Rodrigo Nalio Ramos

"O MICROAMBIENTE SUPPRESSOR NO CÂNCER: EFEITOS LOCAIS E SISTÊMICOS EM MONÓCITOS DE PACIENTES"

Tese apresentada para obtenção de graduação de doutorado pelo:

1) Programa de Pós-graduação em Imunologia no Instituto de Ciências Biomédicas da Universidade de São Paulo – São Paulo – Brasil

   Supervisor: Prof. José Alexandre Marzagão Barbuto, MD, PhD.

2) Escola de doutorado em Biologia Molecular, Integrada e Celular da Universidade de Lyon1 - Lyon – França.

   Supervisor: Christophe Caux, PhD.

São Paulo
2015

Rodrigo Nalio Ramos

"THE IMMUNOSUPPRESSIVE MICROENVIRONMENT IN CANCER: LOCAL AND SYSTEMIC EFFECTS ON PATIENTS' MONOCYTES"

Thesis presented for the obtainment of PhD graduation at:

1) The Graduation Program in Immunology from the Institute of Biomedical Sciences at University of São Paulo – São Paulo - Brazil

   Supervisor: Prof. José Alexandre Marzagão Barbuto, MD, PhD.

2) The Molecular, Integrative and Cellular Biology Doctoral School at University of Lyon-1 – Lyon – France.

   Supervisor: Christophe Caux, PhD.

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS

Candidato(a): Rodrigo Nalio Ramos.

Título da Tese: O microambiente supressor no câncer: efeitos locais e sistêmicos em monócitos de pacientes.

Orientador(a): Prof. Dr. José Alexandre Marzagão Barbuto.

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a ..../................/................, considerou

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Preface

This thesis is a result of about four and a half years of study, experiments and work performed in two different teams: in Prof. Barbuto's Laboratory, at the Department of Immunology from The University of São Paulo (Brazil) and in Dr Caux's Laboratory, at the "Centre de Recherche en Cancerologie de Lyon", also associated to The University of Lyon-1 (France). For these reasons, this manuscript was written in English, according to the previously signed contract of agreement between both Universities, the Principal Investigators and for me.

As you may note, this manuscript of thesis have different elements required from both Universities (From São Paulo and Lyon) and, also, particularities from both laboratories. It is important to mention that aspects of personal preferences will be also found in the manuscript, as an influence of a mixture of Brazilian and French cultures' along the last years.

Briefly, our study brings new findings about the characterization of immune aspects during cancer development, highlighting a possible complementary explanation for tumor scape from immune system. Herein, we characterized Macrophages' features in human tumors; we investigated the effects of tumor microenvironment upon human monocyte differentiation, and, we evaluated the systemic effects of tumor presence on circulatory blood monocytes from cancer patients.

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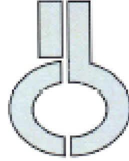

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Cidade Universitária "Armando de Salles Oliveira"
Av. Prof. Lineu Prestes, 2415 - cep. 05508-000 São Paulo, SP - Brasil
Telefone :(55) (11) 3091.7733 telefax : (55) (11) 3091-8405
e-mail: cep@icb.usp.br

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A Comissão de *Ética em Pesquisas com Seres Humanos* do ICB, na sessão realizada no dia 19.10.2011, **APROVOU** o projeto intitulado: *"Estudo de mecanismos envolvidos na modulação da resposta de linfócitos T contra antígenos tumorais por células dendríticas derivadas de monócitos de pacientes com câncer "* sob responsabilidade de execução dos autores Prof. Dr. JOSÉ ALEXANDRE M.BARBUTO e o aluno RODRIGO NALIO RAMOS.

Cabe os pesquisadores executantes elaborarem e apresentar a este Comitê, relatórios anuais (parciais ou final), de acordo com a resolução 196/06 do Conselho Nacional da Saúde, item IX. 2 letra c.

O primeiro relatório deverá ser encaminhado à Secretaria deste CEP em **19.10.2012.**

Atenciosamente,

Prof. Dr. PAOLO M.A.ZANOTTO
Coordenador da Comissão de Ética em
Pesquisas com Seres Humanos - ICB/USP

This thesis is dedicated to:

My wife Evelyn, my loved
and eternal companion, a strong woman who
has been always at my side in the most
difficult and happy moments during this route;

My parents, Moacir and Manuela and
my sister Izabela, unconditionally loved
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“If you want to go quickly, go alone. If you want to go far, go together”

African proverb

ABSTRACT

RAMOS, R. N. **The immunosuppressive microenvironment in cancer: local and systemic effects on patients' monocytes**. 2015. 196 p. Thesis (PhD in Immunology) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015.

Cancer development is currently associated with an immune system failure, mainly due to its dysfunction to sense, recognize and eliminate tumor cells efficiently. In that context, two Antigen-Presenting Cells (APCs) that can be derived from monocytes, the Dendritic Cells (DCs) and the Macrophages (MΦ), have a crucial role in the identification of tissue imbalances and in the stimulation of adaptive antitumor immunity. However, tumor-derived factors modulate those APCs avoiding the optimal priming of the immune responses, culminating in the cancer establishment. Thereby, we investigated here how the tumor microenvironment could modulate the differentiation of monocytes into APCs and its systemic effects on circulating monocytes. Our data revealed that in breast and ovarian cancers, Tumor-Associated Macrophages (TAMs) are the most frequent subpopulation within $CD45^{+}MHCII^{+}$ leukocytes and found in variable frequency as either $CD163^{low}$ or $CD163^{high}$ TAMs. The latter ($CD163^{high}$ TAMs) expressed higher PD-L1 levels and produced elevated IL-10 amounts under LPS activation. Furthermore, a retrospective immunohistochemistry study of breast cancer patients (n=283) with 12.5-year of follow-up reveals a strong correlation between high intra-tumor $CD163^{+}$ TAM and poor patient survival. Additionally, the high frequency of $CD163^{high}$ TAMs was correlated with a low $CD3^{+}$ T cell infiltration. In another experiments, tumor-conditioned medium from primary breast tumors skewed the differentiation of healthy monocytes towards a $CD163^{high}IL\text{-}10^{high}$ phenotype *in vitro*, which not only fail to stimulate but also suppressed naïve $CD4^{+}$ T cell expansion and IFN-γ and TNF-α production *via* IL-10. This acquired phenotype of conditioned-monocytes was associated to the elevated presence of CCL22, M-CSF, TGF-β1, TGF-β3, and VEGF in the tumor microenvironment. Importantly, evaluating the systemic effects of tumors, breast cancer patients' circulating monocytes failed to fully differentiate into M1-MΦ in presence of GM-CSF/IFN-γ and maintained an altered $CD163^{+/-}IL\text{-}10^{+}TNF\text{-}\alpha^{+}$ M2-like phenotype. Likewise, immature DCs differentiated from breast cancer patients' monocytes (Mo-iDCs) expressed high levels of PD-L1, induced lower CD25 expression on T cells and about twice as many $Foxp3^{+}$ Tregs than Th1 or Th2 cells, a phenomenon partially reduced in transwell co-cultures. Moreover, blocking of TGF-β1 and PD-L1 with mAb significantly reduced the induction of $CD4^{+}Foxp3^{+}$ Tregs by patients' Mo-iDCs in co-cultures. Furthermore, fresh monocytes isolated from breast cancer patients blood display an anti-inflammatory functional status by producing higher levels of IL-1RA, IL-10, IL-27, M-CSF, sCD40L and VEGF-A under LPS stimulus when compared to healthy donors' monocytes. Altogether our data suggest that the tumor microenvironment favors the local differentiation of suppressive $CD163^{high}IL\text{-}10^{high}$ MΦ and drives systemic blood monocytes to differentiate into biased MΦ and DCs with suppressive abilities. These findings put forward the importance of new strategies to neutralize cancer-derived factors responsible for $CD163^{high}$ TAMs differentiation and for the modulation of blood circulating monocytes, aiming to improve immunotherapy strategies for cancer patients.

