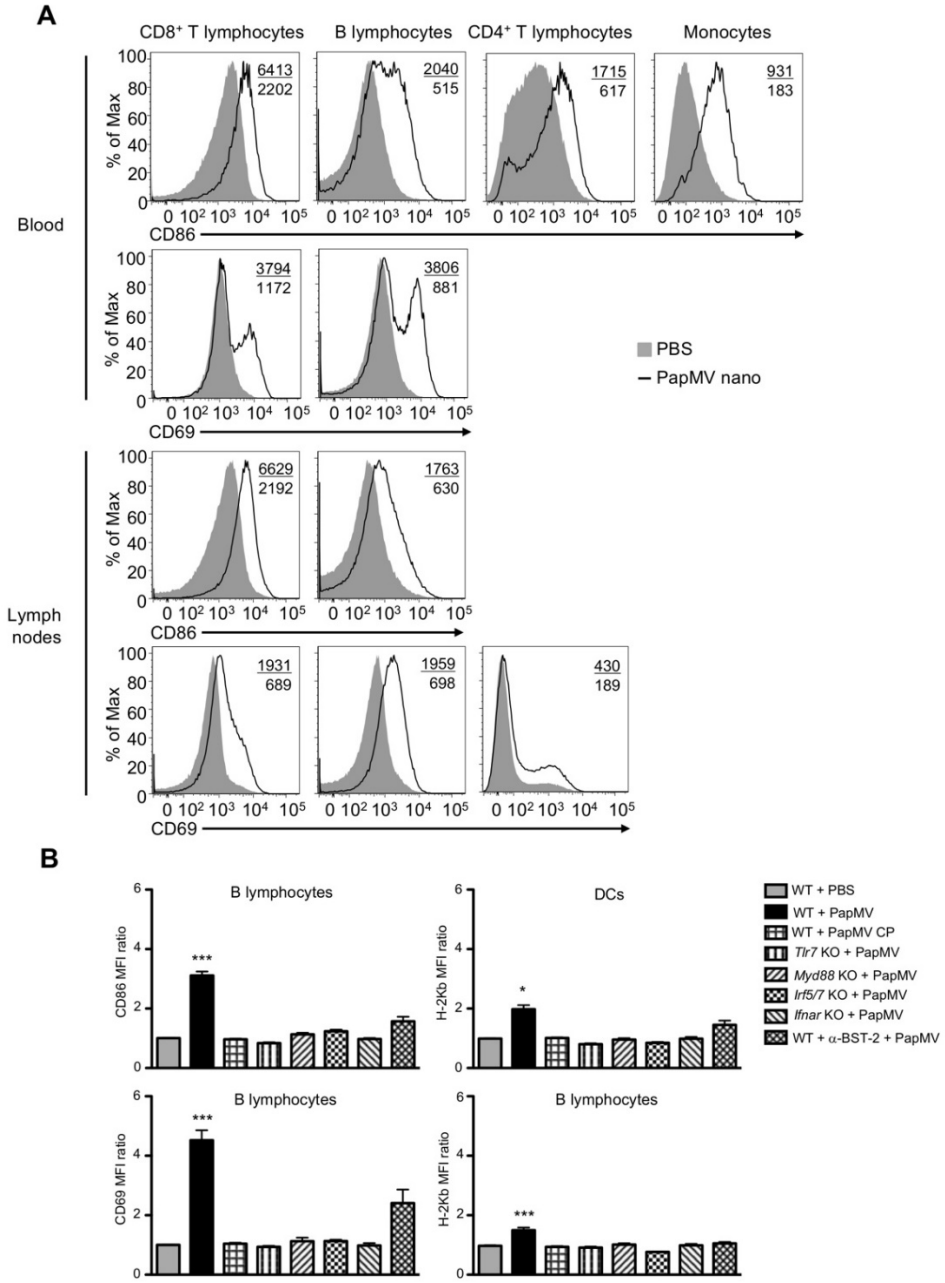

Figure S1. PapMV nanoparticles induce the activation of a broad range of immune cells in mice. Histograms represent flow cytometry analysis of CD86, CD69 and H-2Kb expression on CD8⁺ T cells, CD4⁺ T cells, B cells and monocytes in peripheral lymph nodes (pLN) and blood of WT mice 24 h after i.v. immunization with 100 mg of PapMV (bold lines) or 100 ml PBS (filled histograms). (**B**) Compilation of CD86, H-2Kb and CD69 expression on B cells and DCs 24 h post-immunization. Results are presented as a ratio of the Mean Fluorescence Intensity (MFI) of the analyzed sample on the MFI of the PBS sample. Results are shown as the mean ± SEM. (n = 3; 2-5 mice per group) *$p < 0.05$,***$p < 0.001$.

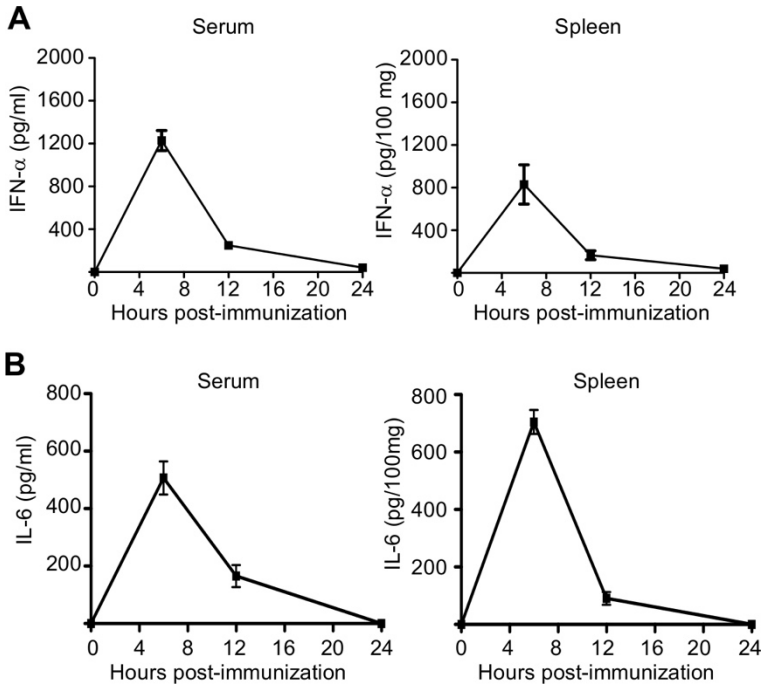

Figure S2. Kinetics of IFN-a (**A**) and IL-6 (**B**) production in serum and spleen of WT mice following immunization with PapMV by ELISA. (n = 3; 2-5 mice per group)

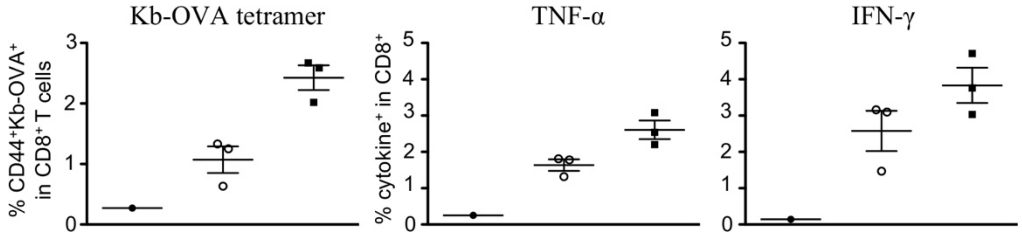

Figure S3. PapMV administration s.c. increases the OVA-specific effector CD8⁺ T cells immune response generated by BMDC-OVA immunization s.c. **(A)** Proportions of Kb-OVA CD8⁺ T cells and (**B**) cytokine producing CD8⁺ T cells detected following splenocyte restimulation 7 days post-immunization. (n = 2; 3 mice per group)

