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Efeito do imunomodulador P-MAPA associado à interleucina-12 no carcinoma de ovário de ratas Fischer 344: abordagem do processo inflamatório e sistema imune

Henrique Spaulonci Silveira

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Biologia Estrutural e Funcional.*

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Palavras-chave: Câncer de ovário; IL-12; Inflamação; P-MAPA; TLR.

Aos meus pais, minha irmã Cristina e a Aline...

Dedico

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SUMÁRIO

LISTA DE ABREVIATURAS

APC: células apresentadoras de antígenos;

CO: câncer de ovário;

IFN-γ: interferon gama;

IL-1: interleucina 1;

IL-2: interleucina 2;

IL-4: interleucina 4;

IL-5: interleucina 5;

IL-6: interleucina 6;

IL-10: interleucina 10;

IL-12: interleucina 12;

IL-17: interleucina 17;

IL-35: interleucina 35;

IP-10: proteína 10 induzida por interferon gama;

MHC: complexo principal de histocompatibilidade;

MIP-1α: proteína 1-alfa inflamatória de macrófagos;

NK: célula exterminadora natural;

P-MAPA: agregado polimérico de fosfolinoleato-palmitoleato de magnésio e amônio proteico;

PARP: poli ADP ribose polimerase

RANTES: células T normais expressas e segregadas reguladas na ativação;

TCD4+: célula T helper positivo;

TCD8+: célula T citotóxica positivo;

Th1: resposta polarizada T helper 1;

TLR: receptor celular do tipo Toll-like;

TLR2: receptor celular do tipo Toll-like 2;

TLR4: receptor celular do tipo Toll-like 4;

TNF-α: fator de necrose tumoral alfa;

Tregs: células T reguladoras;

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RESUMO

O câncer de ovário (CO) é a segunda mais letal de todas as neoplasias ginecológicas e muitas mulheres adquirem quimiorresistência associada ao processo inflamatório. A interleucina-12 (IL-12) é responsável pela resposta inflamatória T helper 1 (Th1) e o agregado proteico de fosfolinoleato de amônio e magnésio e anidrido palmitoleato (P-MAPA) atua como um agente imunoestimulador em cânceres agressivos. Este estudo investigou os tratamentos funcionais com P-MAPA e IL-12 no processo inflamatório e nas células imunes relacionadas ao CO em um modelo *in vivo*. O CO foi induzido quimicamente com 7,12-dimetilbenz(a)antraceno (DMBA) diretamente na bolsa ovariana e, após o desenvolvimento dos tumores, os animais receberam P-MAPA, IL-12 ou P-MAPA combinado com IL-12. A terapia combinatória melhorou as características histológicas características do CO, contribuindo para a redução das células inflamatórias, acúmulo de adipócitos, além de apresentar um tecido mole e móvel, sem aderências e implantes peritoneais. O tratamento com P-MAPA aumentou os níveis de TLR2, TLR4 e TRIF nos OCs, enquanto diminuiu o número de células T reguladoras (Treg) (CD4+ CD25+/FoxP-3+ e CD8+ FoxP-3+). Além disso, a associação de P-MAPA com IL-12 estimulou significativamente as células T CD4+ e CD8+ efetoras nos linfonodos drenantes. Em relação aos mediadores inflamatórios, o P-MAPA aumentou o nível da citocina pró-inflamatória IL-17, enquanto o P-MAPA+IL-12 aumentou os níveis de IL-1β. O tratamento com IL-12 aumentou os níveis das citocinas IL-17, TNF-α, IL-1β e IL-2 e a quimiocina MIP-1α. Concluímos que o P-MAPA regulou positivamente a sinalização de TLR2 e TLR4, possivelmente ativando a via não-canônica, estimulando a resposta imune frente ao tumor. A terapia combinada melhorou a resposta efetiva das células T, contribuindo assim para eliminar as células tumorais. Essas imunoterapias representam uma contribuição adicional para o tratamento da CO.

Palavras-chave: Câncer de ovário, P-MAPA, IL-12, TLR, inflamação, células imunes.

ABSTRACT

Ovarian cancer (OC) is the second most lethal among gynecological malignancies and many women acquire chemoresistance associated with the inflammatory process. Interleukin-12 (IL-12) is responsible for the inflammatory T helper 1 (Th1) response and the protein aggregate of ammonium and magnesium phospholinoleate and palmitoleate anhydride (P-MAPA) acts as an immunostimulatory agent in aggressive cancers. This study investigated the functional treatments with P-MAPA and IL-12 on the inflammatory process and on the OC-related immune cells in an in vivo model. OCs were chemically induced with 7,12-dimethylbenz(a)anthracene (DMBA) into the ovarian bursa, and after developing tumors, the animals were given P-MAPA, IL-12, or P-MAPA combined with IL-12. The combinatory therapy improved the general features of OC, contributing to a reduction in the inflammatory cells, adipocyte accumulation in addition to revealing a soft and mobile tissue with no adherences and peritoneal implants. P-MAPA treatment increased the levels of TLR2, TLR4 and TRIF $_{\text{in OCs}}$ while decreased the number of T regulatory (Treg) cells (CD4+CD25^{+}/FoxP-3^{+} and CD8+FoxP-3^{+}). Additionally, the association of P-MAPA with IL-12 significantly stimulated the CD4+ and CD8+ T cell effector in draining lymphnodes. Regarding to the inflammatory mediators, P-MAPA enhanced the levels of pro-inflammatory cytokine IL-17 while P-MAPA+IL-12 increased IL-1β levels. The IL-12 treatment enhanced IL-17, TNF-α, IL-1β and IL-2 cytokines levels and the chemokine MIP-1α. We conclude that P-MAPA upregulated TLR2 and TLR4 signaling, possibly activating the non-canonical pathway, while attenuating the tumor immunosuppression. Combinatory therapy improved the effector T cell response, thereby contributing to eliminate tumor cells. These immunotherapies represent an additional contribution for the treatment of OC.

Keywords: Ovarian cancer, P-MAPA, IL-12, TLR, inflammation, immune cells.

1.INTRODUÇÃO

1.1. Câncer de ovário: incidência, classificação, quimioresistência e inflamação.

O câncer de ovário (CO), apresenta-se entre os oito tipos de câncer com maior incidência em mulheres sendo o segundo mais letal entre os tumores ligados ao sistema genital feminino (WHO, 2018) (Tabela 1).

Tabela 1- Números estimados de incidência e mortalidade por câncer, em mulheres, em todas as idades, mundialmente em 2018

Tipos de câncer	Incidência (números totais)	Mortalidade (números totais)
Mama	2.088.849	626.679
Colorretal	823.303	396.568
Pulmão	725.352	576.060
Cérvix uterina	569.847	311.365
Tireoide	436.344	25.514
Corpo do Útero	382.069	89.929
Estômago	349.947	269.130
Ovário	295.414	184.799
Fígado	244.506	233.256
Linfoma não-Hodgkin	224.877	102.755

Fonte: Global Cancer Observatory, World Health Organization, 2018

No Brasil, ocorreram 6.150 novos casos de CO entre os anos de 2018 e 2019, o que corresponde a 5,79 casos a cada 100 mil mulheres, tornando o CO o oitavo colocado no ranking de incidência e a terceira causa mais comum de morte por câncer (INCA, 2018) (Tabela 2).

Tabela 2- Distribuição proporcional dos dez tipos de câncer mais incidentes em mulheres estimados para 2018

Tipos de câncer	Casos (números totais)	%
Mama	59.700	29.5%
Colorretal	18.980	9.4%
Cérvix uterina	16.370	8.1%
Pulmão	12.530	6.2%
Tireoide	8.040	4.0%
Estômago	7.750	3.8%
Corpo do Útero	6.600	3.3%
Ovário	6.150	3.0%
Sistema Nervoso Central	5.510	2.7%
Leucemias	4.860	2.4%

Fonte: INCA, Instituto Nacional de Câncer 2018

O aumento no número de óbitos pode ser atribuído ao desenvolvimento tumoral que ocorre no interior da cavidade peritoneal com difícil detecção e de diagnóstico muito tardio (Armstrong et al., 2006; Lengyel, 2010). Mesmo com as terapias atuais, incluindo remoção

cirúrgica e quimioterapia, nos estágios iniciais da doença (I e II), o CO possui taxa de cura entre 70% a 90% dos casos e com 42.9% (Cancer survival rates, 2018) de taxa de sobrevivência (Mathieu, Bedi, Thrower, Qayyum, & Bast, 2018). Porém, os pacientes em estágio III ou IV da doença apresentam somente cerca de 20% de taxa de sobrevida (Armstrong et al., 2006; Kurman & Shih, 2016). A incidência do câncer de ovário aumenta no período pós-menopausa, sendo atribuído em parte, aos fatores associados à senescência ovariana como depleção de oócitos, redução dos esteroides hormonais e aumento dos níveis de gonadotrofinas circulantes (Vanderhyden, 2005). A utilização de contraceptivos orais, laqueadura tubária e amamentação também podem contribuir com a propagação da doença, assim como o acúmulo de mutações nos genes BRCA1/2 e TP53, associados primeiramente ao aumento do risco de câncer de mama (NAROD, 2010; MAVADDAT et al., 2013), e que ao longo dos anos podem atuar como um fator genético que aumentam o risco de CO (Kotsopoulos et al., 2018, 2015) Além disso, o processo ovulatório incessante, associado à inflamação crônica e a exposição aos carcinógenos ambientais contribuem com a oncogênese ovariana (Ness & Cottreau, 1999; Stewart et al., 2004).

Nos últimos anos, análises clínico-patológicas foram realizadas tanto em tumores ovarianos de baixa malignidade quanto em carcinomas invasivos (BURKS; SHERMAN; KURMAN, 1996; SHIH; KURMAN, 2004; DOCHIȚ et al., 2019; LOU et al., 2019). Com base na classificação da lesão cancerosa originada do epitélio ovariano ou de cistos de inclusão, foram observadas as diferenças entre tumor seroso e mucinoso limítrofes (tipo I) e com potencial invasivo (tipo II). Os tumores tipo I apresentam baixo grau de malignidade e incluem o carcinoma seroso micropapilar, carcinoma mucinoso, carcinoma endometrióide e carcinoma de células claras (Lisio, Fu, Goyeneche, Gao, & Telleria, 2019; I.-M. Shih & Kurman, 2005). Em tumores do tipo II, que apresentam alto grau de malignidade, tem como representantes os subtipos: carcinoma seroso, carcinoma mesotelial misto indiferenciados (Kurman & Shih, 2016; Lisio et al., 2019). Aproximadamente 90% dos carcinomas serosos ovarianos são de alto grau (moderado ou pouco diferenciado), compostos por grande massa celular com intensa atividade mitótica, núcleos celulares pleomórficos, e estruturas papiliformes (SMITH SEHDEV; KURMAN, 2003; KURMAN; SHIH, 2008)(Kurman & Shih, 2008; Smith Sehdev et al., 2003). Em modelos experimentais são comuns os subtipos de tumores ovarianos como o adenocarcinoma, cisto adenoma papilar, mesotelioma, tumor de células granulosas (NISHIDA et al., 1998; HOYER et al., 2009).

O CO é considerado um tumor quimioresponsivo à terapia padrão utilizando compostos derivados de platina e/ou taxol como a Cisplatina, Carboplatina, Paclitaxel e o Topotecano que apresentam resultados promissores em resposta ao tipo seroso e endometrióide aumentando a sobrevida dos pacientes (Cloven, Kyshtoobayeva, Burger, Yu, & Fruehauf, 2004; Ho et al., 2004; Kikkawa et al., 2006). Na última década, as terapias alvo para o CO tem sido utilizadas para o combate ao CO, dentre elas estão os inibidores de angiogênese como o Bevacizumab e inibidores de PARP (Poli ADP ribose polimerase) no entanto, mais estudos são necessários para comprovar sua eficácia frente ao CO (Lisio et al., 2019).

Contudo, a maioria dos pacientes desenvolve quimioresistência, que leva à rápida recorrência da doença e os pacientes se tornam incuráveis (Berek et al., 1999). Os mecanismos responsáveis pela quimioresistência ainda são indefinidos, no entanto, a quimioresistencia ligada aos tratamentos com taxol e platina causam diminuição do acúmulo desses fármacos, alteração do transporte e aumento da tolerância a esses medicamentos (Itamochi, Kigawa, & Terakawa, 2008). As células de adenocarcinoma ovariano humano SKOV-3, uma linhagem quimioresistente para cisplatina e paclitaxel, têm sido alvo de estudos envolvendo agentes imunomoduladores. Estudos mostram relação entre inflamação, progressão do câncer e quimioresistência (Balkwill & Coussens, 2004; R. Chen, Alvero, Silasi, & Mor, 2007).

É conhecido que receptores celulares do tipo Toll-like (TLRs) atuam promovendo inflamação local em resposta a infecções por patógenos e moléculas relacionadas, sendo predominantemente expressos por várias células imunes e algumas células tumorais (Girling & Hedger, 2007). Na imunobiologia dos tumores, o papel dos TLRs é controverso. O aumento da sinalização mediada por TLR4 está ligado ao aumento da inibição da apoptose, migração celular, angiogênese e quimioresisteincia ao paclitaxel contribuindo para progressão tumoral e potencial metastático (RAKOFF-NAHOUM; MEDZHITOV, 2009). No entanto, estudos realizados por GARAY et al. (2007) mostraram que ativação de TLR4 é eficaz contra metástases, pois aumentam os níveis de TNF-α promovendo maior permeabilidade dos vasos neoangiogênicos aos agentes citotóxicos, estimula a produção de iNOS levando a apoptose, além de ativar células dendríticas locais. A expressão diferencial de TLR4 nos tecidos tumorais tem despertado grande interesse em pesquisas com câncer. Estudos sugerem que a quimioresistência ao paclitaxel deva ocorrer através de mecanismos ligados ao receptor TLR4 (Kelly et al., 2006). Embora a presença do TLR4 está associada ao infiltrado inflamatório

(Bäckhed & Hornef, 2003), seu papel em células tumorais é pouco compreendido. Quando ativados, os TLRs promovem a cascata de sinalização que desencadeia a inflamação, via adaptadores moleculares denominados MyD88 ou TRIF (**Figura 1**) (Oliva, Bardag-Gorce, Li, French, & French, 2011), o que leva a ativação do fator nuclear-kB (NF-kB) ou do fator de transcrição regulador de interferon (IRF3). O NF-kB regula a transcrição de vários genes relacionados com a resposta imune, adesão celular, proliferação, angiogênese e apoptose (BEINKE & LEY, 2004), sendo que anormalidades da sua regulação podem promover a inflamação e até mesmo o desenvolvimento de CO (G. G. A. Chuffa et al., 2015). As moléculas IL-6, IL-8 e TNF-α são auxiliares no processo neoplásico (Karin, Cao, Greten, & Li, 2002), e um estudo recente realizado em nosso laboratório mostrou aumento da ativação da via de sinalização mediada por TLR4, além da elevação dos níveis de IFN-β, IL-6 e TNF-α pelas células do CO em animais induzido quimicamente com DMBA (G. G. A. Chuffa et al., 2015). A inflamação crônica, com produção desregulada de mediadores inflamatórios, está envolvida em todos os estágios tumorais como crescimento, invasão, migração e metástase; desta forma, sua compreensão se torna necessária para o entendimento da relação de quimioresistência do CO frente aos tratamentos.