Keywords: Breast Cancer. Monocytes. Interleukin 10. Macrophages. Dendritic Cells.

RESUMO

RAMOS, R. N. **O microambiente suppressor no câncer: efeitos locais e sistêmicos em monócitos de pacientes**. 2015. 196 f. Tese (Doutorado em Imunologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2015.

O desenvolvimento do câncer é normalmente associado a desvios no sistema imune, principalmente devido a sua falha em perceber, reconhecer e eliminar células neoplásicas de maneira eficiente. Nesse contexto, duas Células Apresentadoras de Antígenos (APCs), Células Dendríticas (DCs) e Macrófagos (MΦ), têm um papel crucial na identificação de alterações nos tecidos e na estimulação da imunidade adaptativa antitumoral. No entanto, fatores derivados de tumores modulam essas APCs, impedindo a iniciação das respostas imunes e culminando no estabelecimento do câncer. Investigamos aqui como o microambiente tumoral poderia modular a diferenciação de monócitos em APCs *in vitro* e de modo sistêmico. Nossos dados revelaram que em cânceres de mama e ovário, Macrófagos-Associados a Tumores (TAMs) são a subpopulação mais frequente em leucócitos $CD45^{+}MHCII^{+}$, e são encontrados em uma frequência variável de TAMs $CD163^{low}$ ou TAMs $CD163^{high}$. O último, (TAMs $CD163^{high}$) expressaram maiores níveis de PD-L1 e elevada produção de IL-10 sob a ativação de LPS. Além disso, a análise retrospectiva por imunohistoquímica revelou uma forte correlação entre a presença de TAMs $CD163^{+}$ e uma baixa taxa de sobrevida em pacientes com câncer de mama. Ainda, a alta frequência de TAMs $CD163^{high}$ foi correlacionada com um baixo infiltrado de células T $CD3^{+}$. Monócitos saudáveis condicionados por sobrenadantes de tumores de mama tiveram sua diferenciação *in vitro* direcionada para um fenótipo $CD163^{high}IL\text{-}10^{high}$, células capazes de suprimir a expansão de células T naive $CD4^{+}$ e a produção de IFN-γ e TNF-α via IL-10. Esse fenótipo adquirido por monócitos condicionados foi associado à presença de altos níveis de CCL22, M-CSF, TGF-β1, TGF-β3, e VEGF no microambiente tumoral. Interessantemente, avaliando os efeitos sistêmicos dos tumores, monócitos circulantes de pacientes com câncer de mama falharam em diferenciar-se em M1- MΦ na presença de GM-CSF/IFN-γ e mantiveram um fenótipo alterado $CD163^{+/-}IL\text{-}10^{+}TNF\text{-}\alpha^{+}$. De modo similar, DCs imaturas (Mo-iDCs) diferenciadas de monócitos de pacientes com câncer de mama expressaram altos níveis de PD-L1, induziram baixa expressão de CD25 em linfócitos T e induziram duas vezes mais células T reguladoras $Foxp3^{+}$ (Tregs) do que células Th1 ou Th2, fenômeno parcialmente reduzido quando em co-culturas de transwell. Ainda, Mo-iDCs de pacientes ativadas por LPS, ou sob o bloqueio de TGF-β1 ou PD-L1 com mAb apresentaram uma capacidade reduzida em induzir Tregs $Foxp3^{+}$ *in vitro*, mas ainda acima do nível observado em Mo-iDCs de doadores saudáveis. Adicionalmente, monócitos isolados do sangue de pacientes com câncer de mama produziram altos níveis de IL-1RA, IL-10, IL-27, M-CSF, sCD40L e VEGF-A sob a ativação por LPS (24h) quando comparados a monócitos de doadores sadios. Em conclusão, nossos dados sugerem que o microambiente tumoral favorece a diferenciação de MΦ supressivos $CD163^{high}IL\text{-}10^{high}$ e atua sistemicamente no potencial de diferenciação de monócitos sanguíneos os direcionando para MΦ e DCs com habilidades supressoras. Esses achados colocam em evidência a importância de novas estratégias que neutralizem os fatores derivados do câncer responsáveis pela diferenciação de TAMs $CD163^{high}$ e pela modulação sistêmica de monócitos sanguíneos, visando o melhoramento de abordagens imunoterapêuticas para a intervenção clínica de pacientes portadores de câncer.

Palavras-chave: Neoplasias mamárias. Monócitos. Interleucina 10. Macrófagos. Células Dendríticas.

RESUMÉ SUBSTANTIEL (in French)