ARTICLE 4: POTENTIATING CANCER IMMUNOTHERAPY USING PAPAYA MOSAIC VIRUS-DERIVED NANOPARTICLES

Titre en français

Potentialiser l'immunothérapie du cancer par l'utilisation de nanoparticules dérivées du virus de la mosaïque de la papaye

Auteurs

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Contributions des auteurs

MEL : Conception et réalisation du projet, co-écriture du manuscrit

KC : Réalisation du projet

ET : Réalisation du projet

PS : Développement du processus de manufacture du PapMV

DL : Co-écriture du manuscrit

AL : Conception du projet, co-écriture du manuscrit

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Résumé en français

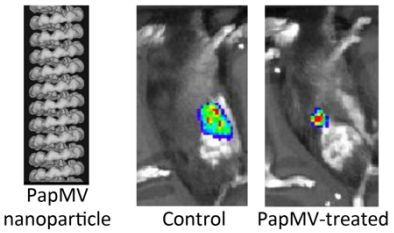
Le développement de nouvelles immunothérapies révolutionne présentement le traitement du cancer. Ces immunothérapies incluent le blocage de points contrôle, l'immunomodulation ainsi que la vaccination thérapeutique. Bien qu'efficaces individuellement, la combinaison de plusieurs approches sera probablement la voie gagnante afin d'atteindre un effet thérapeutique maximal.

À cet égard, les nanoparticules du virus de la mosaïque de la papaye (PapMV) ont démontré un excellent potentiel en tant que molécule immunostimulatrice, adjuvant et plateforme vaccinale grâce à ses capacités à activer la réponse immunitaire innée via l'IFN-α. Dans cet article, nous démontrons que l'administration intra-tumoral du PapMV diminue de façon significative la progression d'un mélanome agressif et prolonge ainsi la survie des souris. Ceci corrèle avec l'augmentation de la production de chimiokine et de cytokines pro-inflammatoire dans la tumeur et l'augmentation de l'infiltration tumoral par des cellules immunitaires. Les proportions de lymphocytes T $CD8^+$ totaux et spécifiques à la tumeur augmente suivant les traitements au PapMV alors que les cellules suppressives dérivées de myéloïdes (MDSC) se font plus rares. De plus, l'administration systémique du PapMV prévient l'implantation de métastases dans les poumons. Il est important de noter que le PapMV améliorer aussi de façon synergique l'effet thérapeutique de la vaccination à base de cellules dendritiques ainsi que le blocage de PD-1 en améliorant la réponse immunitaire anti-tumeur. Cette étude démontre le potentiel immunomodulateur d'une nanoparticule dérivée d'un virus de plant à titre de thérapie contre le cancer qu'il soit utilisé seul ou conjointement avec d'autres immunothérapies en développement.

ABSTRACT: The recent development of novel immunotherapies is revolutionizing cancer treatment. These include, for example: immune checkpoint blockade, immunomodulation or therapeutic vaccination. Although effective on their own, combining multiple approaches will most likely be required in order to achieve the maximal therapeutic benefit. In this regard, the papaya mosaic virus nanoparticle (PapMV) has shown tremendous potential as: i) an immunostimulatory molecule, ii) an adjuvant and iii) a vaccine platform through its intrinsic capacity to activate the innate immune response in an IFN-α dependent manner. Here, we demonstrate that intra-tumor administration of PapMV significantly slows down melanoma progression and prolongs survival. This correlates with enhanced chemokine and pro-inflammatory-cytokine production in the tumor and increased immune-cell infiltration. Proportions of total and tumor-specific $CD8^+$ T cells dramatically increase following PapMV treatment whereas those of myeloid-derived suppressor cells (MDSC) concomitantly decrease. Moreover, systemic PapMV administration prevents metastatic tumor-implantation in the lungs. Importantly, PapMV also synergistically improves the therapeutic benefit of dendritic cell (DC)-based vaccination and PD-1 blockade by potentiating anti-tumor immune responses. This study illustrates the immunostimulatory potential of a plant virus-derived nanoparticle for cancer therapy either alone or in conjunction with other promising immunotherapies in clinical development.

KEYWORDS: Cancer immunotherapy, plant virus-derived nanoparticle, immune checkpoint blockade, immunomodulation, therapeutic vaccination

Table of Contents Graphic



TEXT: In recent years, a growing number of studies have demonstrated the importance of the immune system in controlling cancer development highlighting the possibility of targeting immune cells to improve cancer therapy. Manipulation of the immune system, using immune checkpoint blockade, immunomodulators or therapeutic vaccination, has indeed shown great promise for the treatment of various types of cancer[1-3]. However, the enormous complexity of cancer emphasizes the need of combining multiple treatment approaches in order to achieve the maximal medical outcome. In this regard, plant virus-like nanoparticles (VLPs) offer an attractive means of stimulating immune responses towards tumors due to their intrinsic immunostimulatory properties [4]. For example, VLPs derived from the potato virus X were successfully used to protect mice against a lymphoma challenge through specific antibody production[5]. In addition, cowpea mosaic virus nanoparticles were recently shown to confer therapeutic activity in various poorly immunogenic murine tumor models[6]. Furthermore, we have shown that PapMV nanoparticles possess multiple desirable properties that could be extremely useful for cancer immunotherapy applications. PapMV is recognized by various human and murine immune cells leading to their efficient activation[7-10]. This is achieved through its rapid endocytosis by antigen presenting cells (APC), such as plasmacytoid dendritic cells (pDCs), followed by the release of the non-coding ssRNA contained within the nanoparticle and of its recognition by endosomal TLR7[7, 10, 11]. This leads to the production of interferon-alpha (IFN-α), which is an antiviral cytokine with potent immunomodulatory properties[11]. We have shown that these properties can be exploited to stimulate the development of protective humoral and cellular immune responses against various bacterial and viral infections[8, 9, 11-13]. We demonstrate here that PapMV is also highly efficient at inducing antitumor immunity.

Therapy with high-dose IFN-α has been shown to improve relapse-free and overall survival in melanoma patients[14]. We therefore chose to evaluate the immunotherapeutic potential of PapMV in the B16 syngeneic melanoma-mouse model knowing that the immunomodulatory properties of PapMV largely depend on IFN-α production[11]. Moreover, B16 is weakly immunogenic due to low major histocompatibility complex (MHC) expression making it a highly relevant model for immunotherapeutic studies[15]. PapMV is a nanoparticle possessing a flexible rod-like structure of about 80 nm in length with a neutral charge (figure S1a-c). By conducting *in vitro* stimulation assays, we showed that PapMV had no direct cytotoxic properties towards B16 melanoma cells (figure S1d). However, when PapMV is injected into subcutaneously (s.c.) implanted B16-OVA tumors or systemically, it interacts with several types of immune cells (figure S2a). As shown, all immune cells within the tumor analyzed were found to be associated with PapMV following intra-tumor administration of A647-labelled PapMV. In contrast, PapMV was mainly found within the macrophage population in tumor-draining lymph nodes suggesting that these cells phagocyte PapMV in the tumor and migrate to draining lymph nodes. When PapMV is injected intravenously (i.v.) however, it is predominantly found associated with phagocytic cells from the blood, lungs and spleen such as dendritic cells, B cells and macrophages (figure S2b). Importantly, while we have shown that i.v. injection of PapMV induces systemic IFN-α production by pDCs[11], intra-tumor administration leads to local IFN-α production by macrophages in the tumor and draining lymph node (figure S2c). Thus, PapMV strongly interacts with immune cells and induces IFN-α production following systemic and local administration.