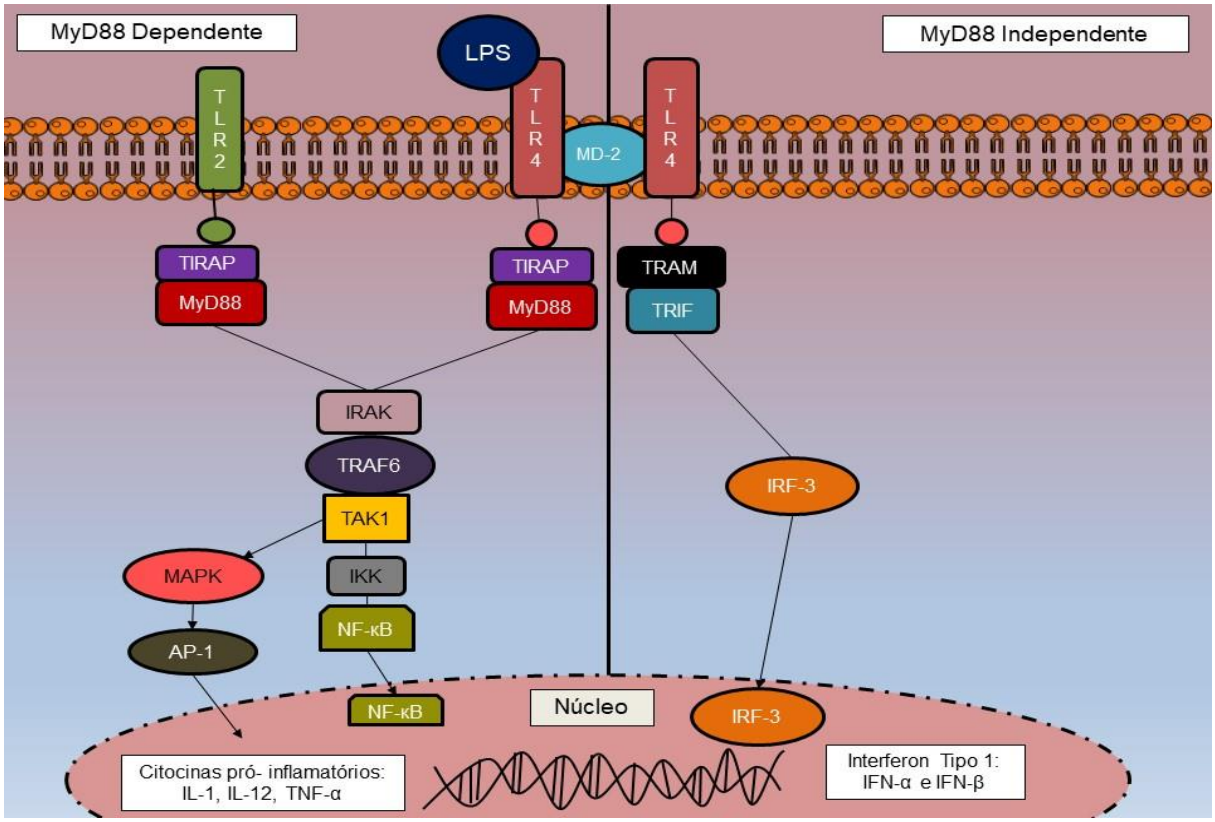
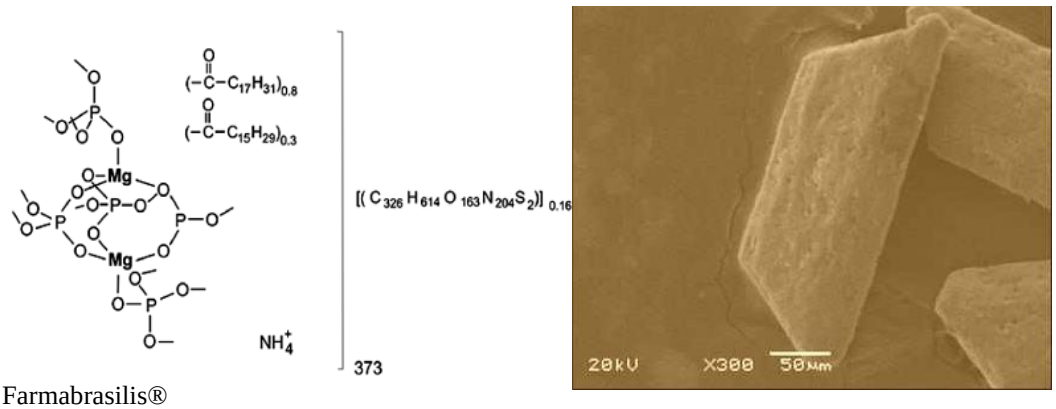


Figura 1: Esquema representativo das vias de sinalização dos receptores TLR2 e TLR4. O receptor TLR2 ativa a cascata de eventos pela via canônica (dependente do MyD88) enquanto o receptor TLR4 o faz pela via canônica e não-canônica (dependente de MyD88 e TRIF). O MyD88, proteína que está associado ao TIRAP,

recruta o TRAF6 e IRAK. O TRAF6 ativa o complexo TAK1e por consequência ativa IKK, um complexo que possui 3 unidades (IKKα, IKKβ e NEMO). Após fosforilação e degradação de Ikβ, o NF-κB p65 é ativado e transloucado para o núcleo, participando da expressão de genes inflamatórios. O TAK1 também pode ativar MAPK, que resulta na ativação e fosforilação de AP-1. O TRIF está associado ao TRAM que por sua vez fosforila o IRF-3 que após transloucar para o núcleo, se liga a genes promotores da transcrição de interferons. LPS: Lipopolissacaríde, MD-2: Proteína mieloide de diferenciação-2. MyD88: proteína de resposta primária à diferenciação associada ao receptor mieloide de interleucina-1. TIRAP: proteína adaptadora que contém o domínio do receptor. TRAF6: fator 6 do fator de necrose tumoral associado ao receptor, IRAK: proteína quinase associada ao receptor de interleucina-1, TAK1: fator de crescimento transformador quinase 1, IKK: Inibidor do fator nuclear κB quinase, NF-κB p65: fator nuclear-κB unidade p65, MAPK: proteína quinase ativada por mitogênio, AP-1: proteína ativadora 1, TRIF:Toll domain-containing adaptor protein-inducing interferon β, TRAM: moléculas adaptadoras relacionadas ao TRIF, IRF-3: fator regulador de Interferon-3. Esquema ilustrativo realizado pelo autor.

1.2. P-MAPA: propriedades antitumorais e oncostáticas e a via mediada por TLRs

O P-MAPA (agregado polimérico de fosfolinoleato-palmitoleato de magnésio e amônio proteico) é um biopolímero em forma de microcristais com propriedades imunomoduladoras, produzida através da fermentação do fungo *Aspergillus oryzae* (**Figura 2**). O P-MAPA foi patenteado pela organização não governamental sem fins lucrativos (Farmabrasilis), e tem sido utilizado experimentalmente em pesquisas envolvendo o câncer de bexiga, apresentando bom prognóstico e baixa toxicidade, atuando no aumento da p53, proteína que possui atividade regulatória sobre os TLRs, e está associada com o grau de agressividade do tumor (FÁVARO et al., 2012; DIAS et al., 2016).



Fonte:Farmabrasilis®

Figura 2. A molécula P-MAPA: Estrutura e imagem por microscopia. A esquerda está representada a forma estrutural e molecular da molécula de P-MAPA na qual é possível identificar grupamentos cetona e elementos como fósforo e magnésio. A direita está representada os micro cristais de P-MAPA (imagem obtida através de microscopia eletrônica de varredura em um aumento de 300X). Barra = 50 µm.

O imunomodulador P-MAPA possui efeitos significativos sobre componentes do sistema imunológico, sendo capaz de estimular os receptores TLR2 e TLR4, e aumentar a produção de linfócitos T, de citocinas como a IL-2 e o IFN-γ, além de promover o aumento da atividade das células natural killers (NKs); também já apresentou aumento na produção e liberação de óxido nítrico pelos macrófagos (FARMABRASILIS, 2008). As citocinas têm a

capacidade de regular as funções de proliferação, apoptose e diferenciação celular, sendo produzidas por vários tecidos e células, incluindo a célula tumoral (Teran et al., 2001). Estudos recentes in vivo e in vitro sugerem que o P-MAPA atua na modulação da resposta imune em tumores malignos, restaurando a imunocompetência celular (Fávaro et al., 2012). Em modelo de CO experimental, o tratamento com P-MAPA associado a cisplatina reduziu o volume tumoral, aumentou a taxa de sobrevida dos animais, e potencializou a via mediada por TLR4 na presença de cisplatina; houve aumento dos níveis de MyD88, TRIF, inibidor de NF-kB alfa fosforilado (p-IkBα), NF-kB p65 citoplasmático e nuclear e interferon-γ (IFN-γ) (de Almeida Chuffa, de Moura Ferreira, Lupi, da Silva Nunes, & Fávaro, 2018). Dado que o P-MAPA apresenta resultados promissores tanto em estudos clínicos como experimentais, novos achados poderão auxiliar nas ações do P-MAPA como terapia complementar, visto que não existe estudos na literatura relacionando o P-MAPA como adjuvante para os tratamentos padrões contra o CO. Há ainda necessidade de investigar a ação do P-MAPA associado com outras moléculas no CO, uma vez que é conhecido que o P-MAPA apresenta baixa toxicidade e pode ser de grande valor para os casos de quimioresistência aos tratamentos convencionais da doença. Recentemente, nosso grupo mostrou que o P-MAPA aumentou a quimiosensibilidade do paclitaxel, e quando associado a IL-12 promoveu redução da migração e invasão das células SKOV-3. Além disso, a combinação de P-MAPA com IL-12 reduziu os níveis de MyD88, IRF3 e NF-kB p65 nas células de CO, mostrando ser efetivo contra uma possível quimioresistência associada a via TLR (LUPI et al. 2019).

1.3. Papel da IL-12 em tumores sólidos

A interleucina-12 (IL-12) é uma citocina relacionada com a imunidade inata e adaptativa atuando também na resposta inflamatória e infecções (Liu et al., 2018; Zhao et al., 2019). A IL-12 é produzida pelas células apresentadoras de antígenos (APCs), linfócitos B, entre outras. A IL-12 atua sobre as células T e NK estimulando a atividade de linfócitos citotóxicos e induzindo proliferação e produção de citocinas, especialmente IFN-γ (Colombo & Trinchieri, 2002a). Além disso, a IL-12 é uma das citocinas responsáveis pela diferenciação da resposta polarizada Th1, que por sua vez, são produtoras de IFN-γ. O IFN-γ aumenta a habilidade das APCs em produzir IL-12 (Ma et al., 1996), atuando como um potente estimulador positivo em várias respostas celulares. A IL-12 é uma molécula heterodimérica, composta de uma cadeia α (subunidade p35) e β (subunidade p40) ligada por pontes dissulfeto (**Figura 3**) (Wolf et al., 1991).

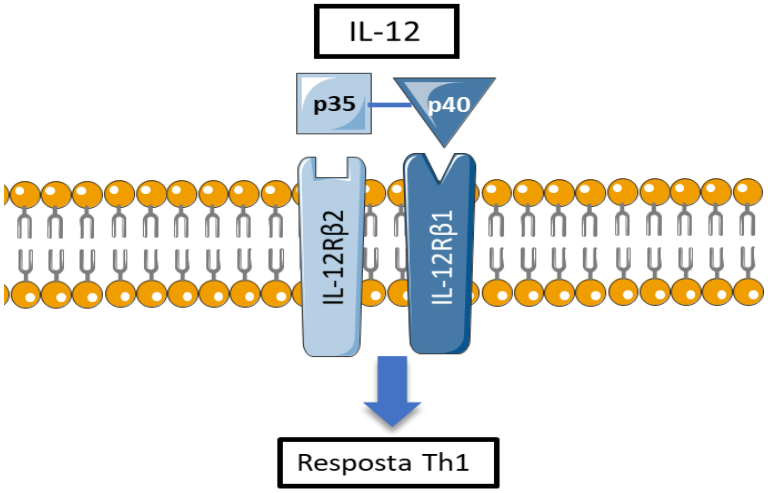


Figura 3. Esquema representativo da IL-12. A IL-12 representada com suas subunidades p35 e p40 unidas por ligações dissulfeto associadas aos seus respectivos receptores de membrana IL-12Rβ2 e IL-12Rβ1 desencadeando assim a resposta Th1.

Foi observado que altas doses de IL-12 levam à indução da imunidade tumoral mediada por células NKs, enquanto baixas doses não mostraram atividade anti-tumoral (Smyth et al., 2002). Outros estudos experimentais têm apontado que a administração sistêmica de IL-12 leva a resposta imune anti-tumor principalmente através da indução de IFN-γ. Especificamente no CO recorrente, o tratamento intravenoso com IL-12 mostrou ser uma estratégia funcional benéfica (Hurteau, Blessing, DeCesare, & Creasman, 2001). Neste estudo, após os ensaios pré-dosagens para limitar a toxicidade, pacientes receberam 250 ng/kg de IL-12 em dose única, seguido por um intervalo de 2 semanas, com ciclos subsequentes de administração durante 5 dias consecutivos, seguido de intervalo de 16 dias, até alcançar a remissão do tumor. Em modelo de CO, o tratamento com IL-12 (0.33 mcg/dia, via i.p., durante 20 dias) resultou em redução no crescimento do tumor com aumento no número de células NK agrupadas no interior do tumor (Silver, Hempling, Piver, & Repasky, 1999). Entretanto, estudos demonstram que o tratamento com IL-12 ainda apresenta considerável toxicidade nos pacientes o que torna necessário mais pesquisas com terapias conjuntas em busca de novos resultados (Lenzi et al., 2007a, 2002). Diante desse cenário, a IL-12 pode ser um importante fator adjuvante na terapia contra diversos tipos de câncer, entre eles o CO.