Introduction et Objectifs

La carcinogenèse est un phénomène qui se produit lentement par la transformation de cellules normales en cellules néoplasiques et par l'adaptation de ces cellules à l'environnement local. Au cours de ce processus, un système immunitaire efficace peut éliminer les cellules néoplasiques, comme suggéré par Paul Ehrlich en 1909. Cependant, chez les patients atteints de cancer, les cellules néoplasiques échappent au contrôle du système immunitaire, sans doute en raison de leur faible immunogénicité et d'une capacité exacerbée à moduler le microenvironnement tissulaire. Dans ce contexte, un micro-environnement très complexe est formé, caractérisé par des modifications locales de pH, des zones d'hypoxie et deneoangiogenèse, avec la génération d'un ensemble unique de signaux qui peuvent modifier les cellules immunitaires locales. Les Cellules Présentatrices d'Antigènes (APCs) infiltrant les tumeurs, notamment les Macrophages (MΦ) et les Cellules Dendritiques (DC), illustrent bien ce phénomène car elles présentent des altérations fonctionnelles, entraînant le développement de réponses immunitaires anti-tumorales inefficaces, ce qui favorise la croissance tumorale et le développement de métastases. En effet, l'infiltration par un nombre élevé de Macrophages-Associés aux Tumeurs (TAM) corrèle à un mauvais pronostic dans plusieurs types tumoraux, parmi lesquels les carcinomes de l'ovaire, et du sein, le cancer du poumon non à petites cellules et les Lymphomes de Hodgkin. Les TAM présentent des fonctions pro-tumorales, telles que i) la production de facteurs pro-angiogéniques (VEGF), ii) la promotion du remodelage tissulaire, iii) la production de cytokines immunosuppressives telles que l'IL-10 et le TGF-β), et iv) la capacité de bloquer les fonctions effectrices des lymphocytes T. Par conséquent, il est à ce jour bien établi que des modifications fonctionnelles des APCs jouent un rôle crucial dans la progression tumorale. Par contre l'impact du microenvironnement tumoral sur les monocytes, précurseurs des MΦ et de certaines populations de DC *in vivo*, est encore mal compris chez l'homme. Dans ce travail de thèse, nous décrivons les effets de ce microenvironnement tumoral sur la différenciation locale et systémique des monocytes en macrophages immunosuppresseuret l'impact de la présence de TAM $CD163^+$ sur la survie des patientes atteintes de cancer du sein.

Resultats

Composition hétérogène en sous-types de TAMs $CD163^{low}$ et $CD163^{high}$ de l'environnement tumoral du cancer du sein, association entre fréquence élevée de TAM $CD163^{high}$ et faible infiltration en lymphocytes T $CD3^+$. Par une analyse de cytométrie de flux multiparamétrique, nous avons démontré que les TAMs, ($CD11b^+HLA\text{-}DR^+CD14^+$), représentent la sous-population de cellules immunes myéloïdes ($CD45^+$) la plus fréquente dans les cancers du sein et de l'ovaire et les deux populations de TAM: M1-like ($CD64^+CD163^{low}$) et M2-like ($CD64^+CD163^{high}$) sont détectables. Nos résultats montrent que parmi les cellules immunitaires ($CD45^+$) vivantes, 51% (± 2,4 SEM) représentent des lymphocytes T $CD3^+$ et 24,7% (± 3 SEM) des TAM $CD14^+$, avec une variation de la population $CD163^{high}$ de 0% à 27,7% (moyenne = 5,8% ± 1 SEM, n = 48). De façon intéressante, la fréquence de TAM

$CD163^{high}$ est corrélée négativement à la fréquence de lymphocytes T $CD3^{+}$. Par ailleurs ces TAM $CD163^{high}$ expriment des forts niveaux de PD-L1, produisent de grandes concentrations d'IL-10 après activation LPS, et n'induisent pas la prolifération de lymphocytes T $CD4^{+}$ naïfs *in vitro*.

Une forte infiltration des tumeurs par des TAMs $CD163^{+}$ est un facteur de mauvais pronostic pour la survie sans rechute des patientes atteintes de cancer du sein. La mise en évidence des TAM $CD163^{+}$ en immunohistochimie sur une cohorte rétrospective de 283 patientes présentant une tumeur primaire de sein non prétraitée avec un recul clinique de plus de 12 ans a permis de montrer une forte corrélation entre une fréquence de TAM $CD163^{+}$ élevée et un risque accru de progression pour les patientes (log-rank $*p<0.05$, n=238). L'analyse selon la classification moléculaire des tumeurs montre que les sous types les plus agressifs (Luminal B, Triple-négative) sont les plus infiltrés par des TAM $CD163^{+}$.

Les monocytes conditionnés par le microenvironnement tumoral présentent une différenciation biaisée en faveur des MΦ suppresseurs $CD163^{high}IL\text{-}10^{high}$. Les monocytes $CD14^{+}$ du sang de donneurs sains sont cultivés pendant 7 jours en présence de surnageant de dilacération *ex-vivo* (SNDil) issus de tumeurs primaires du sein non prétraitées. La différenciation de monocytes en MΦ (SNDil-MΦ) dans ces conditions entraine l'apparition de cellules avec des niveaux de CD163 variable différents (SNDil-MΦ $CD163^{low}$ et SNDil-MΦ $CD163^{high}$) par rapport aux monocytes cultivés en milieu complet (M0-MΦ). Les SNDil-MΦ $CD163^{high}$ qui présentent une réduction de l'expression de CD86 et produisent des grandes quantités d'IL10 ressemblent à des M2-MΦ anti-inflammatoires, différenciées *in vitro* en présence de M-CSF+IL-4. Ces SNDil-MΦ $CD163^{high}$ non seulement ne parviennent pas à stimuler la prolifération des T $CD4^{+}$ naïfs mais inhibent de façon active l'expansion de lymphocytes T $CD4^{+}$ et leur production d'IFN-γ et de TNF-α induite par une activation polyclonale (billes anti-CD3/anti-CD28). Cette fonction suppressive est partiellement médiée par l'IL-10.

Des quantités élevées de CCL22, TGF-β, M-CSF et VEGF dans le microenvironnement tumoral sont associées à la différenciation de SNDil-MΦ de type M2-like $CD163^{high}IL\text{-}10^{high}$. L'analyse du contenu en cytokines/chimiokines des SN-Dil évalués en biologie a été réalisée par la technologie multiplex (Luminex). Les données montrent que l'induction d'expression de CD163 et d'IL-10 sur les SNDil-MΦ de type M2-like est directement corrélée avec des niveaux importants de TGF-β1, TGF-β3 et de CCL22 dans les SN-Dils. En outre, des niveaux élevés de M-CSF et de VEGF sont également observés dans les SNDils favorisant un phénotype $CD163^{high}IL\text{-}10^{high}$. Toutefois, une évaluation plus approfondie en bloquant ces molécules identifiées reste à réaliser pour valider leur rôle dans la différenciation vers des M2-MΦ.

Certaines patientes atteintes de cancer du Sein présentent des monocytes circulants biaisés vers une différenciation en M2-MΦ malgré le cocktail cytokinique (GM-CSF+IFNγ) favorable à une différenciation en M1-MΦ. Les facteurs issus de microenvironnement tumoral pourraient également agir en systémique en altérant les cellules immunes circulantes. Nous avons étudié la capacité de monocytes

circulants de patientes atteintes de cancer du sein à se différencier en MΦ et en cellules dendritiques immatures (Mo-iDCs) *in vitro* en présence des cocktails de différenciation classiques (GM-CSF/IFN-γ pour les M1-MΦ et GM-CSF/IL-4 pour les Mo-iDC). Il apparait que dans 45% des cas les monocytes de patientes sont résistants à la différenciation en M1-MΦ. En fait, les MΦ générés à partir de monocytes de patientes en GM-CSF+IFNγ montrent une fréquence significativement plus élevée de cellules $CD163^+$ (de type M2-MΦ) (moyenne = 42% ± 6,6% SEM) que ceux issus de donneurs sains (moyenne = 18% ± 3,3 SEM). De même, après activation LPS les MΦ générés à partir de monocytes de patientes en GM-CSF+IFNγ produisent plus d'IL-10 que ceux générés à partir de monocytes de donneurs sains (patientes= 227.8pg/ml (±39.6 SEM) *vs* donneurs sains= 48.2pg/ml (±21.7 SEM)).