In order to evaluate the therapeutic activity of PapMV against melanoma, B16-OVA tumors were injected with PapMV on days 7-, 12- and 17-post implantation. As shown in Figure 1a, treatment significantly slowed down tumor progression compared to vehicle treated controls as well as prolonged survival by more than a week in this typically highly aggressive model (Figure 1b). In addition, the percentage of B16 cells expressing Ki67, an activation/proliferation marker, decreased following PapMV administration indicating a reduction in tumor-cell proliferation (Figure 1c). Furthermore, levels of MHC-I surface expression significantly increased suggesting the potential enhancement of tumor immunogenicity (Figure 1d); this is in agreement with the well-characterized effect of IFN-α on MHC-I upregulation[16] as well as with our previous findings which illustrated that systemic PapMV administration increases MHC-I surface expression on various immune cells along with other activation markers and costimulatory molecules[11]. Importantly, the therapeutic effect of PapMV administration on tumor progression and mice survival was also observed with a notably higher efficacy in the less immunogenic parental

B16F10 melanoma line, which does not express the OVA model antigen (Figures 1e-f). To determine the ability of PapMV treatment to induce anti-tumor immune responses, we harvested tumors 6 h after the final administration of PapMV and quantified chemokine and cytokine production in tumor homogenates using a Luminex detection assay. Here we observed that intra-tumor concentrations of chemokines IP-10 (Figure 2a), MIG (Figure 2b) and MCP-1 (Figure 2c) were all significantly increased in PapMV-treated animals compared to controls. Notably, these molecules have been previously shown to be potent chemoattractants especially for T cells, monocytes and DCs[17]. Similarly, the concentrations of pro-inflammatory cytokines IL-1 (Figure 2d) and IL-5 (Figure 2e) were significantly increased in PapMV-treated mice compared to controls; although the levels of IL-6 (Figure 2f) and IFN-α (Figure 2g) were elevated in a subset of animals, these increases did not attain statistical significance. Pro-inflammatory cytokines have also been implicated in immune-cell recruitment and activation within tumor tissue, that drives or inhibits tumor development depending on the nature of the immune infiltrate[18]. Thus we sought to characterize immune-cell infiltration 3 days following the second PapMV administration on day 15 post tumor implantation. Using the leukocyte antigen CD45 as a marker of immune cells, we observed that relative to control mice, PapMV-treated mice featured an elevated proportion of immune cells in tumor homogenates (Figure 2h). Interestingly, proportions of MDSCs significantly decreased in treated tumors (Figure 2i) even though their absolute number did not vary (Figure 2j), possibly due to the large immune infiltrate induced following PapMV administration. MDSCs have been previously shown to favor tumor development through suppression of T-cell responses[19]. We surmise that the decrease in the proportion of MDSCs observed is due to IFN-α, a cytokine that has been demonstrated to inhibit MDSC-mediated immunosuppression[20] and which is highly induced following PapMV administration[11] (Figures S2-3). Next we quantified the level of $CD8^+$ T-cell infiltration in tumors from PapMV-treated mice and found that it was strikingly increased relative to controls (Figures 2j-k). Importantly, proportions (Figures 2l-n) and absolute numbers (Figure 2j) of tumor-specific $CD8^+$ T cells, as quantified by flow cytometry using tetramers specific for the dominant melanocyte self-epitopes of gp100 and TRP2 or the model tumor antigen OVA, also significantly increased in treated mice. Collectively, these results indicate that the intrinsic ability of PapMV to activate the innate immune system leads to increased priming of tumor-specific T-cell responses and resultantly, better tumor control.

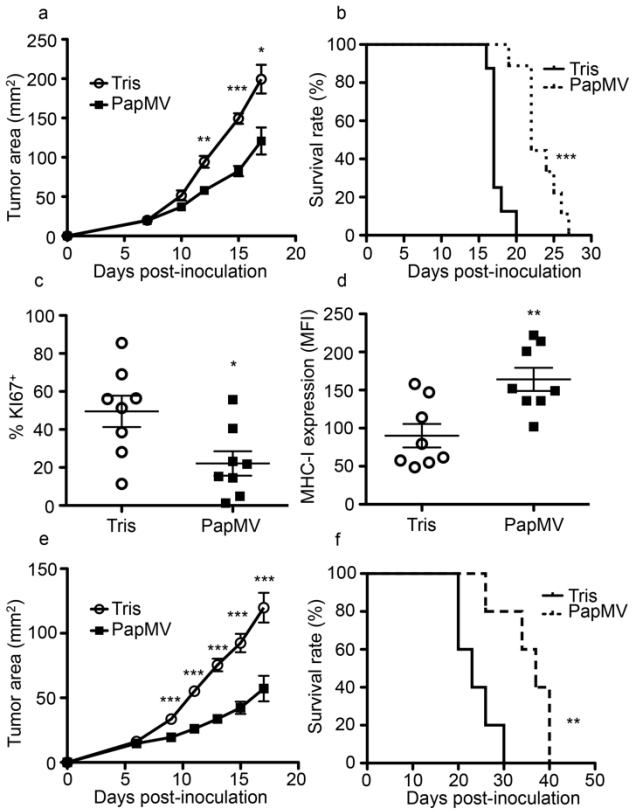


Figure 1. PapMV treatment decreases the growth rate of B16-OVA melanoma tumors and increases survival. C57BL/6 mice were implanted with 2.5-5 x 10^5 B16-OVA (a-d) or B16F10 (e-f) cells s.c. before receiving 100 g of PapMV or Tris 10 mM directly into the tumor on days 7-, 12- and 17-post implantation. **(**a and e) Tumor growth was followed by measuring the tumor diameter and calculation of the tumor area. (b and f) Survival rates of treated mice. Mice were euthanized when tumors reached a diameter of 17 mm. **(**c) Proportions of $Ki67^+$ cells and (d) Mean Fluorescence Intensity (MFI) of MHC-I expression in the $CD45^-$ tumor-cell population measured by flow cytometry on day 15-post implantation. **P* < 0.05, ***P* < 0.01, ****P* < 0.001

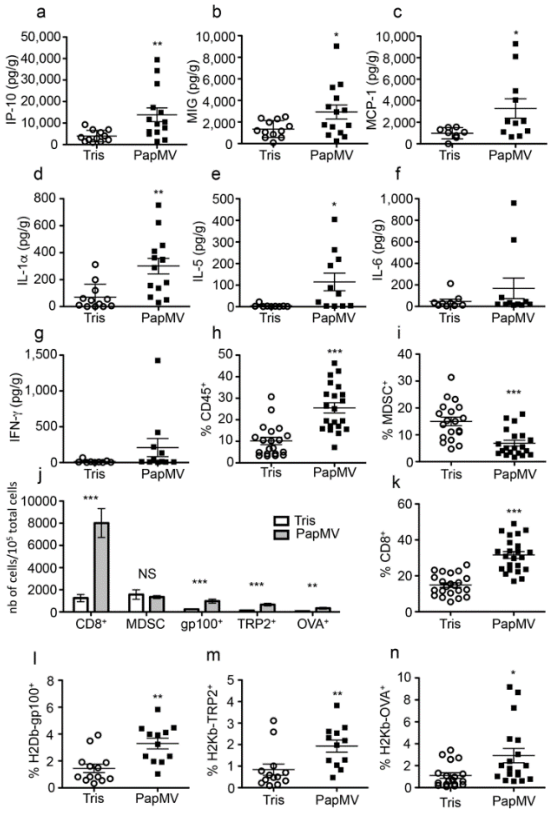


Figure 2. PapMV administration induces chemokine/cytokine production and tumor immune-cell infiltration. C57BL/6 mice were implanted with 5 x 10^5 B16-OVA cells s.c. before receiving 100 g of PapMV or Tris 10 mM directly into the tumor on days 7-, 12- and 17-post implantation. Six hours following the last PapMV treatment, tumors were harvested, homogenized and chemokines/cytokines quantified using Luminex to assay levels of (a) IP-10, (b) MIG, (c) MCP-1 (d) IL-1α, (e) IL-5, (f) IL-6 and (g) IFN-γ. On day 15-post implantation, tumors were harvested to quantify immune-cell infiltration by flow cytometry. (h) Proportions of total $CD45^+$ cells. (i) Proportions of myeloid-derived suppressor cells (MDSC) within the $CD45^+$ cell population. (j) Absolute numbers of different immune-cell populations in the tumor per 10^5 total cells. Proportions of (k) $CD8^+$ T cells, (l) gp100- (m) TRP2- and (n) OVA-specific $CD8^+$ T cells within the $CD45^+$ cell population. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