1.4. Mediadores do processo inflamatório e o CO

A resposta inflamatória é desencadeada por diversas interações entre componentes, tais como células de caráter inflamatório, mediadores químicos presentes na circulação, e por tecidos que sofreram algum dano (Niederhuber, Armitage, Doroshow, Kastan, & Tepper,

2013). No entanto, existe uma dualidade no que diz respeito às funções desses mediadores do processo inflamatório nas ações contra o câncer (Shrihari, 2017). O microambiente tumoral que é formado possui mediadores, os quais, podem tanto ter a ação de inibição tumoral através da ação de células NK, neutrófilos, macrófagos, TCD4+ e TCD8+, quanto promover o crescimento e expansão tumoral através de TNF-α, Tregs, IL-10 e IL-6 (Shrihari, 2017).

As citocinas são polipeptídios produzidos por células do sistema imune e por células que estão no local de lesões através da ativação de proteínas quinases e podem agir de maneira autocria ou parácrina (LIN; CALVANO; LOWRY, 2000; SOMMER; WHITE, 2010). São divididas em interleucinas (IL-1 a IL-35), quimiocinas (citocinas quimiotáticas), interferons (IFNs) e fatores de necrose tumoral (TNFs) (RAEBURN et al., 2002; SOMMER; WHITE, 2010). Essas proteínas participam da proliferação, diferenciação, regulação de outras citocinas e sobrevida de células do sistema imune, podendo atuar como moléculas pró-inflamatórias (Th1, IL-1α, IL-1β, IL-2, IL-12, IL-17 e TNF-α) ou anti-inflamatórias (Th2, IL-4 e IL-10) (CURFS, 1997; COLOMBO; TRINCHIERI, 2002a; AMATYA; GARG; GAFFEN, 2017). Com relação às quimiocinas, estas participam do processo de migração de células do sistema imunológico para que haja a geração de respostas imunes de carater humoral e celular para o combate à patógenos (Griffith, Sokol, & Luster, 2014a). A sua nomenclatura se deve a posição das duas primeiras cisteínas intercaladas com aminoácidos não conservados levando as subclasses CXC, CCL (Rot & von Andrian, 2004); como alvos desse trabalho foram analisadas as quimiocinas CCL3 (MIP-1α), CCL5 (RANTES) e CXCL-10 (IP-10) envolvidas no desenvolvimento do CO, afim de observar os seus níveis frente aos tratamentos propostos.

Em relação ao CO, os mediadores também se relacionam de maneira a auxiliar nos processos de apoptose e supressão, bem como servem de biomarcadoes para a identificação desse tipo de tumor (Shrihari, 2017). O TNF-α, quando se expressa em uma inflamação persistente, leva a danos no DNA, angiogênese, invasão e metastase, além de suprimir as respostas imunes locais (Szlosarek & Balkwill, 2003). O IFN-γ apresenta ações antiproliferativas e imunomoduladoras contra o avanço do CO (Pujade-Lauraine et al., 1996). Segundo WALL et al. (2003), o efeito antiproliferativo do IFN- γ ocorreu em todas as 8 linhagens celulares de câncer de ovário (OAW42, Ovcar-3, Ovcar-4, Ovcar-5, PEO1 (CDDP), PEO14, PEO16, SW626), sendo esse efeito evidenciado pelo aumento dos níveis de PARP clivada. Estudos *in vitro* também apontam que a combinação de monocitos somados ao IFN-γ humano levam a um efeito antitumoral e essa combinação está sendo testada atualmente em

pacientes com CO que receberam uma dose de seus próprios monócitos em associção com o IFN-γ; essa terapia combinando célula e citocina indica uma nova abordagem no âmbito das imunoterapias (JOHNSON; GREEN; ZOON, 2015; GREEN et al., 2018).

No que diz respeito às interleucinas, a IL-1 desencadeia a ativação de genes pró-inflamatórios através dos mediadores intermediários IKK e p38 (Deon et al., 2001). A IL-1β apresenta tanto efeitos anti-tumorais estimulando a resposta Th1 e Th17, quanto sinalizando através de MyD88 e NF-kB para a produção de genes inflamatórios que contribuem para um microambiente favorável ao tumor (Baker, Houston, & Brint, 2019). A IL-2 é uma citocina que estimula o crescimento de células T, a atividade células NK, e induz a diferenciação de células Treg (Liao, Lin, & Leonard, 2011). A imunoterapia que utiliza a citocina IL-2 em associação com o quimioterápico Ricibanil (OK-432), utilizada em pacientes com CO em estágio avançado, apresentou uma taxa de reincidência do tumor menor se comparado ao tratamento com o quimioterápico isolado (Wang, Peng, Su, & Lin, 2018). Corroborando com esse achado, a sobrevida de pacientes com CO reincidente foi aumentada com a aplicação intraperitonial de IL-2 (regime de 16 doses semanais) em associação com docetaxel e carboplatina (MINOR; MOORES; CHAN, 2017).

A IL-4 estimula a diferenciação de linfócitos B e T e possui a função de regular a imunidade adaptativa e humoral. A IL-4 também estimula a mudança de perfil de IgE para IgG e a produção de moléculas MHC de classe II. Além disso, diminui a produção de células Th1, macrófagos, IFN-γ e IL-12 (Hershey, Friedrich, Esswein, Thomas, & Chatila, 1997; Sokol, Barton, Farr, & Medzhitov, 2008). Em um estudo *in vivo*, camundongos com CO implantados que receberam uma citotoxina composta por IL-4 tiveram regressão nos tumores e processos metastáticos e elevação da taxa de sobrevida (Kioi et al., 2005). A IL-17 é uma citocina pró-inflamatória produzida pelas células TCD4+ e compreende seis membros, sendo as isoformas IL-17A e IL-17F produzidas por células com fenótipo Th17; entre suas funções, a IL-17 recruta monócitos e neutrófilos até os locais da inflamação (MIOSSEC; KORN; KUCHROO, 2009; CHIRICOZZI et al., 2011). Em relação ao CO, a IL-17 pode ser utilizada como um biomarcador para a presença da doença, uma vez que ascites provenientes do CO expressam essa citocina de maneira considerável (Y.-L. Chen et al., 2015). A IL-10 é uma citocina que possui ação anti-inflamatória e de imunossupressão uma vez que atenua as repostas efetoras de macrófagos, células T e células NK (Moore, O'Garra, Malefyt, Vieira, & Mosmann, 1993). A quimiocina IP-10 (CXCL-10) tem caráter inflamatório promovendo a quimioatração de macrófagos, células T, células NK, células dendríticas e recrutando

leucócitos para a região danificada. A IP-10 também possui ação antitumoral por inibir a angiogênese em tumores malignos (Dufour et al., 2002).

A RANTES (CCL5) é uma quimiocina produzida a partir de linfócitos T ativados e monócitos e também possui perfil inflamatório atraindo células TCD8+ para o local de inflamações (WERTEL et al., 2011; GRIFFITH; SOKOL; LUSTER, 2014b). Essa quimiocina participa da imunologia do CO uma vez que estimula a migração de linfócitos TCD8+, TCD3+, células Th17 e monócitos para o microambiente do CO (Koshiba et al., 2000; Su et al., 2010). No entanto, a RANTES pode ajudar na diferenciação de células tumorais de CO, e tende a encontrar-se aumentada em pacientes com CO em comparação com pacientes com tumores benignos; seu nível se relaciona com o estágio da doença bem como a extensão da massa tumoral sugerindo participação na progressão tumoral (Tsukishiro, Suzumori, Nishikawa, Arakawa, & Suzumori, 2006). A MIP-1α (CCL-3) é responsável pela migração de macrófagos, células NK e pela interação entre células T e células dendríticas (Bachelerie et al., 2014). No CO, as células NK podem liberar MIP-1α que atraem células dendríticas imaturas para o sítio do tumor e essa liberação é influenciada pela interação entre fatores inflamatórios como IL-12, IL-5 e IFN-α (Wong, Berk, Edwards, & Kalinski, 2013). Desta forma, a observação de possíveis alterações nas concentrações de citocinas e quimiocinas no microambiente do CO pode ser usado como indicativo de resposta aos tratamentos e, de acordo com o subtipo de molécula alterada, pressupor se os mesmos estão exercendo uma ação positiva ou negativa no curso do tratamento da doença.

1.5. Sistema imune e CO

Estudos têm documentado a importância da atuação das células do sistema imune e seus produtos no microambiente tumoral, uma vez que características do infiltrado inflamatório definem seu desenvolvimento e a sua progressão (Onuchic & Chammas, 2010). Experimentos realizados em camundongos imunocomprometidos mostraram não haver diferença entre o desenvolvimento tumoral nesses animais quando comparados aos camundongos selvagens, o que indica a inabilidade do sistema imune em impedir a formação tumoral (Stutman, 1974). Estudos envolvendo mecanismos moleculares de vigilância imunológica comprovaram que a produção endógena de IFN-γ protege o animal contra o crescimento de tumores transplantados e quimicamente induzidos, e a perforina presente em células T citotóxicas e NK está relacionada com a morte da célula tumoral (duPre', Redelman, & Hunter, 2008).

As células T desempenham papel crucial nesses eventos, atuando diretamente nas respostas celulares e na regulação e produção de anticorpos. Os linfócitos T constituem-se de duas principais subpopulações: TCD4+ e TCD8+ ativadas. Em resposta aos antígenos proteicos específicos, os linfócitos TCD4+ auxiliares (Th) podem diferenciar-se em subpopulações de células efetoras com funções distintas e que produzem diferentes grupos de citocinas. As subpopulações bem definidas de linfócitos Th são designadas Th1 e Th2, sendo o IFN-γ, IL-2 e TNF-α as citocinas características das células Th1, e a IL-4, IL-5, IL-10 e IL-13 as citocinas provenientes das células Th2. A IL-12 é a principal indutora da resposta polarizada para células Th1, que por sua vez produzem IFN-γ para promover a diferenciação Th1 enquanto inibe a proliferação das células Th2 (ABBAS et al., 2003;QUINTANS et al., 2019). Nos tumores, as células do sistema imune são recrutadas em decorrência de processos inflamatórios pré-câncer ou por substâncias produzidas pelas próprias células do tumor.

No CO, essas células estão relacionadas com o prognóstico da doença e, além disso, o perfil imunológico pode ser uma ferramenta útil na condução de estratégias funcionais alternativas de tratamento, com destaque para as imunoterapias. No CO epitelial, as células TCD3+ representam a maior população detectada no infiltrado tumoral e em ascites (Santin et al., 2001). Em estágios avançados da doença, a sobrevida é maior em pacientes apresentando tumores com mais de 125 linfócitos/mm^2, variando entre os tipos TCD3+ e TCD8+ (Tomšová, Melichar, Sedláková, & Šteiner, 2008).

Os linfócitos T regulatórios (Tregs) são um subgrupo celular derivados de TCD4+ que modulam de maneira supressora as funções imunológicas de outras células. Grande parte da Tregs estão ligadas a sintese de IL-2 uma vez que expressam a cadeia alfa dos receptores de IL-2, o CD25. O fator FOXP3 (forkhead box P3) é membro da familia de fatores de transcrição Forkhead e possui a capacidade de suprimir a ativação de outras células T; geralmente é produzido por intermédio da ação da IL-2 ao ativar o gene de transcrição STAT5 assim como outros genes ligados a supressão (ABBAS et al., 2003; CAMPBELL; ZIEGLER, 2007; MELO; TAVARES; CARVALHO, 2009; CHURLAUD et al., 2015). Existem quatro tipos de células Tregs: células Th3, TR, Tregs (CD4+, CD25+, Foxp3) e células NK (Umetsu et al., 2003). Além de estarem relacionadas com baixo prognóstico, as células Treg podem bloquear os efeitos protetores dos linfócitos T, suprimindo a produção de IFN-γ e IL-2 pelas células T (ABBAS et al., 2003). Mais recentemente, foi identificado que as células Treg representam 10 a 17% da população de linfócitos T em ascites provenientes de CO (Curiel et al., 2004). Em estudo realizado em pacientes com CO seroso tratados com derivados da

platina e taxol, foi observado que aqueles que tinham taxas de Tregs em maior número antes do início do tratamento apresentaram piora nos resultados da quimioterapia em relação aos que tinham os níveis de Tregs baixos inicialmente (Dutsch-Wicherek et al., 2019). Enquanto a IL-12 estimula o crescimento das células T, o IFN-γ ativa macrófagos e células NK, além de promover diferenciação de linfócitos T e aumentar expressão de moléculas do MHC de classe I e II. O uso de novos agentes imunomoduladores associados aos já existentes poderão indicar novas diretrizes para o tratamento do CO e favorecer a qualidade de vida da paciente.

2. JUSTIFICATIVA

O uso de imunoterapias tem mostrado eficiência na redução dos efeitos danosos em vários tipos de câncer. Além da terapia com P-MAPA não promover citotoxicidade em tecidos/órgãos saudáveis, já revelou ação moduladora sobre alguns mediadores do processo inflamatório em tumores sólidos, podendo favorecer o prognóstico da doença, auxiliar no bem-estar do paciente e, no entendimento dos mecanismos ligados a quimioresistência. A compreensão desses efeitos poderá trazer novas perspectivas para o entendimento do CO. Desse modo, a avaliação do CO, sob o ponto de vista inflamatório, e o papel do P-MAPA associado a IL-12 como estratégia terapêutica na atenuação desse processo se mostram como potenciais abordagens que podem auxiliar no desenvolvimento de novas ferramentas de combate ao CO.

3.HIPÓTESE

A hipótese do estudo é de que o P-MAPA, atuando como um regulador de TLR, e a IL-12, que participa da ativação da resposta Th1, agem como potencializadores da via mediada por TLR2 e TLR4 e que a associação desses componentes estimula diferentes perfis de células imunes associados ao CO.