Egalement, les Mo-iDC différenciées *in vitro* à partir des monocytes de patientes atteints de cancer du sein (GM-CSF+IL-4), expriment de forts niveaux de PD-L1, induisent l'expression faible de CD25 sur les lymphocytes T et multiplie par deux la fréquence de Treg $Foxp3^+$ aux dépends des populations Th1 ou Th2, ce phénomène étant partiellement inhibé dans des cultures en *transwell*. Par ailleurs, après activation par le LPS, les Mo-DC des patientes montrent une diminution de leur capacité à induire des Treg $Foxp3^+$ *in vitro*, mais avec une fréquence plus forte que des Mo-iDC de donneurs sains (Article publié, 1er auteur- Appendix 1). Cette induction de Treg Foxp3 est médiée par TGFβ1 et PD-L1 puisque leur inhibition avec des Ac spécifiques sur les Mo-iDC de patientes réduit considérablement l'induction de Treg $CD4^+Foxp3^+$ dans les co-cultures avec les lymphocytes T $CD4^+$ naïfs (Article publié, 1er auteur - Appendix 2).

Les monocytes de patientes atteintes de cancer du Sein présentent un profil cytokinique immunosuppresseur. De manière intéressante, cette "programmation" altérée des monocytes est déjà détectable en périphérie chez les patientes. En effet, après activation par le LPS pendant 24h, des monocytes circulants provenant de 20 patientes, les surnageants ont été évalués pour la production de 48 médiateurs (cytokines, chimiokines, facteurs de croissance…). Les monocytes de patientes produisent des quantités plus élevées d'IL-10, de VEGF-A, d'IL-27, de CD40L soluble et d'IL-1RA par rapport à ceux de donneurs sains (n=10). Par ailleurs, si l'on sépare en fonction de la capacité des monocytes à se différencier ou non en M1-MΦ, les monocytes des patientes qui présentaient un biais de différenciation vers des M2-MΦ produisent des niveaux significativement plus élevés de CCL5, sCD40L, VEGF-A et IL-10 par rapport aux monocytes de patients qui ont montré une différenciation normale en M1-MΦ.

Conclusions

Nos données suggèrent que des facteurs solubles produits pas le micro-environnement tumoral (telque CCL22, TGF-β, M-CSF et VEGF) favorisent au niveau local la différenciation des monocytes en M2-like MΦ suppresseurs $CD163^{high}IL\text{-}10^{high}$ et agissent en systémique en « programmant » les monocytes circulants qui acquièrent un profil anti-inflammatoire et se différentient MΦ et Mo-iDC présentant des capacités suppressives (Article en préparation, 1er auteur). *In vivo*, l'accumulation des TAMs $CD163^+$ au sein des tumeurs est corrélée à une fréquence diminuée des lymphocytes

T $CD3^+$ et est associé à un impact négatif sur la survie sans progression des patientes atteintes de cancer du sein. Ces résultats mettent en avant l'importance de nouvelles stratégies pour neutraliser les facteurs responsables de la différenciation des TAM $CD163^{high}$ et pour reprogrammer au niveau systémique les monocytes sang, pour améliorer les approches cliniques d'immunothérapie chez les patientes présentant un cancer du Sein.

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LIST OF ABBREVIATIONS

APCs Antigen Presenting Cells
BDCA1 Commercial name for CD1c molecule
BDCA3 Commercial name for CD141 molecule
BT-474 An epithelial cell line from solid breast carcinomas.
CAL-51 cell line from a malignant pleural effusion in breast cancer
CD Cluster of differentiation
CCR7 Chemokine receptor 7
CFSE Carboxyfluorescein Succinimidyl Ester
cRPMI Complete RPMI, RPMI 1640 with 10% of FCS and antibiotics
CTLA-4 Cytotoxic T-Lymphocyte Antigen 4
CTLs citotoxic T lymphocytes
DCs Dendritic Cells
FACS Fluorescence Activated Cell Sorting
FCS Fetal Calf Serum
Foxp3 *Forkhead Box* P3 (Transcription Factor of regulatory T cells)
GATA-3 Transcription Factor of T helper 2 lymphocytes
GM-CSF Granulocyte-macrophage colony-stimulating factor
HLA-DR Human Leukocyte Antigens -DR
HIF-1α Hypoxia-inducible factor 1-alpha
IFN Interferon
IHC Immunohistochemistry assay
IL Interleukin
INCA Instituto Nacional do Câncer/Institute Nacional du Cancer
iTreg Induced regulatory T cells
LPS Lipopolysaccharide
mAb Monoclonal Antibody
MCF-7 An invasive breast ductal carcinoma cell line
mDC Myeloid Dendritic Cells
MFI Median Intensity of Fluorescence
MHC- I Major Histocompatibility Complex molecule of class I
MHC- II Major Histocompatibility Complex molecule of class II
Mono Monocytes
Mo-iDCs Monocyte-derived Immature Dendritic Cells
Mo-mDCs Monocyte-derived Mature Dendritic Cells

MΦ	Macrophage
NFκB	Nuclear Factor κB
nTreg	Natural Regulatory T cells
PBMCs	Mononuclear Peripheral Blood Cells
pDC	Plasmocytoid Dendritic Cells
PD-L1	Programmed death-ligand 1, also called CD274 or B7-H1
PD-L2	Programmed death-ligand 2, also called CD273 or B7-DC
PGE2	Prostaglandin E2
RoR-γ	Transcription Factor of T helper 17 lymphocytes
sCD40L	Soluble CD40 ligand
SN-Dil	Supernatant from primary dilacerated tumors
SNDil-MΦ	Macrophage conditioned by supernatant from dilacerated tumors
SN-Tum	Supernatant from cultured tumors by 48h
SNTum-MΦ	Macrophage conditioned by supernatant from cultured tumors
SK-BR-3	A Mammary Adenocarcinoma cell line
TAMs	Tumor-Associated Macrophages
T-Bet	Transcription Factor of T helper 1 lymphocytes
TGF-β	Transforming growth factor beta
Th1	T helper 1 lymphocyte
Th2	T helper 2 lymphocyte
Th3	Regulatory T cell that produce TGF-*β*
Th17	T helper 17 lymphocyte
TNF-α	Tumor Necrosis Factor-alpha
Tr1	Regulatory T cell that produce IL-10
Tregs	Regulatory T lymphocytes
TSLP	Thymic stromal lymphopoietin
TSW	Transwell system
WHO	World Health Organization
VEGF	Vascular endothelial growth factor