The most significant advantage of using non-infectious virus-derived nanoparticles for cancer immunotherapy, as opposed to live oncolytic viruses or nucleoside analogs, possibly lies in their utility for systemic delivery applications. For understandable safety concerns, it would be prudent to avoid using live viruses for such applications wherein non-infectious nanoparticles would provide a viable alternative. Moreover, the use of nucleoside analogs as agonists of TLR7/8 induce adverse systemic side effects *in vivo* limiting their use to topical applications[21]. In contrast, i.v. administration of PapMV does not lead to significant systemic cytokine production except from IFN-α and IL-12p40, which bear desirable anti-tumor adjuvant properties[22, 23] (Figure S3). This is an important consideration to make given that systemic treatment is probably the most

appropriate means of preventing metastasis development. To investigate the suitability of this approach, PapMV was administered systemically to mice followed by i.v. administration of B16-OVA cells 6 h later to evaluate the induction of metastatic implantation in the lungs as previously described[24]. Mice were euthanized 17 days post-inoculation to assess metastasis development in harvested lungs. PapMV-treated mice showed radically reduced numbers of tumor nodules compared to controls with some mice being practically free of metastasis (Figure 3a). To provide a more quantitative assessment of the anti-metastatic effect of PapMV in this experimental setting, treatment with PapMV was performed 6 h before or 2 days after i.v. administration of B16-OVA cells expressing luciferase (B16-OVA-oFL) followed by luciferase quantification in lung homogenates 21 days later. Systemic delivery of PapMV before tumor injection provided significant protection against metastasis implantation in the lungs (Figure 3b). Remarkably, treatment with PapMV 2 days after metastasis implantation also provided significant therapeutic benefit. The protective anti-metastatic effect correlated with an elevated proportion of tumor-specific T cells in lung-draining mediastinal lymph nodes (Figure 3c) again indicating the development of PapMV-induced immune mediated protection. As expected and in accordance with our previous study[11], the beneficial effect of PapMV was highly dependent on TLR7 (Figure S4a). Moreover, treatment with ssRNA or capsid monomers from PapMV either locally or systemically did not provide any significant therapeutic (Figures S4a-b) or immune stimulatory (Figures S4c-i) activity confirming the requirement for PapMV to be in a nanoparticle structure to exhibit adjuvant properties. Remarkably, we also observed a greater therapeutic benefit from PapMV compared to equivalent amounts of R837, a TLR7 agonist already in clinical use for cancer treatment (Figures S4a-i).

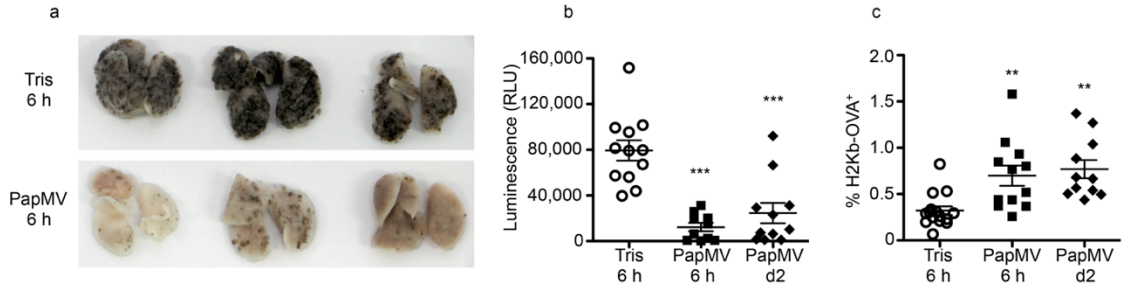


Figure 3. Systemic PapMV administration reduces B16-OVA metastasis implantation in the lungs. (a) C57BL/6 mice were injected i.v. with Tris (top) or PapMV (bottom) 6h before i.v. inoculation with B16-OVA cells. Mice were euthanized 17 days post B16-OVA injection to observe lung nodule formation. (b) Mice were injected with Tris or PapMV 6 h before or 2 days after (d2) B16-OVA-oFL (B16-OVA cells expressing luciferase) inoculation. Luminescence was quantified in lung homogenates 21 days post tumor-cell inoculation by measuring Relative Light Units (RLUs). (c) Flow cytometry analysis of OVA-specific $CD8^+$ T cells in mediastinal lymph nodes at day 21-post B16-OVA-oFL inoculation. $^{**}P < 0.01$, $^{***}P < 0.001$.

The efficacy of PapMV for cancer immunotherapy is supported by its ability to increase the number of tumor-specific T cells and enhance their effector function[11]. Of note, various immunotherapy approaches are currently being developed to either favor the expansion of tumor-specific T cells or increase their effector capacity and some of these have already been translated into clinical application[1]. In light of these trends, we sought to determine whether administration of PapMV in concert with immunotherapeutic approaches in clinical development could drive enhanced anti-tumor activity. First, we tested its capacity to augment the effectiveness of a DC-based vaccine when used as an adjuvant. Here, mice were treated with PapMV directly into s.c. B16-OVA tumors 7 days after implantation after which an s.c. injection of DCs pulsed with the immunodominant ovalbumin peptide, SIINFEKL, was administered in the opposite flank 6 h later. Upon administration of an identical vaccine boost at day 14, we observed increased proportions of OVA-specific $CD8^+$ T cells in the blood of PapMV-treated mice compared to mice treated with unpulsed DCs or OVA-pulsed DCs in the absence of adjuvant (Figure 4a). Notably, this is consistent with our previous findings in which we used a systemic DC-OVA vaccination approach and observed that PapMV significantly increased proportions of OVA-specific splenic-T cells[11]. More importantly, PapMV treatment dramatically reduced tumor growth compared to DC-OVA vaccination in the absence of PapMV adjuvant (Figure 4b and 4c), which translated into a significant increase in the survival rate of treated animals with one mouse completely clearing the tumor (Figure 4d). To determine the mechanism of protection in the surviving mouse, B16-OVA-oFL cells were injected i.v. 45 days post tumor clearance and metastasis development was evaluated 21 days later. No signal was detected upon quantification of luciferase activity thus confirming the establishment of protective immunological memory following PapMV treatment (data not shown). These data clearly demonstrate the adjuvant properties of PapMV, which can be applied towards increasing the therapeutic potential of DC-based vaccination strategies. As previously mentioned, another recent and extremely promising immunotherapeutic approach is the development of immune checkpoint blockade protocols especially against the PD-1 immunoregulatory pathway[25]. *In vivo* blockade of PD-1 or of its receptor PD-L1 has been shown to impede the inhibitory effect of this pathway on T cells thus potentiating the development of a more effective immune response[26]. Therefore, we evaluated the synergistic effect of PapMV treatment in combination with PD-1 blockade. Mice receiving PapMV combined with an anti-PD1 blocking antibody showed a significant increase in proportions of $CD8^+$ T cells specific for the tumor antigens gp100 (Figure 4e) and TRP2 (Figure 4f) compared to anti-PD-1 treatment alone which by itself, did not significantly increase tumor-specific T-cell numbers when compared to groups receiving the isotype control. Moreover, tumor-specific $CD8^+$ T-cell proportions in tumors