4. OBJETIVO GERAL

Avaliar o efeito dos tratamentos com o imunomodulador P-MAPA e IL-12 isoladas, e associação de P-MAPA com IL-12, sobre o processo inflamatório e o perfil das células do sistema imune associada ao CO induzido quimicamente em ratas Fischer 344.

4.1 OBJETIVOS ESPECÍFICOS

1. Avaliar os aspectos histopatológicos do CO após os tratamentos funcionais;

2. Imunolocalizar os receptores TLR2 e TLR4 e quantificar os receptores TLR2, TLR4 e os fatores imunológicos MyD88, TRIF, IRF3 e NF-kB envolvidos no processo inflamatório;

3. Quantificar os mediadores do processo inflamatório (citocinas pró e anti-inflamatória) presente no soro dos animais com CO;

4. Analisar e quantificar as células do sistema imune (linfócitos TCD4, TCD8 e Tregs) nos linfonodos drenantes do CO.

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CAPÍTULO 2

Artigo submetido ao periódico científico "*Life Sciences*"

P-MAPA activates TLR2 and TLR4 signaling while its combination with IL-12 stimulates CD4+ and CD8+ effector T cells in ovarian cancer

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Short-title: P-MAPA and IL-12 stimulate effector T cells in ovarian cancer.

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Abstract

Ovarian cancer (OC) is the first most lethal among gynecological malignancies and many women develop chemoresistance associated with the inflammatory process. Interleukin-12 (IL-12) is responsible for the T helper 1 (Th1) response and the P-MAPA acts as an immunostimulatory agent in aggressive cancers. We investigated herein the effects of P-MAPA and IL-12 on the inflammatory and immune responses in a chemically-induced OC model. OCs were induced with 7,12-dimethylbenz(a)anthracene into the ovarian bursa, and the animals were given P-MAPA, IL-12, and their combination as treatment. The combinatory therapy improved the general OC features, reducing inflammatory cells and adipocyte accumulation, in addition to revealing a soft and mobile tissue with no adherences and peritoneal implants. P-MAPA treatment increased the levels of TLR2, TLR4 and TRIF in OCs while decreasing the number of regulatory T (Treg) cells. Additionally, the association of P-MAPA with IL-12 significantly increased the number of CD4+ and CD8+ T effector cells in draining lymph nodes. Regarding the inflammatory mediators, P-MAPA enhanced the levels of the pro-inflammatory cytokine IL-17 while P-MAPA+IL-12 increased the levels of IL-1β. Treatment with IL-12 enhanced the cytokine levels of IL-17, TNF-α, IL-1β, and IL-2 in addition to the chemokine MIP-1α. We conclude that P-MAPA upregulated TLR2 and TLR4 signaling, possibly activating the non-canonical pathway, while attenuating the tumor immunosuppression. Also, the combination of P-MAPA with IL-12 improves the antitumor immunoresponse, opening a new therapeutic approach for fighting OC.

Keywords: Ovarian cancer, P-MAPA, IL-12, TLR, inflammation, immune cells.

1. Introduction

Ovarian cancer (OC) is the most lethal cancer related to the female reproductive system (Siegel, Miller, & Jemal, 2019). This may be attributed to the nature of OC developing inside the peritoneal cavity where the symptoms are generics and hard to be detected (Armstrong et al., 2006). Although surgical removal and chemotherapy are efficient to treat OC at an early stage (Mathieu et al., 2018), the disease become incurable at advanced stages. OC exhibits a good response to platinum- and taxane-based therapies, displaying 80% efficiency for the serous and endometrioid subtypes (Cloven et al., 2004; Kikkawa et al., 2006). However, most patients develop chemoresistance during the treatment resulting in a high recurrence rate (Berek et al., 1999). One of the emerging strategies for OC treatment includes new targets of immunotherapies (Bronte et al., 2014), which are associated with chemosensitivity and tumor regression (de Almeida Chuffa et al., 2018).

P-MAPA (protein aggregate magnesium and ammonium phospholinoleate-palmitoleate anhydride), is a subproduct of the Aspergillus oryzae fermentation, a biopolymer with immunomodulatory properties that exhibits antitumor effects in different experimental models (de Almeida Chuffa et al., 2018; Fávaro et al., 2012; Garcia, Seiva, et al., 2016). More recently, it has been shown that P-MAPA associated with cisplatin reduced the OC volume and increased the survival rate of OC-bearing models. In addition, P-MAPA stimulated the expression of proteins involved in the inflammatory response to OC (de Almeida Chuffa et al., 2018). Alternatively, P-MAPA has shown a significant effect on immune system components stimulating TLR2 and TLR4 receptors, increasing T lymphocyte production, and enhancing the activity of natural killer cells (NKs) [11]. We recently reported that P-MAPA enhanced the sensitivity to paclitaxel and is capable of altering several important proteins associated with OC progression (Júnior et al., 2019; Lupi et al., 2019). However, its effective role on immune cells infiltrated in OC needs to be further explored. Interleukin-12 (IL-12) is a cytokine of the innate and adaptive immunity produced by the antigen-presenting cells (APCs) (Cohen, Shea, Heffron, Schmelz, & Roberts, 2016; Trinchieri, 2003). It especially stimulates the production of interferon-γ (IFN-γ) resulting in a polarized T helper 1 (Th1) immune response while stimulating CD8+ T and NK cells to sensitize cytotoxic response [15,16]. Although OC patients showed a tumor regression after IL-12 therapy, one of the barriers to be overcome is related to its high toxicity when administered at high levels (Lenzi et al., 2007a).

Toll-like receptors (TLRs) are active molecules that participate in the host defense by recognizing molecular patterns associated with pathogens. The TLR2 and TLR4 are involved in the OC inflammatory processes and activate signaling pathways via TIR domain-containing adaptor inducing interferon-β (TRIF) or myeloid differentiation factor 88 (MyD88). These pathways are related to chemoresistance, cytokine/chemokine production and cell proliferation (R. Chen et al., 2007; Kiziltas, 2016). TLRs have a dual role in cancer. TLR4 activation is effective against metastases due to the increase in TNF-α levels which promote permeability to cytotoxic agents, stimulation of iNOS production and activation of local dendritic cells (Garay et al., 2007). On the other hand, increased TLR4-mediated signaling is associated with tumor progression and metastatic potential of OC (Vlad et al., 2010). According to KE et al. [22], the tumor-associated macrophages (TAM) mediated the elevation of MyD88-dependent TLR pathway in OC cells indicating the promotion of cellular invasion and tumor progression. In SKOV-3 cells, P-MAPA reduced the TLR2 levels while the combination of P-MAPA with IL-12 significantly reduced migration and invasion capacity of cells; these effects seem to be dependent on the downregulation of MyD88, interferon regulatory factor 3 (IRF3), and nuclear factor kappa B (NF-kB p65) (Lupi et al., 2019). Meanwhile, an increase in TLR expression by the immune cells can lead to a beneficial action after creating an inflammatory environment that hampers OC progression (de Almeida Chuffa et al., 2018).

Immune responses to OC involve the ascitic infiltration by CD3+T cells, which represent the major population of immune cells (Santin et al., 2001). Otherwise, regulatory T cells (Tregs) are a population that modulates the function of other important cells (Umetsu et al., 2003), being associated with lower OC prognosis by suppressing the production of IFN-γ and IL-2 by T cells while inhibiting antitumor responses (Pedros, Canonigo-Balancio, Kong, & Altman, 2017). There is an interaction between inflammation and immune system regarding their functions against cancer (Shrihari, 2017). Depending on types of cytokines and chemokines secreted by the cells, the OC can be reduced or expanded in size and aggressiveness (Ingersoll et al., 2015; Lane, Matte, Garde-Granger, Bessette, & Piché, 2018; Mustea et al., 2008). Since IL-12 has a central role in establishing an effective antitumor Th1 response and P-MAPA is able to stimulate TLR2 and TLR4 receptors, we investigated the in vivo effect of their combination against OC developed in a chemically induced model of rat.

2. Materials and Methods

2.1. Animals, OC induction and treatments

Forty adult female Fischer 344 rats (60-days-old, weighing ±200g) were used in the experiment. The animals were purchased from the Multidisciplinary Center for Biological Investigation (CEMIB) at University of Campinas (UNICAMP), being individually housed in polypropylene cages with laboratory-grade pine shavings as bedding and kept in a room under controlled temperature and luminosity (22 ± 1ºC with a 12/12h light/dark cycle). Standard rodent chow (3074 SIF, Purina Ltda., Campinas, SP, Brazil) and filtered tap water were provided *ad libitum*. This experimental protocol was previously approved by the Animal Ethical Committee of the Institute of Bioscience/UNESP (CEUA), Campus of Botucatu, SP, Brazil (Permit number: 950).

Sixty-day aged animals were anesthetized with 10% ketamine and 2% xylasine during the estrous phase. After washing with chlorhexidine and 70% (v/v) ethanol, the skin and abdominal wall were incised to access the fat pad surrounding the ovaries. The bursa of the left ovaries was injected with a single dose of 100µg of 7,12-dimethylbenz(a)anthracene (DMBA; Sigma Chemical Co, St Louis, MO) dissolved in 10 µL of sesame oil (vehicle) [30-32]. Control right ovaries were injected with the vehicle only (sham-surgery). After the procedures, the ovaries were placed back to the cavity, and muscles, fascia, and skin were tightly closed using 3-0 silk suture (Ethicon Inc., Mexico, MX). Antibiotic benzylpenicillin potassium was given to the animals in the post-operatory stage and all tumors were monitored by volume and total size following four months.

The animals were divided into four experimental groups (n=10/group): Control (CO), composed by rats induced to OC with DMBA and treated with saline solution; P-MAPA, composed by rats induced to OC with DMBA and treated with P-MAPA; IL-12, composed by rats induced to OC with DMBA and treated with recombinant IL-12 (rrIL-12); P-MAPA+IL-12, composed by rats induced to OC with DMBA and treated with P-MAPA and IL-12 simultaneously. P-MAPA was administered via i.p. at 5 mg/kg body weight dissolved in 0.20 mL of 0.9% (v/v) saline (vehicle), twice a week for 60 days totalizing 16 injections/animal (Fávaro et al., 2012). The animals treated with rrIL-12 (Genetics Institute, Andover, MA) received 300 ng/kg, via i.p., diluted in 0.60 mL phosphate-buffered saline (PBS), once a week for 60 days, according to Lenzi et al., [17] (Figure 1). All treatments were administered in the morning (between 09:00-10:00 h). After 180 days, the animals were anesthetized with ketamine and xylasine and euthanized by decapitation for sample collection.

2.2. OC histopathology

After euthanasia, the OCs (n=7/group) were harvested and half of the tissues were fixed in 10% buffered formalin followed by dehydration, clearing, and paraffin embedding (Oxford Labware, St. Louis, USA). The tissue was serially sectioned at 4-µm thick, and section was stained with hematoxylin and eosin. Samples were histopathologically analyzed and reported by a pathologist, and OCs were subtyped according to the features of the neoplasms. Animals displaying evident OC had a portion of the tissue removed, rapidly frozen, and stored at -80˚C for further analysis.

2.3. Immunohistochemistry

For immunohistochemical analysis, OC sections (n=7/group) were deparaffinized in xylene and hydrated in graded series of ethanol. Antigen retrieval was performed in microwave oven using 0.01 M sodium citrate buffer, pH 6.0, for 15 min. After blocking peroxidase activity, the tissues were incubated in 3% bovine serum albumin (BSA) for 1h, and then exposed to primary antibodies (1:100; Abcam, Cambridge, UK) in a humidified chamber overnight at 4º C: rabbit polyclonal anti-TLR2, mouse monoclonal anti-TLR4, rabbit polyclonal anti-MyD88, rabbit polyclonal anti-TRIF, rabbit monoclonal anti-interferon regulatory factor 3 (IRF3), and rabbit polyclonal anti-nuclear factor kappa B subunit p65 (NF-kB). After reactions, the slides were subsequently washed in TRIS-buffered saline plus Tween 20 (TBS-T buffer) and incubated with secondary antibody (Polymer Anti-Mouse IgG or Anti-Rabbit - DAKO® CYT) at room temperature for 1h. Finally, tissues were reacted with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) for 5 min, and counterstained with hematoxylin. Negative controls were used by omitting the primary antibody. The immunohistochemistry data (IHC) were analyzed by pathologists under a Zeiss AxiophotII microscope (Carl Zeiss, Oberkochen, Germany).

2.4. Western blot analysis

OC samples (n=5/group) were removed and stored at -80º C. Serous papillary tumors were dissected and homogenized using 10x RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA) supplemented with protease inhibitors (Sigma Chemical Co.). After protein extraction, aliquots of 1:10 (v/v) Triton X-100 were added to the homogenates and samples were agitated for 2h. The lysates were then centrifuged at 21,912 g for 20 min at 4ºC to remove cell debris, and total protein was quantified by Bradford assay. 40-µg protein samples were dissolved in 1.5X Laemmli buffer and used for SDS-PAGE (Bio-Rad

Laboratories, Hercules, CA, USA) in preformed 4-12% acrylamide gradient gels. After electrophoresis was carried out on TRIS-glycine running buffer system (60mA for 2h), the proteins were electro-transferred to nitrocellulose membranes, and then blocked with 3% BSA for 1h. After non-specific blocking, proteins were incubated with primary antibodies (1:500; Abcam, Cambridge, UK) at 4°C overnight: anti-TLR2, anti-TLR4, anti-MyD88, anti-NF-kB p65, anti-TRIF, anti-IRF3. After removing unbound antibodies, the membranes were washed 3x and then incubated for 90 min with respective secondary antibodies (Sigma-Aldrich, St. Louis, MO) diluted 1:20,000 in 1% BSA. After washes with TBS-T solution, positive reactions were obtained using ECL kit (Thermo Fisher Scientific, MA). The blots were calculated using individual samples and were represented as the mean optical density (band intensity/β-actin). β-actin was used as the endogenous control for each blot.

2.5. Cytokine and Chemokine assay

Cytokines and chemokines levels were determined in the serum (n=10 samples/group) using a MilliPlex® Map Kit (EMD Millipore, Darmstad, Germany) with a standard detection kit according to the manufacturer's protocol. The rat cytokine/chemokine Panel I kit (Cat.#RECYTMAG-65K) included the following analytes: interleukin IL-1α, IL-1β, IL-2, IL-4, IL-12, IL-17, human interferon-inducible protein 10 (IP-10/CXCL-10), macrophage inflammatory protein-1 (MIP)-1α, interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α), and regulated upon activation normal T cell expressed and secreted (RANTES/CCL5). The levels of the analytes varied from 2.4 to 50,000 pg/ml and the intra-assay CV was <10%, interassay CV <15%. Fluorescence intensity was measured using MAGPIX system (Luminex® Corporation, Austin, TX, USA).