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1 INTRODUCTION

1.1 Cancer as a complex disease

Cancer is the name given to a large group of malignant proliferative diseases that nowadays constitute the second cause of death worldwide, which was responsible for circa 8 million deaths in 2012 (WORLD HEALTH ORGANIZATION - WHO). Among the different types of cancer, breast cancer appears as the main cause of death for women in the world, representing the first and second causes of cancer deaths in developing and developed countries (INCA – Brazil), respectively with 521.000 deaths of breast cancer registered in 2012 in the world (WHO). In Brazil, about 57,000 new cases of female breast cancer were diagnosed in 2014, representing around 20% of total cases of cancer (INSTITUTO NACIONAL DO CANCER - INCA - Brazil). Likewise, data from the “Institut National du Cancer” in France, registered about 48,000 new cases of female breast cancer in 2012 (INSTITUT NACIONAL DU CANCER - INCA - France), highlighting the worldwide relevance of this disease.

Malignant neoplasias are multi-factorial disorders, which incidence has been showing an increase year by year in developed countries, suggesting that it might be associated with modern habits (JEMAL et al., 2004; RADICE; REDAELLI, 2003). Most organs and tissues are subjected to the development of neoplasia and several characteristics are used to define the disease (cellular origin, tissue organization, vascularization, local and systemic spread, chromosomal and genetic alterations, but also tumor infiltration by leukocytes). With the advances in our knowledge of the biology of cancer, there is an increasing tendency to reclassify this disease based on its molecular characteristics rather than its morphology (which predominated till recently).

Genetic insults occur throughout the life and, combined with environmental factors, can lead to cancer initiation and/or promotion. Well known external agents like UV radiation, tobacco, alcohol and diet are frequently linked to cancer development, acting directly or indirectly as promoters of the disease (ROSSI et al., 2014; TSAI et al., 2010; WARREN et al., 2014). Thus, cancer is a genetic anomaly characterized by the abnormal differentiation of cells that lose their proliferation control, frequently have defects in their mechanisms of apoptosis and a high genetic instability. In order to generate a tumor, however, the neoplastic cell has to acquire

the ability to induce angiogenesis, a process that can be considered as a turning point in carcinogenesis (FOLKMAN et al., 1989). From that point, those genetically unstable cells, proliferating independently from tissue regulation, may acquire the definitive hallmark of cancer: the ability to invade other tissues. The “final” step in the malignant differentiation of the neoplastic cell is the acquisition of the metastatic potential that will allow its growth at distant sites and organs (review by HANAHAN; WEINBERG, 2000).

Within this general pathway, specific genomic alterations have been associated with cancer development. For breast cancer, BRCA1, p53, and Her2/neu expression have been described as the most important genomic targets of alterations in patients and are useful molecules to predict tumor development and the choice of treatment (MA et al., 2014; SONG et al., 2014).

1.2 Cancer Immunosurveillance

It is necessary to note that carcinogenesis is a silent phenomenon, which happens slowly, but not only in the neoplastic cells: tissues surrounding the tumor are also gradually modified during the process. Throughout oncogenesis, a very complex and typical microenvironment is formed, characterized by local pH alterations; zones of hypoxia; angiogenesis; inflammation with recruitment/accumulation of a distinct profile of immune cells. Besides that, several mechanisms of cancer control probably are turned on, and one of the most important is the presence of an efficient immune system, able to survey and eliminate the newly formed neoplastic cells (BURNET, 1957; DUNN et al., 2002). Although the first idea of immunosurveillance was conceived by Paul Ehrlich in 1909, only later in the 1950s the official hypothesis was postulated by Macfarlane Burnet (1957) and Lewis Thomas (1959), speculating the participation of lymphocytes as sentinels capable to recognize and destroy tumors. Only later, after the 1970s, when athymic nude mice lineages were used as models (STUTMAN, 1974 and 1979), it emerged the participation of adaptive immunity in tumor responses, however not convincing enough to confirm Ehrlich’s hypothesis. Even mouse models were not well established in that time, these preliminary findings corroborated observations in humans, where individuals with primary immunodeficiencies (GATTI; GOOD, 1971)

and patients treated with immunosuppressive drugs after transplantation (SHEIL, 1986) showed higher risk to develop cancer. The immunosurveillance premise was confirmed later by models showing that IFN-γ and perforin deficient mice and RAG2 knockout mice (KAPLAN et al., 1998; SHANKARAN et al., 2001; STREET et al., 2001) presented increased frequency and growth of chemically-induced or spontaneous tumors. Interestingly, even considering the crucial role of the immune system in the elimination of tumors, the process of inflammation has been lately considered as advantageous for tumor growth, at least in certain tumor models (HANAHAN; WEINBERG, 2011). Several mechanisms of tumor evasion have been described in the past century, but the role of the inflammation and of the immune system in the natural history of tumors has been “reinserted” in the studies just recently (HANAHAN; WEINBERG, 2011). Moreover, differently from infections, the development of malignant neoplasias is normally characterized as a silent and very slow process where non-self antigens are presented in low levels, failing to trigger an immune response.

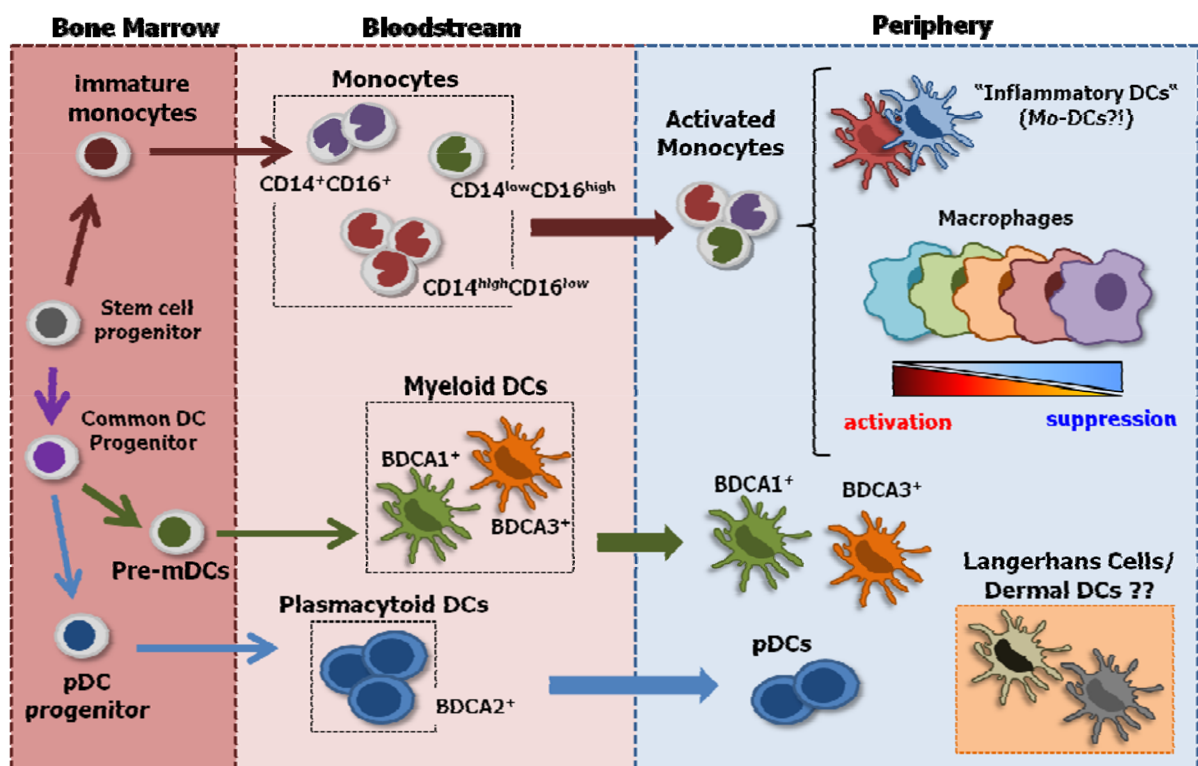
1.3 The Immune System: Human Antigen-Presenting Cells