of mice treated with PapMV combined with anti-PD-1 antibodies were not significantly higher than those that we previously observed with PapMV treatment alone (Figure 2l and 2m). These results indicate that on its own, PD-1 blockade does not favor immune-cell infiltration in tumor tissue and that the ability of PapMV to induce immune-cell infiltration is not enhanced by PD-1 blockade. This conclusion is in agreement with clinical data indicating that the therapeutic benefit of PD-1 blockade is mainly achieved in the presence of pre-existing anti-tumor T-cell immunity[27]. However, in concert, the two agents work synergistically towards improved tumor immunity as shown by the reduction in tumor growth (Figure 4g) and increased survival (Figure 4h) compared to PD-1 blockade alone. Furthermore, the combined efficacy was also improved over that of PapMV alone (Figure 1a and 1b). This is consistent with a recent study using a mouse melanoma model describing an increased therapeutic effect when anti-PD-1 blockade was combined with poly(I:C) administration[28]. The synergistic effect is likely the result of increased immune-cell infiltration and tumor-specific T-cell priming, through PapMV's capacity to increase antigen presentation[9, 10], alongside the well-known action of PD-1 blockade on increasing T-cell function. In agreement with this hypothesis, we observed a significantly increased proportion of effector $CD8^+$ T cells producing granzyme B and IFN-γ when the two treatments were combined (Figure 5a and 5b) which translated into a significant reduction of tumor progression (Figure 5c).

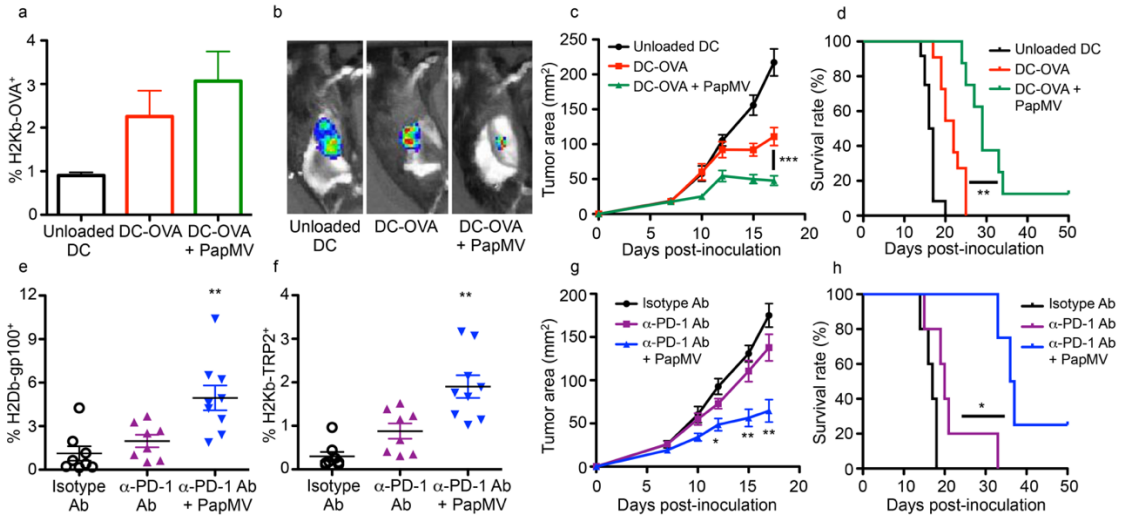


Figure 4. PapMV administration potentiates the therapeutic effect of dendritic cell-based vaccination and PD-1 blockade. C57BL/6 mice were implanted s.c. with B16-OVA (a, c, d) or B16-OVA-oFL cells (b). On day 7 post implantation, mice were treated with PapMV directly into tumors followed 6 h later by s.c. vaccination with DCs pulsed with the immunodominant peptide from ovalbumin SIINFEKL in the opposite flank. An identical vaccine boost was given on day 14. (a) Proportions of OVA-specific $CD8^+$ T cells in the blood and (b) *in vivo* imaging of mice with B16-OVA-oFL tumors on day 14-post implantation. (c) Tumor growth was followed by measuring the tumor diameter and calculation of the tumor area. (d) Survival rates of treated mice. Mice were euthanized when tumors reached a diameter of 17 mm. In a separate set of experiments, mice were implanted s.c. with B16-OVA tumors and treated with 100 μg

PapMV on days 7-, 12- and 17-post implantation. On days 7, 11 and 15, mice received i.p. administrations of 250 μg of anti-PD-1 blocking antibody or isotype control. (e) Proportions of gp100- and (f) TRP2-specific $CD8^+$ T cells within the $CD45^+$ cell population on day 15-post implantation. (g) Tumor growth was followed by measuring tumor diameter and calculation of the tumor area. (h) Survival rates of treated mice. *P < 0.05, **P < 0.01, ***P < 0.001.

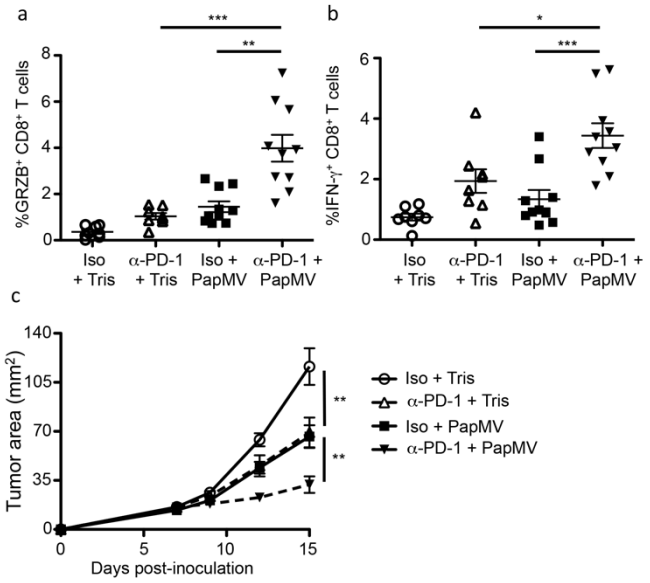


Figure 5. Combined PapMV and anti-PD-1 antibody treatment increases the functionality of infiltrating $CD8^+$ T cells. C57BL/6 mice were implanted s.c. with B16-OVA cells and treated with 100 μg PapMV on days 7- and 12-post implantation. On days 7- and 11-, mice received i.p. administrations of 250 μg of anti-PD-1 blocking antibody or isotype control. (a) Proportions of granzyme B and (b) IFN-γ positive $CD8^+$ T cells within the $CD45^+$ cell population on day 15-post implantation. (c) Tumor growth was followed by measuring the tumor diameter and calculation of the tumor area. *P < 0.05, **P < 0.01, ***P < 0.001.

It is important to note that the therapeutic effects observed with PapMV were obtained without having to vaccinate against any tumor epitopes. Indeed, treatment with PapMV alone induced the development of $CD8^+$ T-cell responses against endogenous tumor epitopes possibly by breaking immune tolerance towards these antigens or by significantly enhancing pre-existing anti-tumor immune responses. This confers a significant advantage to PapMV over classical therapeutic vaccination approaches that require identifying and selecting the most appropriate patient-specific antigenic tumor peptides for vaccine design[29]. Another advantage is conferred by the ease with which PapMV can be engineered to express peptides on its surface, which provides an opportunity to further increase its therapeutic potential by generating nanoparticles displaying various tumor epitopes potentially strengthening even more specific anti-tumor immune responses generated[9, 30]. Furthermore, nanoparticles derived from plant viruses offer significant benefits over other immunomodulatory molecules in clinical development such as endosomal TLR

agonists. Indeed, unlike RNA or nucleoside analogues that induce undesirable side effects through indiscriminate cellular target and entry as well as exhibit short half-lives[31-33], plant virus-derived nanoparticles are safe, immunogenic, stable and predominantly target antigen-presenting cells[4]. Moreover, in contrast to other plant virus particles, PapMV carries a non-coding ssRNA making it environmentally safe.