2.6. Specimen collection and cell isolation

Representative immune cells were obtained from the lumbar lymph nodes (n=7/group) directly associated with OC. They were minced into small pieces of 3 mm^2 and single-cell suspensions were filtered through 70 µm cell strainers (BD Falcon, San Jose, CA, USA). Afterwards, immune cells were suspended in Hanks' balanced salt solution (HBSS modified) and were centrifuged into 1.5 mL conical tubes at 10,000 rpm for 10 sec at 4ºC, and then resuspended in buffered PBS for cell counting in Neubauer chamber. A total of 10^6 cells were set as a parameter for cytometry analysis.

2.7. Flow cytometry

The fluorochrome-coupled monoclonal antibodies (mAbs) were anti-CD3 (FITC), anti-CD4 (PE-Cy7), anti-CD25 (PE) (BD Bioscience, San Jose, CA, USA), and anti-CD8 (PerCP-eFluor 710), anti-Foxp3 (APC) (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Freshly isolated immune cells were washed and incubated with specific mAbs for surface markers and were kept for 20 min in a dark chamber. For the intracellular detection of Foxp3, cells were first fixed in suspension and then permeabilized before adding the mAb, in accordance with the manufacturer's instructions (eBioscience^{TM} Fixation/Permeabilization concentrate). Thereafter, the cells were incubated with anti-FoxP3 for 30 min at room temperature. Briefly, cells were centrifuged and washed twice with 300 μL cytometry buffer (PBS containing 0.5% BSA and 0.02% sodium azide). After washings, cells were resuspended in 300 μL cytometry buffer and acquired in a FACSCanto^{TM}II cytometer (BD Biosciences) using the FACSDiva software. Isotype controls were used in this experiment. Data were analyzed using the FlowJo, vX.10.6 (Tree Stars Inc.).

2.8. Statistical analysis

Data were performed using analysis of variance (One-way ANOVA) for one factor and presented as the mean ± standard deviation (SD). Significant results were subjected to *post-hoc* Tukey test. For non-parametric distribution, Kruskal-Wallis test complemented with Dunn was used. Statistical significance was set at $P < 0.05$ for all analyses. Results were analyzed and constructed using *GraphPad Prism 5.0* scientific graphing software (GraphPad Software, San Diego, CA).

3. Results

3.1. IL-12 alone or associated with P-MAPA reduces the OC-related inflammatory cells and adipocytes infiltration.

OC tissue harvested from the control animals displayed a complex solid tumor mass with large cysts containing extensive amount of fat surrounding the ovary (Figure 2 A and E). The left ovaries of P-MAPA-treated animals showed small cysts filled with serous fluid (Figure 2 B and F). Although the left ovaries of the animals treated with IL-12 and P-MAPA+IL-12 showed a cystic cavity, its morphological aspect revealed a soft and mobile tissue with no adherences and peritoneal implants (Figure 2 C, D, G, H). The right ovaries

were inoculated with vehicle only, and presented integrity and typical morphology with normal follicular and luteal activity (sham-surgery).

The ovarian tumors were serous papillary adenocarcinomas. As evidenced in the Figure 1 I, the untreated OCs had well-formed papillae with infiltration of inflammatory cells and presented higher accumulation of adipose tissue between the cells. After P-MAPA treatment, we observed an increase in tumor-infiltrating lymphocytes together with adipose cells (Figure 2 J). Tumor tissue of animals treated with IL-12 or P-MAPA+IL-12 showed the same pattern of papillae organization resembling juxtaposed epithelial tufted with hypercromasia. Also, these OCs presented low number of inflammatory cells and low accumulation of adipose cells (Figures 2 K and L). Since fat infiltration has recently been associated with poor prognosis in OC, the reduction of this content seems to be a protective effect against tumor aggressiveness.

3.2. P-MAPA stimulates TLR2 and TLR4 in OC

To investigate the efficiency of treatments on TLR2 and TLR4 expressions, OC samples were analyzed by immunohistochemistry and western blotting. P-MAPA-treated animals showed increased TLR2 immunostaining in the OC cells compared with control and IL-12-treated animals (Figure 3 A). P-MAPA further increased the expression of TLR2 in the cells of serous papillary OC (2.8-fold increase, 3.3-fold increase, and 4.2-fold increase vs. control, IL-12 and P-MAPA+IL-12-treated animals, respectively; Figure 3 B and C). Treatment with IL-12 seems to suppress the increase of TLR2 by P-MAPA. In addition, P-MAPA positively regulated the TLR4 levels in OC cells (3.7-fold increase and 4.5-fold increase vs. control and IL-12-treated animals, respectively; Figure 3 A-C). These results indicate the role of P-MAPA as a TLR2 and TLR4 agonist.

3.3. TRIF is upregulated by P-MAPA in OC

We investigated the involvement of P-MAPA and IL-12 on downstream molecules of canonical (via MyD88 activation) and non-canonical (via TRIF activation) pathways. P-MAPA and IL-12 did not change the expression of MyD88 or IRF3 (Figure 4 A-C; $P>0.05$), while NF-kB levels seem to be upregulated by P-MAPA, IL-12 and P-MAPA+IL-12 treatments (Figure 4 A-C). These alterations are particularly related to the P-MAPA effects. In addition, P-MAPA increased the TRIF levels and promoted a strong immunoreaction in the epithelial cells of OC (8.2-, 2.6- and 12.5-fold increases vs. control, IL-12 and P-MAPA+IL-

12 groups, respectively; Figure 4 A-C); although the IRF3 levels were unchanged by the treatments, P-MAPA positively regulated TRIF which may be involved in the activation of non-canonical TLR pathway. We further observed that IL-12 has antagonizing actions on the increase of TRIF levels by P-MAPA.

3.4. P-MAPA and IL-12 differently regulate the levels of cytokines and chemokines in the animals with OC.

To determine which cytokines and chemokines are circulating in OC-bearing animals and how P-MAPA and IL-12 can regulate its production, we investigated the levels of IL-1α, MIP-1α, IL-4, IL-1β, IL-2, IL-12, IFN-γ, IL-17, IP-10, TNF-α and RANTES (Figure 5). As expected, IL-12 levels were higher in animals treated with IL-12 alone than in the other groups (1.60-fold increase vs. control, 1.36-fold increase vs. P-MAPA, and 1.22-fold increase vs. P-MAPA+IL-12). Animals treated with the combination of P-MAPA and IL-12 also showed higher IL-12 levels than control (1.31-fold increase vs. control). Treatment with IL-12 resulted in high secretion of MIP-1α (2.1-fold increase vs. control), IL-2 (3.6-fold increase vs. control, 2.8-fold increase vs. P-MAPA, and 2.6-fold increase vs. P-MAPA+IL-12 groups), IL-17 (1.71-fold increase vs. control) and TNF-α (1.46-fold increase vs. control, 1.26-fold increase vs. P-MAPA, and 1.22-fold increase vs. P-MAPA+IL-12 groups); P-MAPA treatment also promoted a significant increase in IL-17 levels (1.35-fold increase vs. control). Treatments with IL-12 and P-MAPA+IL-12 induced secretion of IL-1β (1.11- and 1.40-fold increase respectively to control and P-MAPA groups). IP-10 was reduced only after IL-12 treatment (0.72-fold reduction vs. control). Finally, levels of IL-1α, IL-4, IFN-γ and RANTES were not significantly changed in OC-bearing animals.

3.5. P-MAPA reduces CD4+ Tregs whereas the combination of P-MAPA with IL-12 stimulates CD4+ effector T cells in OC.

After analyzing the profile of freshly isolated cells from the OC-related lymph nodes, we verified whether the frequency of CD4+CD25+Foxp-3+ T lymphocytes (Tregs) and related cell subpopulations are altered by P-MAPA and IL-12. The gates for each cell analysis on CD4+CD25+Foxp-3-Foxp3+ were constructed and different frequencies were set for comparison (Figure 6 A-E). The percentage of CD4+ T was reduced after treatment with P-MAPA combined with IL-12 (30.2% and 35% lower than control and IL-12 groups, respectively; Figure 6 F). P-MAPA treatment significantly reduced the frequency of Tregs

(37.5% lower than control group); however, IL-12 and its combination with P-MAPA had no significant effect on this cell. The relative mean intensity of Foxp-3+ expression in the CD4+CD25+ cells was unchanged following the treatments. Although we are unable to define the immune response, an increased frequency of effector CD4+ T cells (CD25+Foxp-3-) was observed after treatment with P-MAPA (3.4 times higher than control); this response seems to be Th17-mediated since IL-17 levels were also higher after P-MAPA treatment, which may be beneficial for overall animal survival. Moreover, IL-12 (12 times higher than control group) promoted an elevation in the number of effectors CD4+ T cells and this effect was intensified by the combination with P-MAPA (12.5 times higher vs. control group) (Figure 6 F).

3.6. P-MAPA reduces CD8+ suppressor cells whereas the combination of P-MAPA with IL-12 stimulates CD8+ T cell effector in OC.

We evaluated the frequency of CD8+CD25+Foxp-3+ T lymphocytes (T suppressor phenotype) using the gates for analysis of CD8+CD25+Foxp-3-Foxp3+ within the frequencies for comparison (Figure 7 A-E). Total percentage of CD8+ T was unchanged after treatments. The number of CD8+ suppressor cells varied after the P-MAPA treatment showing a significant reduction in this cell population (24% lower than control group; Figure 7 F). The relative mean fluorescence of Foxp-3+ gated on the CD8+/CD25+ cells was similar, however P-MAPA therapy exhibited a more homogenous result. P-MAPA was also effective in stimulating the effector CD8+ T cells (12 times higher than control; Figure 7 F), which could suggest a differentiation into the cytotoxic response in OC microenvironment. Notably, the combination of P-MAPA with IL-12 promoted a significant increase in the number of effectors CD8+ T cells (21 times higher than control; Figure 7 F).

4. Discussion

We observed that P-MAPA therapy is able to promote both TLR2 and TLR4 activation while reducing CD4+ Treg and CD8+ T suppressor cells; in addition, the combination of P-MAPA with IL-12 results in increased number of effectors CD4+ and CD8+ T cells in OC (Figure 8). These findings are in agreement with a recent study using P-MAPA combined with cisplatin documenting that the combinatory treatment raises the levels of TLR2 and TLR4 in an in vivo model of OC (de Almeida Chuffa et al., 2018). In another research with bladder cancer, P-MAPA also induced the TLR2 and TLR4 activation, thus

increasing the IFN-γ signaling mediated by TRIF [33]. In addition to acting as immune regulators, P-MAPA and IL-12 efficiently modulate proteins associated with OC progression which indicate them as potential agents affecting OC cells; some of these proteins were recently identified and are involved in tight junction, focal adhesion, DNA methylation, RNA metabolism, cell signaling, cell senescence, mitochondrial processes and ribosomal function (Júnior et al., 2019).

Regarding to TLR signaling, the TLR2 and TLR4 stimulation was also documented in a study involving the P-MAPA both in vivo and in vitro in a bladder cancer model; notably, P-MAPA acted as an adjuvant in antitumor therapy [9,33]. Although no significant difference has been observed in other downstream molecules, P-MAPA upregulated TRIF expression in OC via the late activation of NF-kB. This might be regulating the inflammatory process and controlling the tumor progression. It has been observed a biological effect of IL-12 treatment on the increase of IFN-γ levels, thus leading to an enhancement of its synthesis by IL-12 stimulation [34]. The combinatory therapy of P-MAPA and IL-12 did not changed the TLR2- and TLR4-mediated signaling pathways, despite the biological effect in upregulating TLR4 levels was noted. The animals treated with IL-12 and P-MAPA+IL-12 presented a morphological appearance of OC with mobile tissue and no adherences or peritoneal implants. P-MAPA already demonstrated a considerable effect in the histopathological recovery of bladder cancer [35]; in our study, this effect was more evident after the combination with IL-12 where the adenocarcinomas evidenced papillae organization resembling juxtaposed epithelial tufted with a considerable reduction in the number of inflammatory cells and low amount of adipose tissue.

The adipocytes can be observed as components of the tumor microenvironment in colon, ovary, breast, and stomach tumor cells, which promote the secretion of hormones, growth factors, and interleukins (e.g., IL-6 and IL-1β) [36,37]. In accordance with Nieman and colleagues [37], a significant growth in ovarian tumor was observed when human adipocytes was co-cultured with ovarian cancer cells in in vivo and in vitro experiments; the lipolysis generates energy to the proliferation of OC cells. Further analysis involving tissue lysates from OC-associated adipocytes revealed an increase in chemoresistance to paclitaxel; this resistance was attributed to the downregulation of APAF1 protein [38]. Considering the majority of OCs observed in the present study are serous papillary adenocarcinomas, we identified a reduction in the accumulation of infiltrated adipocytes after P-MAPA+IL-12 and IL-12 treatment, which might indicate an improvement in the prognosis and a possible role

against the chemoresistance. The decrease in inflammatory infiltrate also corroborates with previous findings of the immunomodulatory action of P-MAPA [8,9,39].

The interleukins and chemokines, which participate in a complex signaling machinery of many types of immune functions, are responsible for either induce tumor progression or regression (Shrihari, 2017). The IL-1β and IL-17 are cytokines possessing pro-inflammatory functions in OC, acting in several immunological processes [40,41]. The increase in IL-17 levels was also observed by Droeser et al. [42] who analyzed biopsies of patients with OC in stages between II to IV and, after surgical removal, the treatment with IL-17 increased the chemosensitivity to cisplatin. Of note, P-MAPA might work to promote similar chemosensitivity as a result of IL-17 stimulation and could be used as additive treatment for recurrent OC or for reducing the chemoresistance in OC patients.

In non-cancer cells, IL-1β cytokine can stimulate the NF-kB and IRF7 through a MyD88-dependent pathway with TRAF6 promoting the upregulation of IL-6, TNF-α, IFN-α, IFN-β and IFN-γ expression [43]. The activity of IL-1β in OC samples has been associated with poor prognosis [40,44]; furthermore IL-1β was secreted by OC cells suppressing the p53 expression and the genomic integrity [45]. In our study, the P-MAPA+IL-12 treatment increased the IL-1β levels in the serum of animals possibly indicating a stimulation of this process against the OC progression. As expected, IL-12 levels were increased in the IL-12 and P-MAPA+IL-12 treated groups whereas P-MAPA itself does not interfere with the secretion of IL-12.