The immune system is made up of diverse cells and specialized tissues responsible for the homeostasis in a well-orchestrated function. Specialized cells, the Antigen-Presenting Cells (APCs), are strategically distributed in tissues and organs, where they are able to quickly sense and identify the environmental imbalances, identify pathogens or damage, and stimulate immunity. Several subpopulations of cells have been described in humans, including Monocytes, Dendritic Cells, and Macrophages, which build very heterogeneous scenery of antigen presentation (scheme 1).

The APCs’ sensitivity to environmental modifications is critical for the initiation of immune responses, and is possible due to their large repertoire of pattern recognition receptors (PRR), extracellular and intracellular, which are able to identify molecular patterns associated with pathogens and/or tissue damage (PAMPs and DAMPs, respectively). Continuously, APCs internalize and process large molecules into smaller ones that will be presented to T lymphocytes in the context of specialized molecules – belonging to the Major Histocompatibility Complex products, when the

presented molecules are proteins, and to the CD1 family, when they are lipids. The consequence of this presentation will depend on the signals received from the environment by the APCs via their PRR. When the tissue, where the APC captured the potential antigens, contains enough molecular patterns signaling damage/danger, the APCs undergo a process of maturation that allows them to trigger an adaptive immune response.

Scheme 1 – Monocytes, DCs and Macrophages subsets in humans



1.3.1 Dendritic Cells

Dendritic cells (DCs) are considered the most important subpopulation of APCs with unique abilities to activate and stimulate naïve T lymphocytes (BANCHEREAU et al., 2000). Diverse DC subsets have been identified in mouse and humans during the past decades, and their dual role in the balance between immunity versus tolerance is increasingly recognized. In healthy tissues, immature DCs capture and process antigens, which, presented to T cells will lead to tolerance;

however, when DCs recognize a tissue imbalance, they acquire a mature phenotype during their migration to the draining lymph node, where they can stimulate (naïve) T cells, thus triggering the adaptive response to the antigens they present (MELLMAN; STEINMAN, 2001). During the maturation process DCs show an increased expression of the CCR7 chemokine receptor (GUERMONPREZ et al., 2002; YANAGIHARA et al., 1998) and up-regulate the expression of co-stimulatory (CD80, CD86 e CD40) and MHC molecules (class I and II), crucial signals that will directly regulate the quality and the intensity of T cell responses (BANCHEREAU et al., 2000; CAUX et al., 1994a; CAUX et al., 1994b). DCs consist of a very heterogeneous group of cells in mice and humans that may share similar functions but are not completely defined. In the literature, human DCs were characterized and divided in two major populations in peripheral blood: the plasmacytoid DCs (defined as $BDCA2^{+}$) and myeloid/conventional DCs (defined as $BDCA1^{+}$ or $BDCA3^{+}$).

Human plasmacytoid DCs (pDCs), further characterized by the expression of the BDCA2 marker (CD303), have their origin in the bone marrow and can be found in the circulation and in several tissues, where they respond to viral infections with the production of high levels of IFN-α (reviewed by MATHAN, 2013). Some authors have described a role for pDCs in the induction and proliferation of regulatory T cells *in vivo* and *in vitro* (OCHANDO et al., 2006; OUABED et al., 2008; SHARMA et al., 2007; TAKAGI et al., 2011) and, also, in the activation of Th17 responses in experimental autoimmune encephalomyelitis (ISAKSSON et al., 2009) and in mouse models of cancer (GUERY et al., 2014)

Human myeloid/conventional DCs (mDCs) are also derived from the bone marrow and found at low concentrations in the blood, lymphoid organs, and other tissues. These cells are further subdivided into two distinct subsets: $BDCA1^{+}$ ($CD1c^{+}$) cells are apparently the best inducers of T $CD4^{+}$ and cytotoxic responses, whereas $BDCA3^{+}$ ($CD141^{+}$) cells, have been described as more efficient to cross-present antigens. Recent studies have shown that human $BDCA3^{+}$ mDCs, though present in lymphoid tissues at very low frequencies, are highly effective in the cross-presentation of tumor and necrotic antigens for the induction of T $CD8^{+}$ activation (BACHEM et al., 2010; JONGBLOED et al., 2010; SEGURA et al., 2013a). In turn, $BDCA1^{+}$ mDCs may be considered as the better equipped DC subset to sense tissue imbalances, mainly due to their wide expression of Toll-like receptors (HÉMONT et al., 2013). These, when engaged, lead to an efficient maturation of mDCs, the

production of IL-12 and the expression of high levels of co-stimulatory molecules, favoring the differentiation of T cells towards the Th1 profile (NIZZOLI et al., 2013).

Additionally, diverse strategies allowed the differentiation *in vitro* of myeloid DCs from circulating precursors, like $CD34^+$ cells - in presence of GM-CSF and TNF-α (CAUX et al., 1996) - or blood monocytes - with GM-CSF and IL-4 (SALLUSTO; LANZAVECCHIA, 1994) - generating monocyte-derived DCs (Mo-DCs). The possibility of Mo-DCs generation has opened a large spectrum of possibilities to study and exploit DCs in immunotherapeutic protocols for infections and cancer (BANCHEREAU et al., 2005; BARBUTO et al., 2004). It is worth noting that some researchers do not consider Mo-DCs as an *in vivo* existing population in humans (NAIK, 2008). However, more recently, an elegant study based on gene signature revealed that human Mo-DCs generated *in vitro* may, indeed, be equivalent to the inflammatory DCs *in vivo*, a DC subset that arises in inflammatory conditions. Inflammatory DCs, defined as $CD14^+BDCA1^+FC\varepsilon RI^+$, were found in synovial and ovarian ascites fluids and share some functional abilities with monocyte/macrophages, but were uniquely able to expand Th17 lymphocytes *ex-vivo* (SEGURA et al., 2013b). All in all, one can say that DCs are extremely important in the activation and modulation of immunity, mainly by their ability to prime naïve T cells, but their origin and development, in humans, is only starting to be unraveled (BRETON et al., 2015; LEE et al., 2015).