In conclusion, PapMV's intrinsic capacity to activate the innate immune system can be harnessed to advance anti-tumor T-cell responses providing a novel immunotherapy approach with a strong translational potential for cancer treatment. PapMV is presently being tested in a phase 1 randomized, controlled, dose escalation trial when used as adjuvant for the seasonal flu vaccine to determine its safety, tolerability and effectiveness[34]. Results from this trial could thus provide a strong basis for future immunotherapy trials in cancer patients.

Methods

PapMV nanoparticles

PapMV nanoparticles were provided by Folia Biotech Inc. (Quebec city, Quebec, Canada) and produced as described in our previous study[12]. In all experiments, nanoparticles were diluted in Tris 10mM prior to be used. LPS contamination was always < 50 endotoxin units/mg protein and considered as negligible.

Dynamic light scattering

The size of nanoparticles was recorded with a ZetaSizer Nano ZS (Malvern, Worcestershire, United Kingdom) at a temperature of 10°C at a concentration of 0.1 mg/ml diluted in 10mM Tris pH8 or PBS buffer.

Electron microscopy

Electron micrograph were taken on a FEI Technai Spirit G2. We used negative staining with uranyl acetate 2% dissolved in 10mM Tris/HCl pH 8.0.

Cell culture

B16F10 and B16-OVA cells were kindly provided by Dr. Adrian Ochsenbein (Bern University, Bern, Switzerland) and Dr. Richard Vile (Mayo Clinic, Rochester, MN) respectively and cultured in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum and 5 mg/mL of G418 to select for OVA expression. B16-OVA cells were transfected with an SR-α vector containing the optimized firefly luciferase gene (oFL) kindly provided by Dr. Patrick Hwu (MD Anderson Cancer Center, Houston, TX) and selected using 1 µg/ml puromycin to generate the B16-OVA-oFL cell line. Bone marrow derived dendritic cells were generated as described previously[11].

In vitro cytotoxic assay

B16-OVA cells were incubated with 100 μg/ml PapMV and viable cells were enumerated after 24, 48 and 72 h of stimulation.

Mice

Six- to 10-wk-old, female C57BL/6 mice were purchased from Charles River Laboratories. All animal experiments were reviewed and approved by the Institut national de la recherche scientifique animal care committee. To establish s.c. tumors, 2.5-5 x 10^5 B16-OVA, B16-OVA-oFL or B16F10 in 100 μl PBS were injected in the right flank of mice. *In vivo* visualization of B16-OVA-oFL was performed using the Xenogen IVIS 100 *in vivo* imaging system by injecting 2 mg D-luciferin intraperitoneally to tumor bearing mice.

PapMV distribution experiment

PapMV was labeled with Alexa 647 using the Molecular Probes® Protein Labeling Kit (Thermo Fisher Scientific Inc.) following the manufacturers' instructions. Alexa 647-labeled PapMV was injected i.t. or i.v. and mice were sacrificed two to four hours post-immunization for flow cytometry analysis.

Immunotherapy treatments

When tumors were palpable, at day 7-post inoculation, immunotherapy treatments were initiated. For treatments using PapMV alone, 100 μg PapMV or 100 μl Tris 10 mM was injected directly into the tumor on days 7- and 12-post inoculation for immune response analyses on day 15 and on days 7-, 12- and 17-post inoculation for tumor progression and survival studies. When PapMV was combined with BMDC vaccination, 100 μg PapMV was injected directly into the tumor on days 7- and 14-post inoculation in combination with s.c. injection in the left flank of 1.25 x 10^6 BMDC loaded with the $OVA_{257–264}$ peptide (SIINFEKL) 6h later. For PapMV treatments combined with anti-PD1 blockade, intra-tumor injections of 100 μg PapMV on days 7 and 12 were combined with i.p. administration of 250 μg anti-PD-1 blocking antibody or isotype control (BioXcell) on days 7- and 11-post inoculation and immune response analyses were conducted on day 15-post inoculation. Tumors were collected, dissociated and digested with collagenase D and DNAse I before red blood cell lysis was performed prior to flow cytometry analysis. For tumor progression and survival studies, a third administration of anti-PD-1 or isotype control antibody was given on day 15 while a third PapMV treatment was given on day 17-post inoculation. Tumor growth was followed by measuring tumor diameter using a caliper and calculation of the tumor area. Mice were euthanized when tumors reached a diameter of 17 mm.

Lung nodule establishment assay

Mice were injected i.v. with 100 μl Tris or 100 μg PapMV 6 h before tumor inoculation with 5 x 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. For metastasis quantification, mice were injected i.v. with 100 μl Tris or 100 μg PapMV 6h before or 2 days after tumor inoculation with 1 x 10^6 B16-OVA-oFL and euthanized on day 21-post inoculation. Lungs were homogenized in 1 ml Reporter Lysis Buffer (Promega Corp) for luminescence quantification while mediastinal draining lymph nodes were harvested for tetramer staining analysis by flow cytometry.

Luminex cytokine analyses

Mice were injected s.c. in the right flank with 5 x 10^5 B16-OVA cells. At day 7-post inoculation, 100 μg PapMV or 100 μl Tris 10 mM were injected directly into the tumor. Treatment was repeated at days 12- and 17-post inoculation. 6h after the last injection, tumors were harvested, weighed and homogenized in 1 ml PBS. Supernatants were used to evaluate cytokine production using a Luminex screening assay kit (R & D systems) following the manufacturers' instructions. Chemokine/cytokine concentrations are reported in pg per gram of tumor. Chemokine/cytokine production was evaluated in the blood of mice, 6 h after 100 μg PapMV i.v. injection and are reported in pg/ml of blood.

Flow cytometry analyses and antibodies

Tumors were recovered from mice and dissociated using frosted slides (VWR International). Cells were digested with 1mg/ml collagenase D (Roche) and 100 μg/ml DNAse I (Roche) for 15 minutes at 37°C and passed through a 100 μm cell strainer (BD Biosciences). Red blood cell lysis was then performed using a 0.83% NH_4Cl solution. Single-cell suspensions were finally washed with FACS buffer (PBS, 1% BSA, 0.1% sodium azide). Flow cytometry analyses of mouse surface antigens in tumor-infiltrating cells was performed with the following antibodies: anti-CD44 (IM7), -CD11b (M1/70), -CD8 (53-6.7), gr-1 (RB6-8C5), -F4/80 (BM8), (BioLegend), -CD45 (30-F11) (BD Biosciences), and H-2Kb (AF6-88.5.5.3) (eBioscience). Staining was performed for 20 min at 4℃. PE-coupled gp100–H2Db, TRP2-H2Kb and OVA-H2Kb tetrameric complexes were generated as previously described[35] and staining was performed at 37℃ for 15 min. Intra-nuclear staining of Ki67 (solA15) was performed using Foxp3 / Transcription Factor Staining Buffer (eBioscience), according to the manufacturer's instructions. Intra-cellular staining for IFN-γ (XMG1.2) (BioLegend) and Granzyme B (NGZB) (eBioscience) was performed after a 5 h restimulation with OVA (2 mg/ml) in the presence of brefeldin A (10 mg/ml) while IFN-α production was evaluated after 4 h incubation with brefeldin A (10mg/ml) (RMMA-1) (PBL assay science) using fixation/permeabilization buffer (BioLegend), according to the manufacturer's instructions.

Flow cytometry analyses were performed on a BDLSR Fortessa flow cytometer (BD Biosciences) and data was analyzed using the FlowJo software (Tree Star Inc.).