The synthesis of IL-2 is often induced by Th1 lymphocytes and is associated with activation of NK cells which results in cytotoxic activity and antitumor action [46]. Also, administration of IL-2 associated with the chemotherapeutic Ricibanil (OK-432) to patients with advanced OC presented a lower rate of tumor recurrence compared with chemotherapy alone [47]. Corroborating this finding, the survival of patients with relapsing OC increases after intraperitoneal application of IL-2 (dose regimen for 16 weeks) in combination with docetaxel and carboplatin directly into the tumor [48]. In agreement with the role of IL-2, our data revealed that IL-12 stimulates the synthesis of IL-2 levels, thus fostering the antitumor effect of IL-2 in OC treatment. Treatment with IL-2 also increases the level of MIP-1α (CCL-3), a chemokine released by IL-18-activated NK cells in OC patients, thus promoting the recruitment of immature dendritic cells [49]. The levels of IP-10 (CXCL-10) were decreased with IL-12 treatment, both of these chemokines having chemoattractant activity for recruiting macrophages, NK cells, and T cells with potential antitumor activity [50].

A high number of Treg cells are associated with immunosuppression in OC due to the inhibition of other immune cell activities such as T and B lymphocytes, DCs, monocytes, macrophages, and NK cells [51,52]. Our results showed that P-MAPA consistently decreased the number of CD4+ Foxp3+ (Treg) and CD8+Foxp3+ (TCD8+ suppressor) cells whereas P-MAPA+IL-12 stimulated the levels of CD4+Foxp3- and CD8+ Foxp3- effector T cells. Recently, CD4+Foxp3+ cells have suppressive effects on CD8+ T cells in OC and the accumulation of CD8+ effector T cells is associated with increased survival rate in patients [53,54]. In this context, the immunomodulatory agents may contribute to the control of growth and aggressiveness of OC. On the one hand, Treg cells act by regulating the immunological activities while preventing their possible damages; on the other hand, they suppress the immune responses in OC [55,56], favoring the tumor immune escape. The number of Tregs tends to increase as the OC progress, and this must be considered as important parameters during disease monitoring among patients [56]. Consistently, P-MAPA actively works by reducing the immunosuppression whereas P-MAPA in combination with IL-12 lead to higher number of effector cells in tumor region, such as CD4+ and CD8+ T lymphocytes.

5. Conclusions

In summary, we demonstrated that combination of P-MAPA with IL-12 ameliorates the histopathological features of OC. Notably, P-MAPA therapy induced the upregulation of TLR2 and TLR4 signaling which are partially involved in the cytokine secretion associated with antitumor activity. While P-MAPA stimulates the synthesis of pro-inflammatory mediators (e.g, IL-17), its combination with IL-12 increased the production of other important cytokines such as IL-1β. Furthermore, P-MAPA significantly reduced the immunosuppression by lowering Tregs concentration and combinatory therapy increased effector TCD4+ and TCD8+ response, thereby enhancing immunosurveillance against OC. These immunotherapeutic agents may represent additional strategy in the treatment of OC, especially for planning therapies to reinforce OC immunostimulation.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

HSS, LALJ, LGAC: conceived the hypothesis of the study, collected and analyzed the data, and drafted the manuscript. WJF, GGR, RK, ISN: participated in its design and intellectual conception of the study, and in the acquisition of data. Authors read and approved the final version of this manuscript.

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Data Availability Statement

The data supporting the findings of this current study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. Schedule of the experimental procedures. (A) OC induction using DMBA as carcinogen. Experimental groups: Control, P-MAPA, IL-12 and P-MAPA+IL-12; surgical resection of the reproductive organs. (B) Time lapse division according to the OC induction, tumor development, treatments and euthanasia. (C) Period of P-MAPA and IL-12 administration.

Figure 2. The reproductive organs of the rats evidencing the alterations in the left ovarian tissue. (A,E) Left ovary (tumor) of control group animals showing rigid mass and fat accumulation. (B,F) Left ovary (tumor) of the animals treated with P-MAPA displaying a cyst containing serous secretion. (C,G) Left ovary (tumor) of the animals treated with IL-12 showing soft and mobile tissue with cavity. (D,H) Left ovary (tumor) of the animals treated with P-MAPA and IL-12 showing soft and mobile tissue without abdominal adhesions. In the treated animals, peritoneal implants were not observed. The right ovaries are controls (vehicle only) for tumor induction (sham-surgery) and show intact tissue with normal activity. Micrographs represent serous papillary carcinoma (the most incident subtype) found in the control (I), P-MAPA (J), IL-12 (K) and P-MAPA+IL-12 (L) groups. Focal inflammatory infiltrate (*), black arrowhead: adipose cells, black arrow: juxtaposed epithelial tufted. Bar = 50 μm, H&E.

Figure 3. (A) Immunohistochemical analysis of the TLR2 and TLR4 receptors in the control, P-MAPA, IL-12, and P-MAPA+IL-12 groups (black arrow, Bar = 50 μm). (B) Representative profile of the TLR2 and TLR4 receptors in extracts of 50 μg pooled proteins of 5 samples/group. (C) Optical densitometry analysis of the TLR2 and TLR4 receptors after normalization with β-actin. Data are expressed as the mean ± SD; * $P < 0.05$.

Figure 4. (A) Immunohistochemical analysis of the MyD88, NF-kB, TRIF, and p-IRF3 in the control, P-MAPA, IL-12, and P-MAPA+IL-12 groups (black arrow, Bar = 50 μm). (B) Representative profile of the MyD88, NF-kB, TRIF and p-IRF3 factors in extracts of 50 μg pooled proteins of 5 samples/group. (C) Optical densitometry analysis of the MyD88, NF-kB, TRIF and p-IRF3 after normalization with β-actin. Data are expressed as the mean ± SD; * $P < 0.05$.

Figure 5. Multiplex analysis of cytokines and chemokines in OC-bearing animals in response to P-MAPA and IL-12 treatments. Concentrations of IL-12, MIP-1α, IL-2, IL-17, TNF-α, IL-1β, IP-10, IL-1α, IL-4, IFN-γ and RANTES were evaluated in the serum of animals (n=10/group). All data are expressed as the mean ± SD. *P< 0.05. The samples were assayed in duplicate and in the same run. One-way ANOVA complemented by Tukey test. IL-12: interleukin 12; MIP-1α: macrophage inflammatory protein 1-alpha; IL-2: interleukin 2; IL-17: interleukin 17; TNF-α: tumor necrosis factor α; IL-1β: interleukin 1β; IP-10: human interferon-inducible protein 10; IL-1α: interleukin 1α; IL-4: interleukin 4; IFN-γ: interferon gamma; RANTES: regulated on activation, normal T cell expressed and secreted; MDC: macrophage-derived chemokine.

Figure 6. Frequency of Treg lymphocytes (CD4+/CD25+/FoxP-3+) in draining lymphnodes of animals with OC. (A) Graphs demonstrate the gates of population analysis of Treg cells. From left to right: gate singlet was based on single cell analysis. The gate analysis of lymphocytes was based on the size and granularity of the cell population; CD3+ gate represents the selection of the population of T lymphocytes; CD4+ gate shows the remaining molecules analyzed, namely CD25+/FoxP-3 or CD25+/FoxP-3+ (Treg); finally, the construction of the CD25+/FoxP-3+ gate was based on the labeling of the control isotype of FoxP-3. (B) Control group; (C) P-MAPA group; (D) IL-12 group; (E) P-MAPA+IL-12 group. (F) Statistical analysis of the different frequencies within the CD4+ T population among the experimental groups. MFI: mean fluorescence intensity. ANOVA complemented by Tukey; *P<0.05, **P<0.01.

Figure 7. Frequency of CD8+ T suppressor lymphocytes (CD8+/CD25+/FoxP-3+) in draining lymphnodes of animals with OC. (A) Graphs demonstrate the gates of population analysis of T suppressor cells. From left to right: gate singlet was based on single cell analysis. The gate analysis of lymphocytes was based on the size and granularity of the cell population; CD3+ gate represents the selection of the population of T lymphocytes; CD8+ gate shows the remaining molecules analyzed, namely CD25+/FoxP-3- or CD25+/FoxP-3+ (T suppressor); finally, the construction of the CD25+/FoxP-3+ gate was based on the labeling of the control isotype of FoxP-3. (B) Control group; (C) P-MAPA group; (D) IL-12 group; (E) P-MAPA+IL-12 group; (F) Statistical analysis of the different frequencies within the CD8+ T population among the experimental groups. MFI: mean fluorescence intensity. ANOVA complemented by Tukey; * P<0.05.

Figure 8. Schematic illustration of the effects of P-MAPA and IL-12 therapies in the OC microenvironment. Different molecules have distinct shapes and colors, and up and down arrows indicate whether the treatment increased or decreased the molecular function and level. Although IRF3, MyD88, and NF-kB levels was not quite significantly altered by treatments, P-MAPA positively regulated the expression of TLR2 and TLR4 in addition to increasing TRIF levels, which may results in non-canonical TLR signaling pathways. The P-MAPA also increased the number of CD4+/FoxP-3- and CD8+/FoxP-3- T effector cells and IL-17 levels while decreasing the number of CD4+/FoxP-3+ and CD8+/FoxP-3+ T cells, both related to immunosuppressive effects. The IL-12 treatment raised CD4+/FoxP-3- cell population and elevated the levels of IL-1β, IL-2, IL-17, MIP-1α, and TNF-α, while decreasing IP-10 level. Finally, the combinatory treatment with P-MAPA and IL-12 stimulated a differentiation into CD4+/FoxP-3- and CD8+/FoxP-3- T effector cells in addition to promoting elevation in IL-1β. P-MAPA: protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride; TLR2: toll-like receptor 2; TLR4: toll-like receptor 4; TRIF: TIR-domain-containing adapter-inducing interferon-β; IRF-3: interferon regulatory

factor 3; MyD88: myeloid differentiation factor 88; NF-kB: nuclear factor kappa B; IL, interleukin; MIP-1α, macrophage inflammatory protein 1α; TNF-α: tumor necrosis factor α; IP-10: interferon gamma-induced protein 10.

FIGURE 1

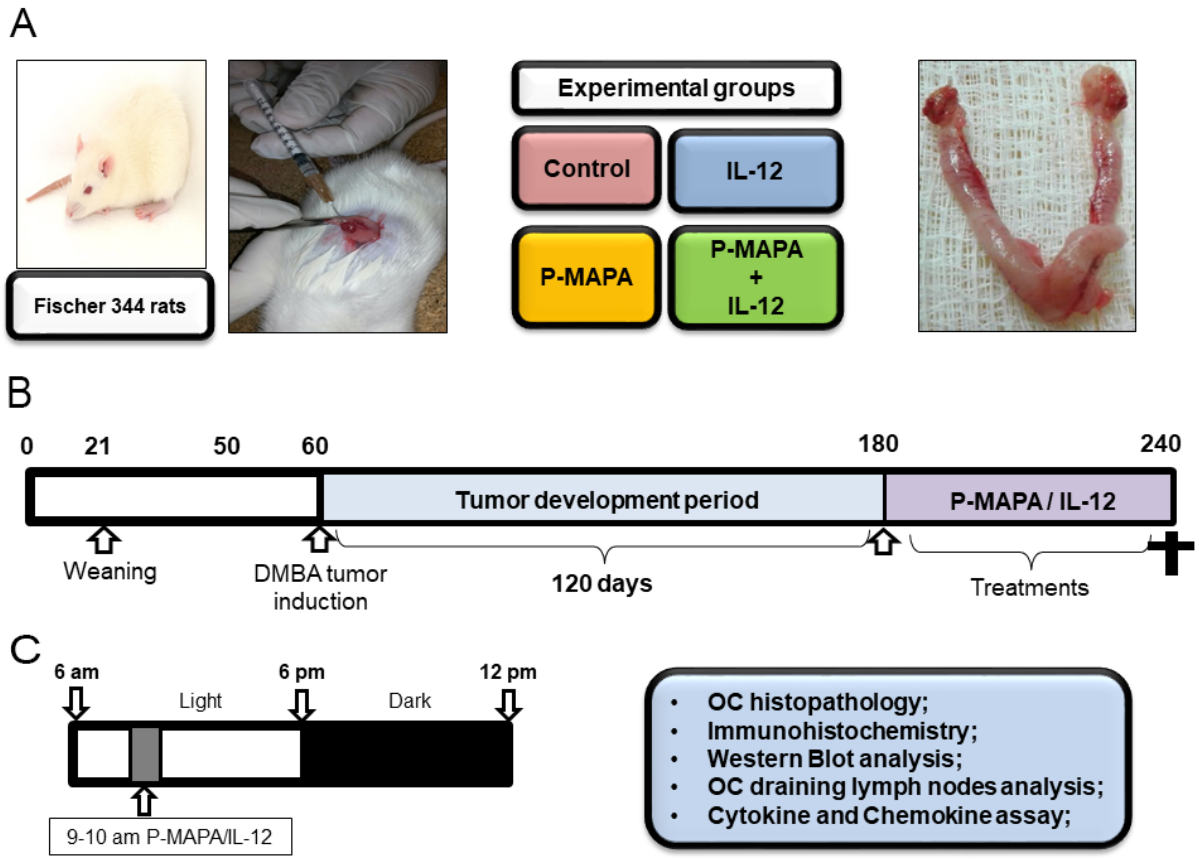

A
Fischer 344 rats
Experimental groups
Control
IL-12
P-MAPA
P-MAPA + IL-12
B
0
21
50
60
180
240
Tumor development period
P-MAPA / IL-12
Weaning
DMBA tumor induction
120 days
Treatments
C
6 am
Light
6 pm
Dark
12 pm
9-10 am P-MAPA/IL-12
OC histopathology;
Immunohistochemistry;
Western Blot analysis;
OC draining lymph nodes analysis;
Cytokine and Chemokine assay;