1.3.2 Macrophages

Though DCs are the major inducers of naïve T cell responses, other well-known APCs, the macrophages (MΦ), are equally critical for lymphocyte activation in tissues. Macrophages have an essential role in the modulation of tissue microenvironment, fundamentally by their ability of phagocytosis and clearance, by the large quantity of cytokine they secrete and by their spectral plasticity. During an inflammatory process, newly arrived monocytes can be rapidly recruited to tissues, where they differentiate into macrophages, contributing to local immunity, while resident macrophages can live long in tissues, up to decades, and are deeply committed to maintain tissue equilibrium, regulating the intensity of inflammation, and acting in tissue remodeling (GORDON; MARTINEZ, 2010). Thus, macrophages in

tissues may derive from two distinct differentiation pathways: one giving rise to the resident MΦ, which, in mice at least, seem to emerge at the fetal stage, from hematopoietic precursors in the liver and have a low rate of renewal (reaching up to 30 years in humans); the other pathway is detected during infections or inflammatory processes, when blood monocytes migrate into tissues and differentiated into MΦ. Though heterogeneous, MΦ share some functional characteristics, even when localized in distinct tissues, where they receive different names: Alveolar Macrophages, Peritoneal Macrophages, Kupffer cells, Microglia, Osteoclast, etc (Reviwed by EPELMAN et al., 2014). These cells are involved in the control of infections (GORDON, 2003; RUSSEL et al., 2009), in the resolution of acute inflammation (SERHAN; SAVILL, 2005), and in the regulation of the metabolic responses to tissue stress (HOTAMISLIGIL; ERBAY, 2008). Through their broad range of functions and dynamic plasticity, macrophages are also implicated in several chronic pathological conditions including diabetes and atherosclerosis (MEDZHITOV, 2008; TABAS, 2010).

MΦ seem to be weak inducers of naïve T cell activation, a phenomenon that, *in vivo*, could be due to their poor competence to migrate to lymph nodes for antigen presentation, in contrast to DCs. On the other hand, MΦ present a large spectrum of morphological and functional plasticity, which is affected by local tissue conditions and by their cell-to-cell interactions during the immune responses. Diverse authors have described MΦ as a bi-functional population that can be classified as M1-MΦ (inflammatory) or M2-MΦ (anti-inflammatory), assuming similar parameters to those used to define Th1 and Th2 responses. However, this classification may be an oversimplification of their biology. To define the two polarized subtypes, tissue localization, surface markers, and the profile of produced cytokines are used (SICA; MANTOVANI, 2012). Human M1 macrophages show high expression of CD86 and HLA-DR, and produce diverse pro-inflammatory molecules as IL-12, TNF-α, CXCL9 and iNOS. On the other side, M2 anti-inflammatory macrophages are usually defined by their elevated expression of the scavenger receptor CD163 and by the production of typical anti-inflammatory cytokines, as IL-10 and TGF-beta, and the angiogenic factor VEGF (SICA; MANTOVANI, 2012). Nonetheless, it is important to highlight that this clearly bipolar behavior is observed when MΦ are differentiated *in vitro*, under well-defined conditions (JAGUIN et al., 2013; LACEY et al., 2012). The plasticity of MΦ *in vivo* is much more complex. It must be fine tuned to fit the needs of tissues

subjected, for example, to chronic infections or tumor development (MOSSER; EDWARDS, 2008), as the present work will demonstrate.

1.3.3 Monocytes

As the previous paragraphs have demonstrated, monocytes are an important blood cell, generated in the bone marrow and present in peripheral blood with a half-life of 1-2 days. Though monocyte recruitment to the tissues occurs during infections or inflammatory diseases, their contribution to the homeostatic tissue population (e.g. resident Macrophages) without diseases is minimal after birth (reviewed by AUFFRAY et al., 2009). These cells are heterogeneous and can be divided in three distinct subpopulations: one major subset, defined as CD14 positive but with low CD16 expression ($CD14^{++}CD16^{neg/low}$, called classical monocytes); one minor subset that express low or no CD14, but high CD16 ($CD14^{low}CD16^{++}$, called non-classical monocytes); and one transient or intermediate subpopulation identified by the double expression of intermediate levels of CD14 and CD16 ($CD14^{+}CD16^{+}$) (PASSLICK et al., 1989).

The $CD14^{++}CD16^{neg/low}$ monocytes represent about 90% of total blood monocytes, express high levels of the chemokine receptor 2 (CCR2) and low levels of the chemokine receptor CX3CR1. It is the only subset able to produce IL-10 rather than TNF-α after LPS activation *in vitro* (SKRZECZYŃSKA-MONCZNIK et al., 2008; WEBER et al., 2000; ZIEGLER-HEITBROCK et al., 1992). In contrast, human $CD14^{low}CD16^{++}$ monocytes secrete high levels of TNF-α in response to LPS (actually, they are the highest producers when compared to the other subpopulations), a characteristic that gave them the name of inflammatory monocytes (BELGE et al., 2002; SKRZECZYŃSKA-MONCZNIK et al., 2008). Transient monocytes ($CD14^{++}CD16^{+}$), on the other hand, secrete intermediate levels of both IL-10 and TNF-α, depending on the stimulus. Furthermore, these cells express the Fc gamma receptors CD64 and CD32 and have high phagocytic activity (GRAGE-GRIEBENOW et al., 2001). Studies in literature have reported that monocytes expressing high levels of CD16 are increased in the peripheral blood of patients with acute inflammation (MIZUNO et al., 2005) and infectious diseases (HORELT et al., 2002), but are dramatically reduced in subjects submitted to

glucocorticoid treatment (FINGERLE-ROWSON et al., 1998). It is interesting to mention that the complete absence of $CD16^+$ monocytes from the circulation is not necessarily associated with disease (FRANKENBERGER et al., 2013). Thus, a dynamical plasticity among subsets of monocytes is readily detectable (ZIEGLER-HEITBROCK; HOFER, 2013), but their contribution to tissue Macrophage/DC subpopulations in the time-course of human diseases remains poorly understood.