Statistical analyses

For pairwise comparisons, data were analyzed for statistical significance using Student's t test. Statistical significance was determined as $P < 0.05$. Survival curves were plotted according to the Kaplan–Meier method, and statistical significance in the different treatment groups was compared using the log-rank test.

ASSOCIATED CONTENT

Supporting Information

Supporting Information Available : Additional data for the physicochemical and cytotoxic properties of PapMV, PapMV distribution following intra-tumor and intravenous injection, pro-inflammatory cytokine production and control experiments performed with PapMV ssRNA, monomers, R837 and TLR7 KO mice. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

M.-È. L. and A. L. conceived and designed the experiments: M.-È. L., E. T. and K. C. performed the experiments; P. S. developed the PapMV manufacturing process; M.-È. L., D. L., and A. L. co-wrote the paper. All authors have given approval to the final version of the manuscript.

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Conflict of Interest

Denis Leclerc is the founder and a shareholder of Folia Biotech. Inc., a Canadian biotech company with the mandate to commercialize the PapMV technology.

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ABBREVIATIONS

PapMV, Papaya mosaic virus; APC, Antigen presenting cells; MDSC, Myeloid derived suppressor cells; DC, dendritic cell; IFN, interferon; MHC, major histocompatibility complex; TLR, Toll like receptor; LPS, lipopolysaccharide; PD-1, programmed cell death 1; PD-L1, programmed death ligand; IP-10, Interferon gamma-induced protein 10; MCP1, monocyte chimoattractant protein 1; MIG, Monokine induced by gamma interferon.

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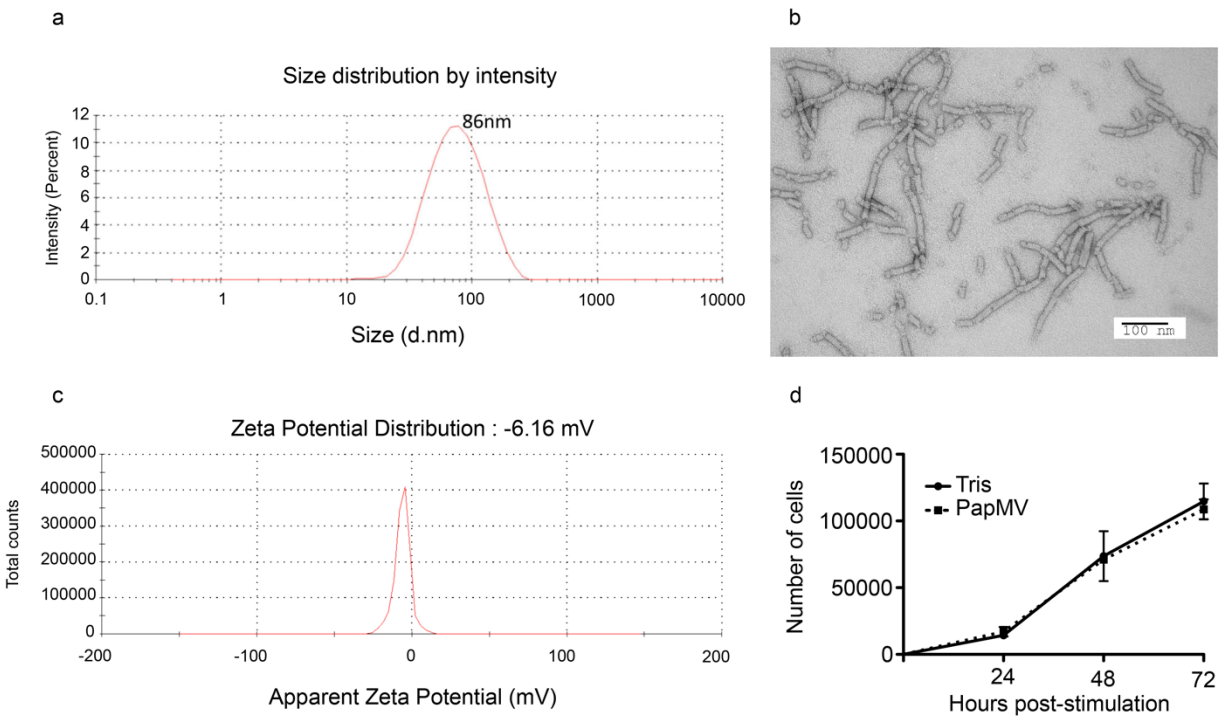

Figure S1. Physicochemical and cytotoxic properties of PapMV. (a) Size measured by dynamic light scattering (DLS) (b) electron micrograph and (c) zeta potential distribution of PapMV nanoparticles. (d) B16-OVA cells in culture were incubated with 100 μg/ml PapMV and cell numbers were determined at various time points following stimulation.