FIGURE 2

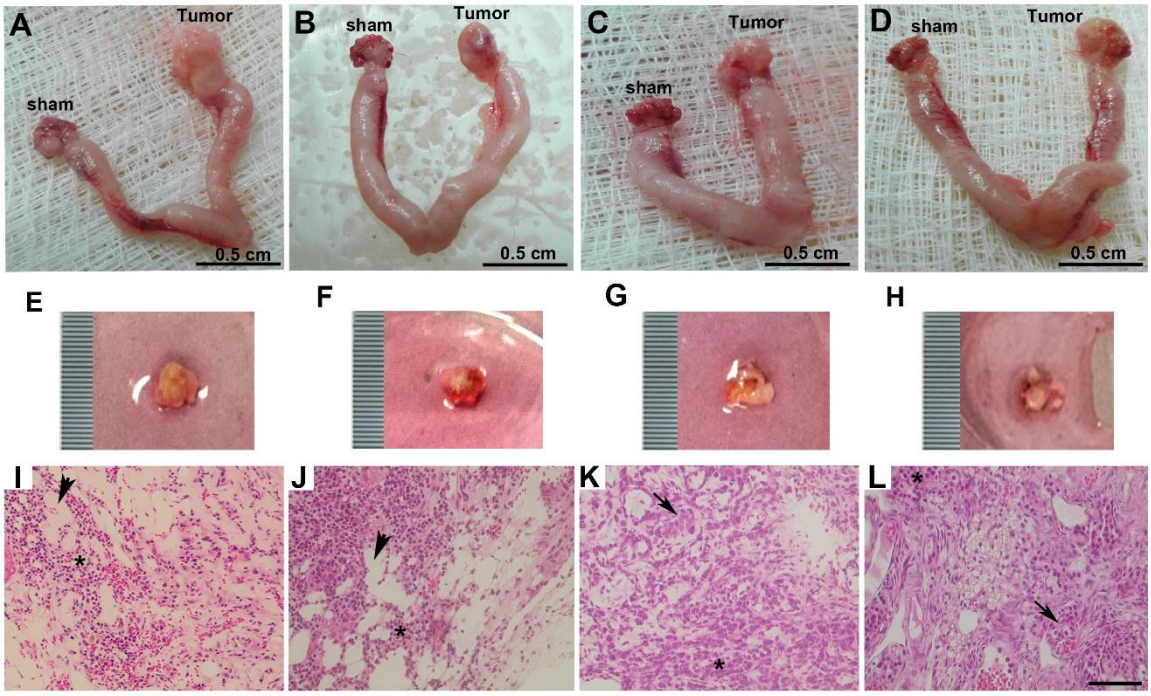

A
Tumor
sham
0.5 cm
B
sham
Tumor
0.5 cm
C
Tumor
sham
0.5 cm
D
sham
Tumor
0.5 cm
E
F
G
H
I
J
K
L

FIGURE 3

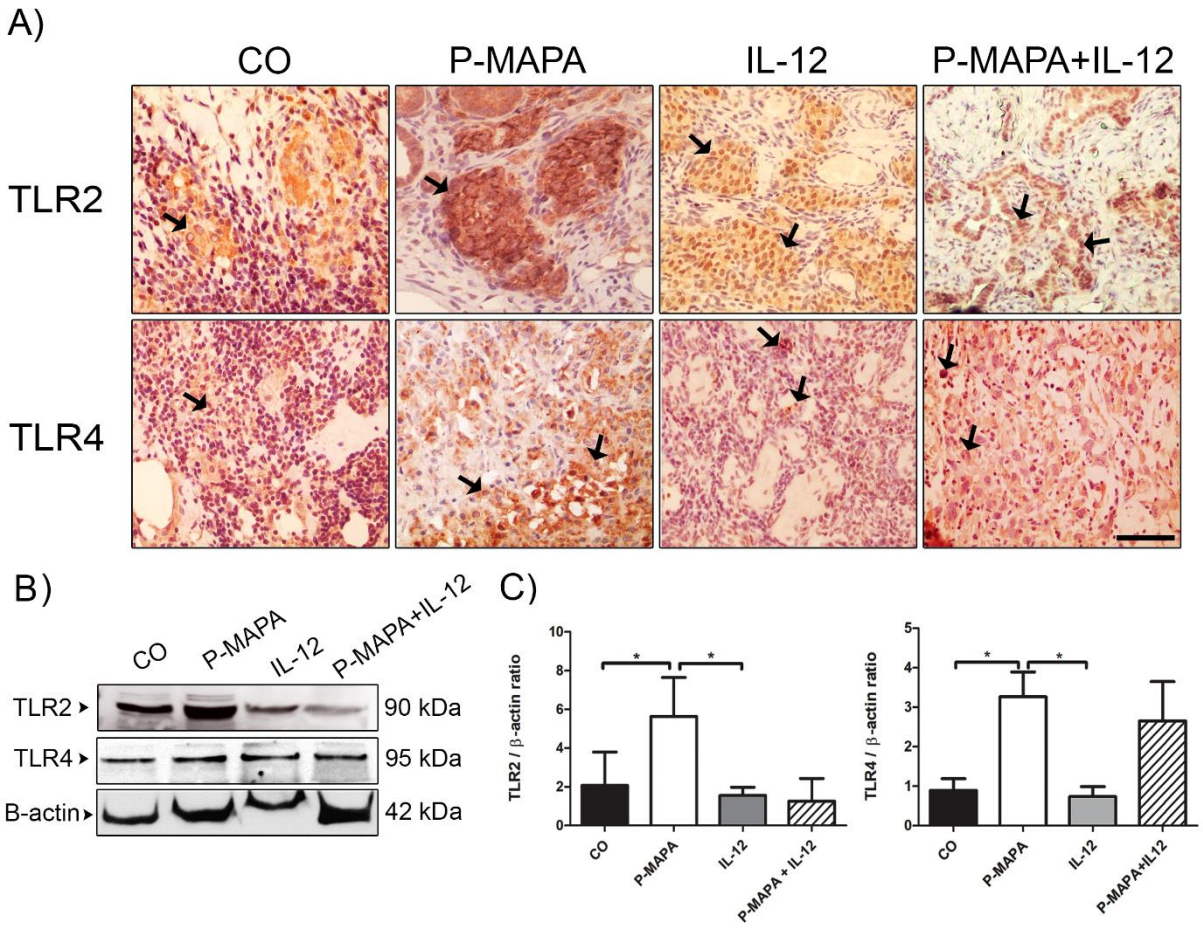

A)
CO
P-MAPA
IL-12
P-MAPA+IL-12
TLR2
TLR4
B)
CO
P-MAPA
IL-12
P-MAPA+IL-12
TLR2
TLR4
B-actin
90 kDa
95 kDa
42 kDa
C)
TLR2 / β-actin ratio
TLR4 / β-actin ratio
CO
P-MAPA
IL-12
P-MAPA + IL-12
P-MAPA+IL12

FIGURE 4

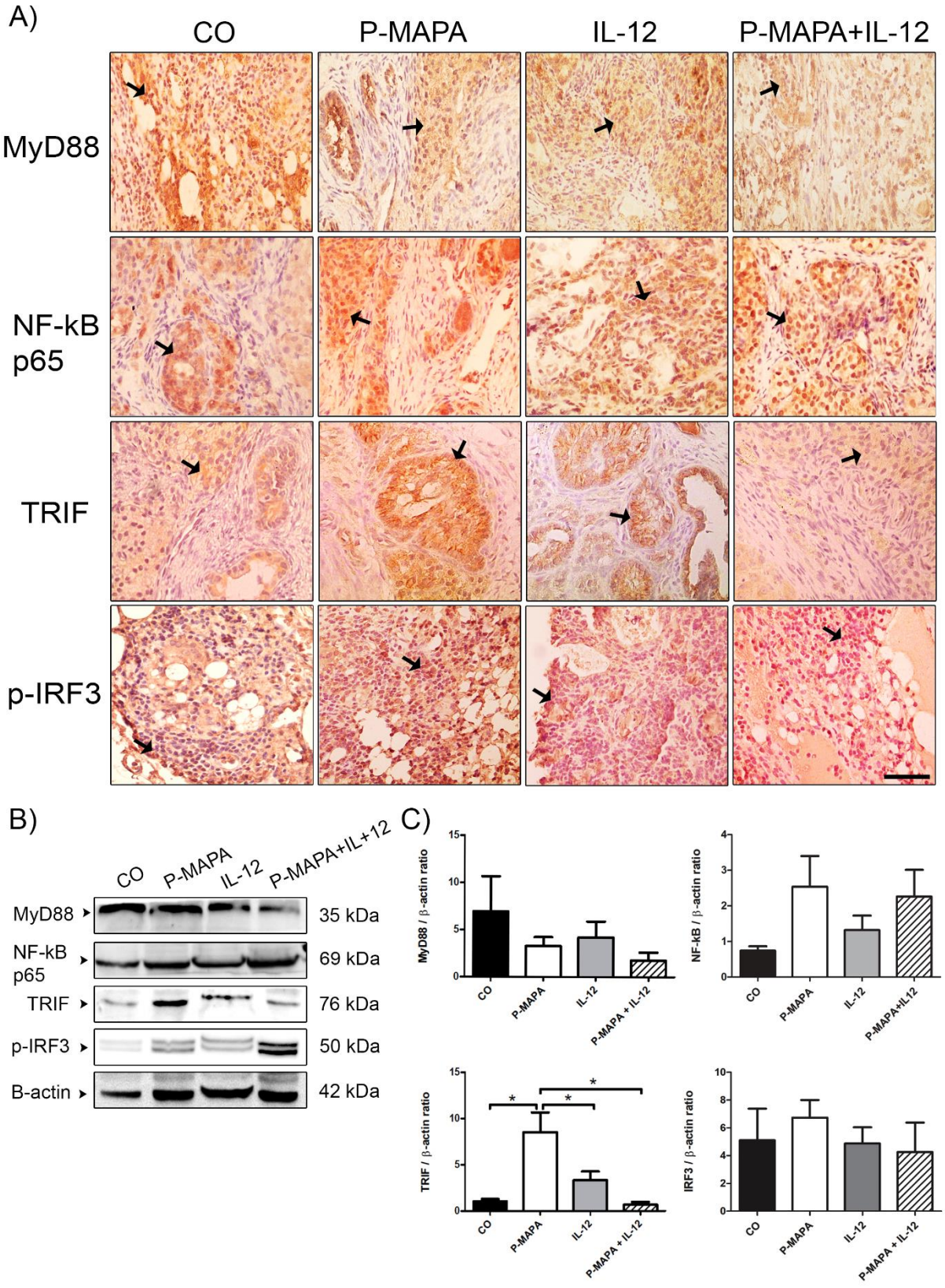

A)
CO
P-MAPA
IL-12
P-MAPA+IL-12
MyD88
NF-kB p65
TRIF
p-IRF3
B)
CO
P-MAPA
IL-12
P-MAPA+IL+12
MyD88
NF-kB p65
TRIF
p-IRF3
B-actin
35 kDa
69 kDa
76 kDa
50 kDa
42 kDa
C)
MyD88 / β-actin ratio
NF-kB / β-actin ratio
TRIF / β-actin ratio
IRF3 / β-actin ratio
CO
P-MAPA
IL-12
P-MAPA + IL-12
P-MAPA+IL12

FIGURE 5

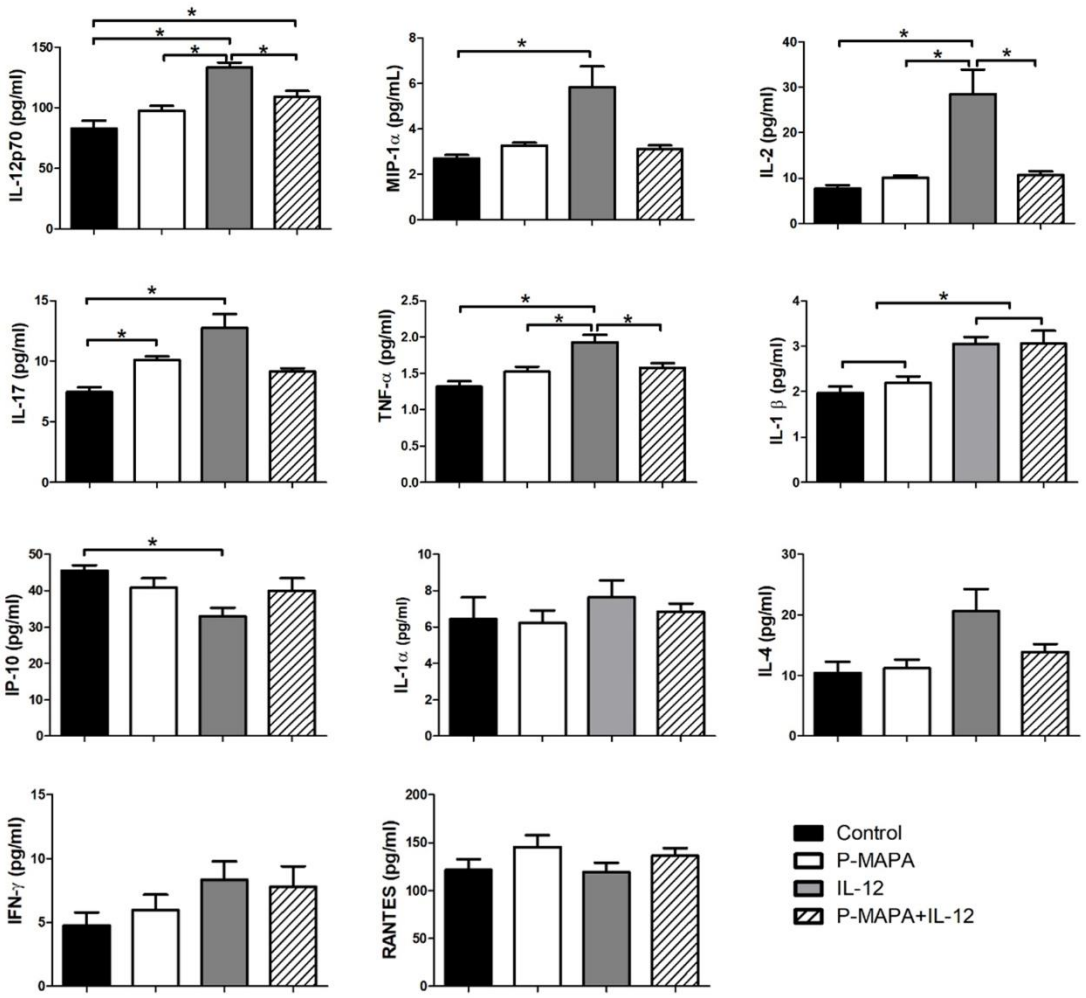

IL-12p70 (pg/ml)
MIP-1α (pg/mL)
IL-2 (pg/ml)
IL-17 (pg/ml)
TNF-α (pg/ml)
IL-1 β (pg/ml)
IP-10 (pg/ml)
IL-1 α (pg/ml)
IL-4 (pg/ml)
IFN-γ (pg/ml)
RANTES (pg/ml)
Control
P-MAPA
IL-12
P-MAPA+IL-12