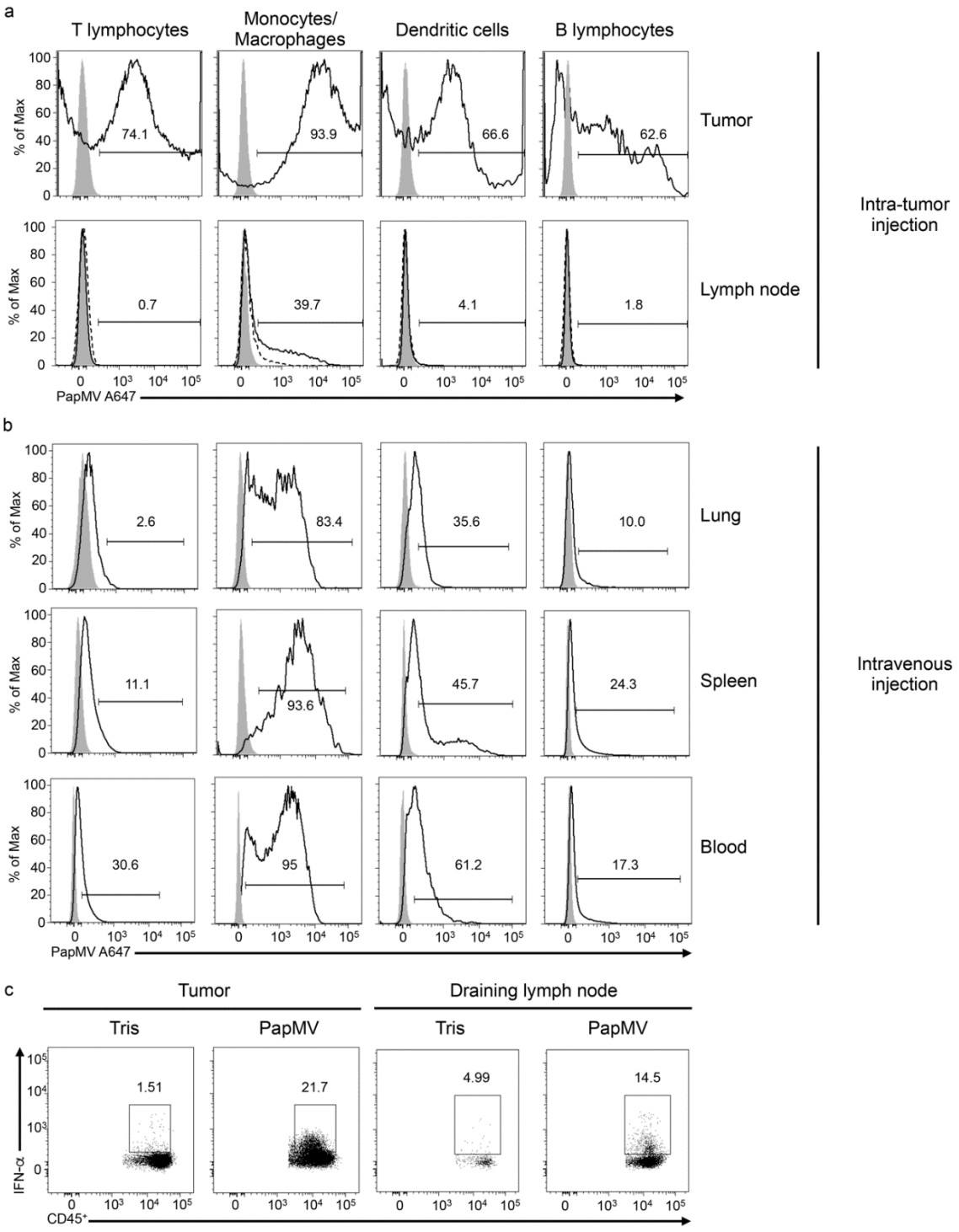

Figure S2. PapMV is predominantly found associated with phagocytic cells following administration. Representative overlay histograms showing the distribution of PapMV nanoparticles in the tumor (upper graph) and draining (bold lines) and non-draining (dash lines) lymph nodes (lower graph) in different immune cell populations after intra-tumor (a) or intravenous (b) injection in comparison with naïve mice (filled histograms). (c) Flow cytometry analysis of IFN-α production by F4/80$^+$CD11b$^+$ macrophages in the tumor and draining lymph node 4h after PapMV injection in the tumor.

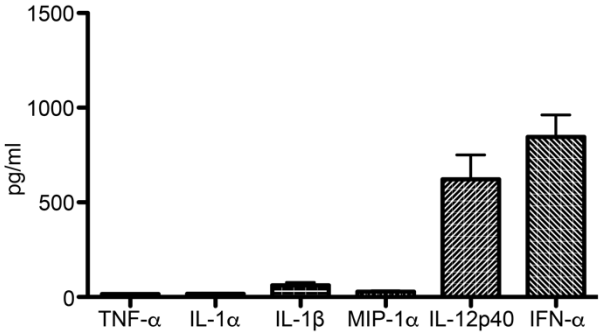

Figure S3. Systemic administration of PapMV induces the production of IL-12p40 and IFN-α but not of other pro-inflammatory cytokines. Luminex quantification of TNF-α, IL-1α, IL-1β, MIP-1α, IL-12p40 and IFN-α production in the blood 6 h after intravenous administration of PapMV.

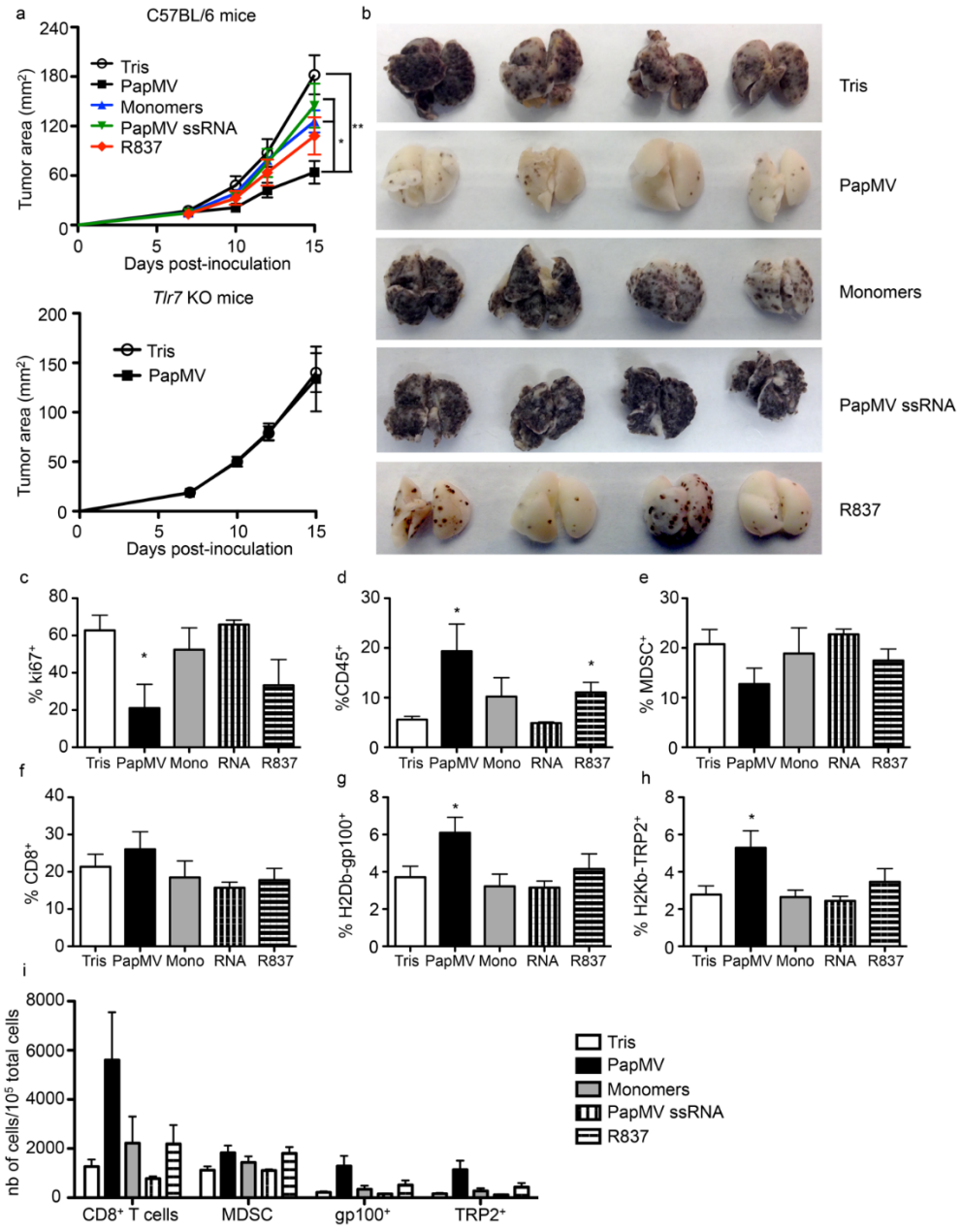

Figure S4. TLR7- and nanoparticle-dependent therapeutic and immune stimulating properties of PapMV. C57BL/6 mice were implanted subcutaneously with 5 x 10^5 B16-OVA cells. On d7, mice were injected directly into the tumor with 100 µl Tris 10mM, 100 µg PapMV (5 µg of ssRNA equivalent), 95 µg monomers, 5 µg PapMV ssRNA or 5 µg R837. Treatments were repeated on d12 post implantation. **(**a) Tumor growth in C57BL/6 (upper panel) or *Tlr7* KO mice (lower panel) was followed by measuring the tumor diameter and calculation of the tumor area. (b) C57BL/6 mice were injected i.v. with 100 µl Tris 10mM, 100 µg PapMV (5 µg of ssRNA equivalent), 95 µg monomers, 5 µg PapMV ssRNA or 5 µg R837 6 h before i.v. inoculation with B16-OVA cells. Mice were euthanized 17 days post B16-OVA injection to observe lung nodule formation. On d15 post implantation, tumors were harvested to evaluate tumor cell proliferation and to analyze immune-cell infiltration by flow cytometry. (c) Proportions of $Ki67^+$ cells in the $CD45^-$ tumor-cell population. (d) Proportions of total $CD45^+$ cells or (e) myeloid-derived suppressor cells (MDSC), (f) $CD8^+$ T cells, (g) gp100- and (h) TRP2-specific $CD8^+$ T cells within the $CD45^+$ cell population. (i) Absolute numbers of different immune-cell populations in the tumor per 10^5 total cells. **P* < 0.05, ***P* < 0.01