FIGURE 6

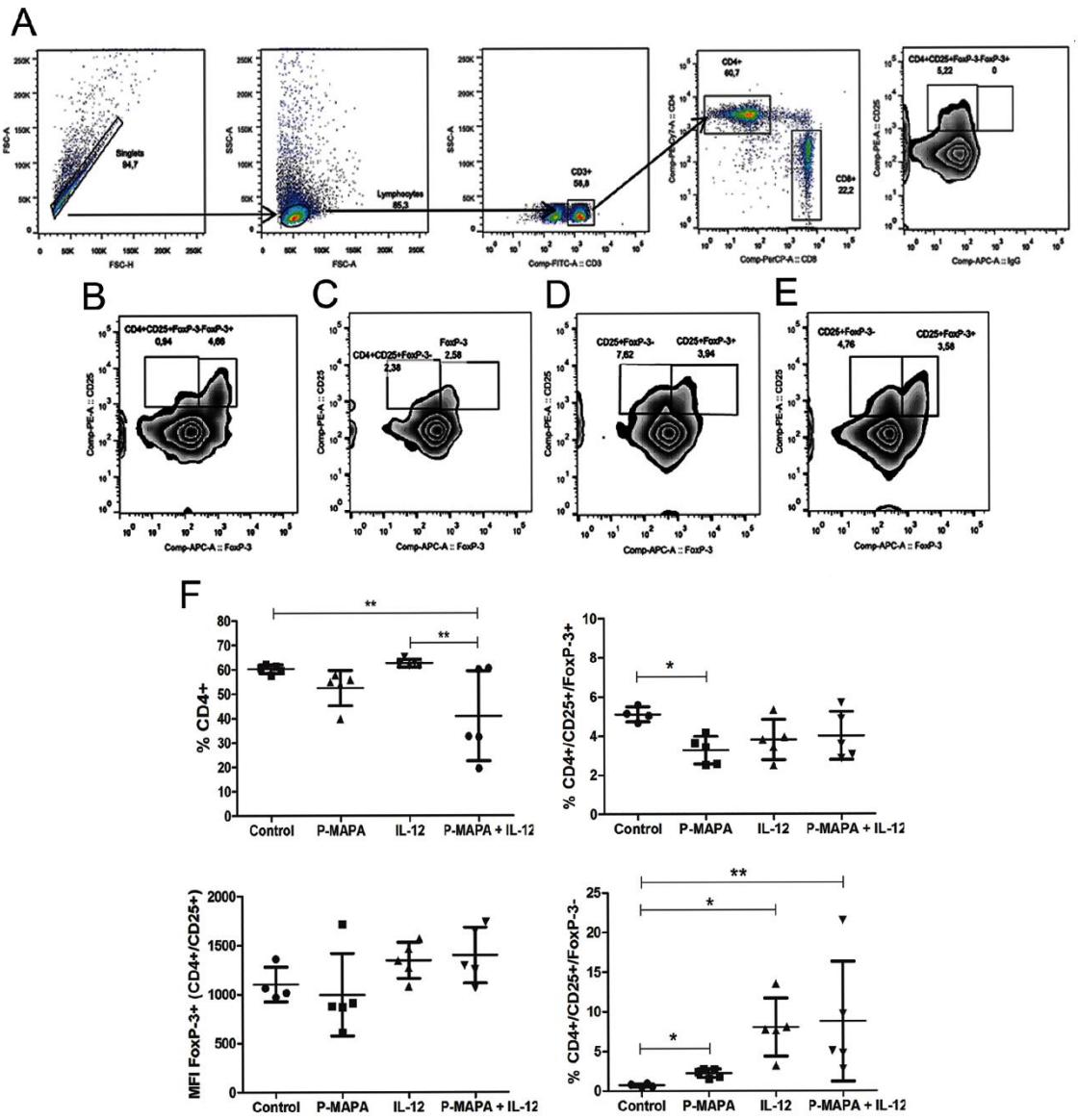

A
Singlets
94,7
Lymphocytes
85,3
CD3+
58,8
CD4+
60,7
CD8+
22,2
CD4+CD25+FoxP-3-FoxP-3+
5,22
0
B
CD4+CD25+FoxP-3-FoxP-3+
0,94
4,66
C
CD4+CD25+FoxP-3-
2,38
FoxP-3
2,58
D
CD25+FoxP-3-
7,62
CD25+FoxP-3+
3,94
E
CD25+FoxP-3-
4,76
CD25+FoxP-3+
3,58
F
% CD4+
% CD4+/CD25+/FoxP-3+
MFI FoxP-3+ (CD4+/CD25+)
% CD4+/CD25+/FoxP-3-
Control
P-MAPA
IL-12
P-MAPA + IL-12

FIGURE 7

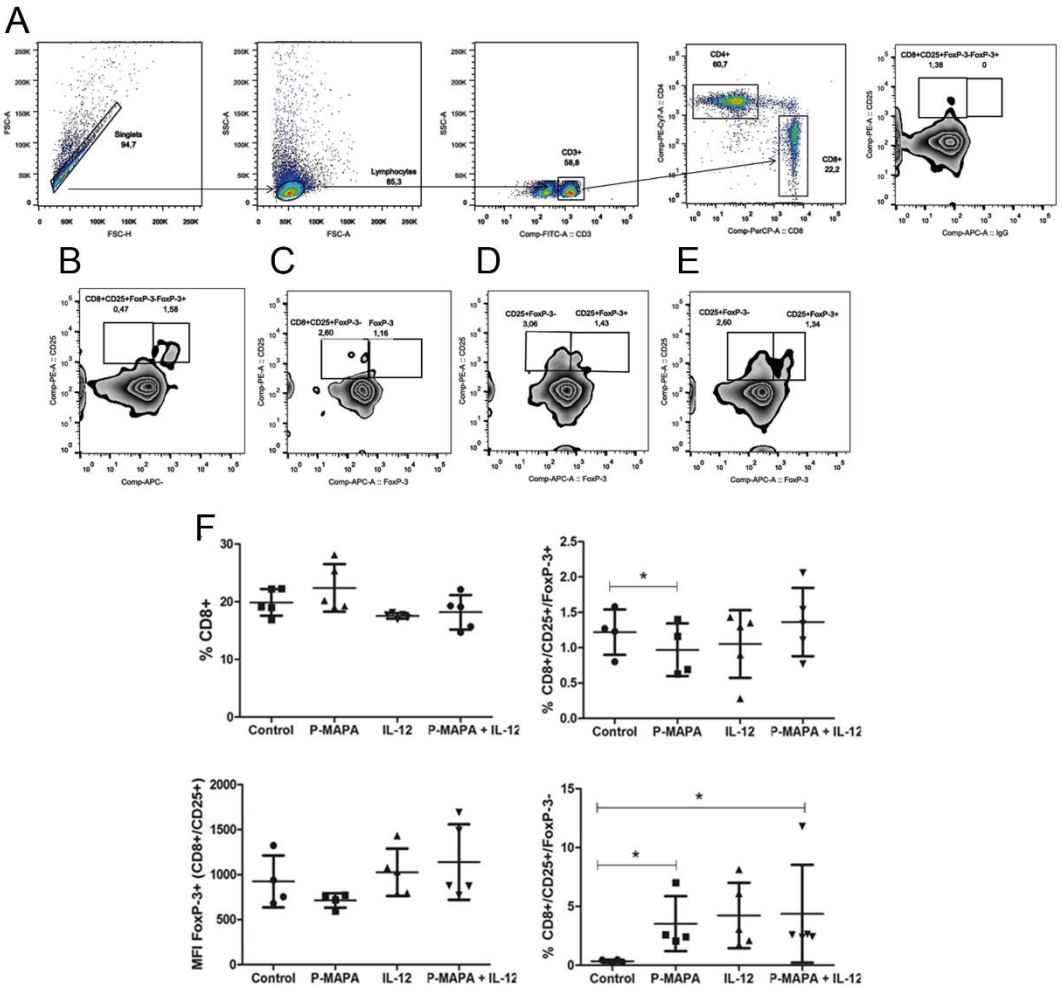
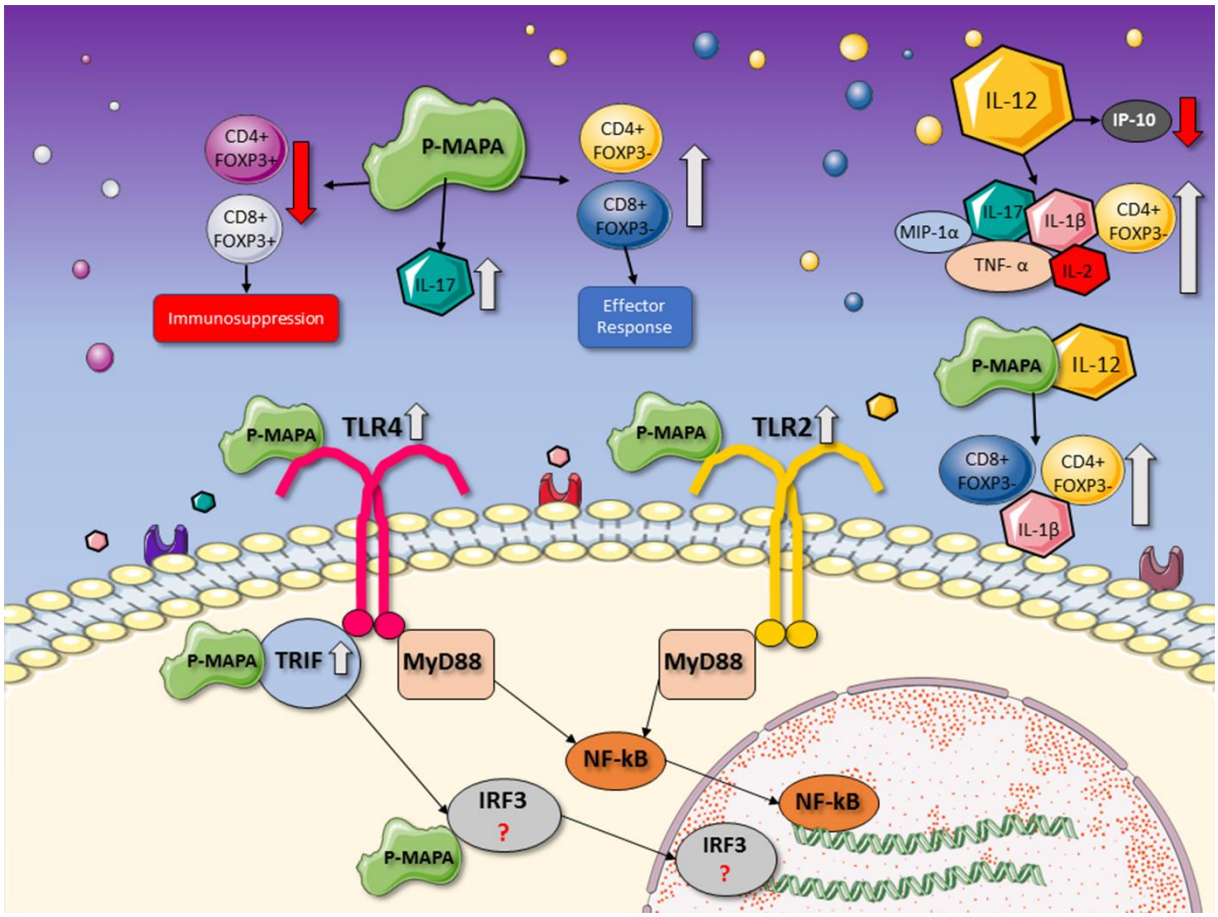

A
Singlets
94,7
Lymphocytes
85,3
CD3+
58,8
CD4+
60,7
CD8+
22,2
CD8+CD25+FoxP-3-FoxP-3+
1,38
0
B
CD8+CD25+FoxP-3-FoxP-3+
0,47
1,58
C
CD8+CD25+FoxP-3-
2,60
FoxP-3
1,16
D
CD25+FoxP-3-
3,06
CD25+FoxP-3+
1,43
E
CD25+FoxP-3-
2,60
CD25+FoxP-3+
1,34
F
% CD8+
% CD8+/CD25+/FoxP-3+
MFI FoxP-3+ (CD8+/CD25+)
% CD8+/CD25+/FoxP-3-
Control
P-MAPA
IL-12
P-MAPA + IL-12

FIGURE 8


CD4+ FOXP3+
CD8+ FOXP3+
Immunosuppression
P-MAPA
IL-17
CD4+ FOXP3-
CD8+ FOXP3-
Effector Response
IL-12
IP-10
MIP-1α
IL-17
IL-1β
CD4+ FOXP3-
TNF- α
IL-2
P-MAPA
IL-12
CD8+ FOXP3-
CD4+ FOXP3-
IL-1β
P-MAPA
TLR4
P-MAPA
TLR2
P-MAPA
TRIF
MyD88
MyD88
NF-kB
NF-kB
IRF3 ?
IRF3 ?
P-MAPA

ANEXO 1

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Efeito do imunomodulador P-MAPA associado a Interleucina-12 no Câncer de Ovário: abordagens in vivo sobre o processo inflamatório e sistema imune", Protocolo nº **950-CEUA**, sob a responsabilidade de **Luiz Gustavo de Almeida Chuffa, Luiz Antonio Lupi Júnior e Henrique Spaulonci**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Finalidade:	( ) Ensino	(X) Pesquisa Científica
Vigência do Projeto:	Início: 30/3/2017	Término: 30/3/2019
Espécie/linhagem:	*Rato Fischer 344*	
Nº de animais:	*48*	
Peso:	*180g*	Idade: *2 meses*
Sexo:	*Fêmea*	
Origem	*CEMIB – Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório – UNICAMP – Campinas/SP – CNPJ: 046.068.425/0001-33*	

Botucatu, 03 de março de 2017.

Prof. Dr. Bruno Cesar Schimming
Presidente da CEUA

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Comissão de Ética no Uso de Animais IBB-Unesp