1.4 The Immune System: Stimulation of T lymphocyte subsets

Besides the role these three cell populations (DCs, MΦ, and monocytes) play in the inflammatory process, they are also critical for the generation and evolution of adaptive immune responses. It is well known that APCs, through a series of signals, generate a combinatory "code" that primes T cells and starts the adaptive immune response. The activation of naïve T cells depends on the engagement of its T cell receptor (TCR), interacting with the MHC class I or II molecules plus antigenic peptide complexes, and a combination of co-stimulatory signals (frequently termed "second signal"). This activation can be further modulated by the various cytokines in the microenvironment, and significantly by those produced by DCs, resulting on lymphocyte polarization and expansion. These interactions occur in the secondary lymphoid organs and are essential for the conversion of naïve $CD4^+$ T lymphocytes into function-committed T cells, which coordinate the overall immune response, through the stimulation of other immune cells. Actually, $CD4^+$ T lymphocytes may acquire different cytokine secretion profiles and, thus, be separated into four major subsets: T helper (Th) 1 cells, Th2, Th17 and regulatory T cells (Tregs).

Th1 cells are usually induced by the combination of signals delivered by high levels of CD80/CD86 on the APCs and IL-12, are characterized by the expression of the transcription factor T-bet, and secrete high levels of IFN-γ. This subset is frequently associated with effective responses to intracellular bacteria and pathogen destruction. It also induces the activation of T $CD8^+$ lymphocytes, Natural Killer (NK) cells and pro-inflammatory macrophages (OESTREICH; WEINMANN, 2012).

Th2 cells, on the other hand, are mainly induced by IL-4 signaling and typically express the intra-nuclear factor GATA-3. Th2 lymphocytes secrete IL-4, IL-5 and IL-13, cytokines that are usually involved in allergic responses and in the elimination of

helminths, phenomena that involve the activation of mast cells and eosinophils (HO, 2009).

Th17 were described more recently and seem to be induced by TGF-β plus IL-6, in cooperation with IL-23 and IL-1β signaling. The transcription factor that characterizes these cells is the ROR-γt and their most typical product is IL-17 (A through F isoforms). These cells seem to be needed for effective immune responses against extracellular pathogens and fungi (ZIELINSKI et al., 2012). Some authors further correlate Th17 cells with chronic tissue inflammation, sometimes cooperating with Th1 cells during the development of several autoimmune diseases (ANNUNZIATO et al., 2012).

Not all T cell subsets, however, are involved in antigen elimination - a fundamental T cell subpopulation is that of the regulatory T cells (Tregs). These are characterized by the expression of the nuclear transcriptional factor Foxp3 and can be further divided into natural Tregs (nTregs), which are generated in the thymus and those induced in the periphery, the induced Tregs (iTregs). nTregs represent about 5-10% of total $CD4^+$ circulating T lymphocytes in humans and are the consequence of an alternative differentiation pathway for thymocytes with a high affinity for self-peptide-MHC complexes. This differentiation pathway seems to rely, in humans, upon migratory DCs activated by the thymic stromal lymphopoietin (TSLP), which create a microenvironment supportive for the induction of Foxp3 in immature $CD4^+CD8^-$ thymocytes, contributing to their positive selection (SAKAGUCHI et al., 2010). In addition, nTregs are extremely important for the maintenance of self-tolerance and immune homeostasis, since individuals with IPEX, a syndrome characterized by Foxp3 deficiency, present serious autoimmune disorders (BARZAGHI et al., 2012). Though iTregs also express Foxp3 and, thus, should be absent in these patients, the role of this latter subpopulation could be, at least in part, overtook by other peripherally induced T cells, like Tr-1 and Th3 cells, which also have suppressive abilities, due to the production of IL-10 and TGF-beta, respectively (FARIA; WEINER, 2005; RONCAROLO et al., 2006).

On the other hand, iTregs are generated in the periphery by the conversion of conventional $CD4^+$ T cells into $CD127^{low}CD25^{high}Foxp3^+$ regulatory T cells. For this conversion it seems that stimulatory signals, from the TCR engagement, added to inhibitory signals, like those delivered by TGF-β and/or IL-10, and, very likely, many others, derived from local APCs, combine, driving the cells through a still

incompletely understood pathway. As mentioned before, other subpopulations of T cells with suppressive abilities have been described in literature.

Actually, other subsets of $CD4^+$ T helper cells are likely to be identified as the investigation of specific conditions progresses, a situation that can be exemplified by the recent description of Th9 and Th22 cells involved in patients with ulcerative colitis (GERLACH et al., 2014) and multiple sclerosis (ROLLA et al., 2014), respectively. Indeed, it is important to point that the profiles of T cell responses are not static in the course of infections or inflammation, but represent a dynamical and cooperative balance between innate and adaptive elements that can lead to immunity or disease. In this dynamical balance, the functional status of DCs, MΦ, and Monocytes is essential, since they are very effective sensors of tissue homeostasis and disequilibrium and able “translators” of the microenvironment to the adaptive immunity.

Even if not addressed in our present work, another important aspect of the immune system is its ability to develop humoral responses. Besides their obvious role in the production of antibodies, whose roles in tumor immunity are not negligible, B lymphocytes are also able to present antigens via MHC-II and, thus, might affect more closely the issues addressed in the present work. Nevertheless, these possible roles will not be further discussed, but should be, eventually integrated in a view that would lead to the full comprehension of tumor-immune system interactions.

1.5 The Immune System under tumor development: new players for a new game

So, the immune system is an effective participant in the maintenance of physiological equilibrium in the organism. When this is disrupted by an infection, immune sentinels, in general, are fast to identify the situation and to trigger immunity. However, the development of tumors is normally recognized late, probably due to its low immunogenicity and high capacity to hide the tissue microenvironment changes induced by its presence. This is most evident in the analysis of DCs and MΦ within tumors, whose functional alterations resulting in ineffective anti-tumor immune responses, contributing not only for the persistence but also for the growth and tumor metastasis. Actually, during cancer development, tumor and stromal cells promote

the migration/expansion of immunosuppressive regulatory T lymphocytes (Treg) (FAGET et al., 2011; GOBERT et al., 2009), the accumulation of anti-inflammatory Tumor-Associated Macrophages (TAMs) (BISWAS; MANTOVANI, 2010; POLLARD, 2004), and cause alterations in DC biology at the activation and functional levels (BALEEIRO et al., 2008; SISIRAK; FAGET et al., 2012).

Several studies have associated Tregs accumulation with tumor immune escape mechanism in cancer (CURIEL, 2007; ZOU, 2005; ZOU, 2006). Some authors consider this fact as a major obstacle in the development of cancer immunotherapy (DUNN; OLD; SCHREIBER, 2004; SAKAGUCHI, 2005; ZOU, 2005). Coherently, other authors during the past decades have described the profile of infiltrating immune cells in different human tumors as an important predictive factor for disease progression (FRIDMAN et al., 2013). Indeed, the profile of tumor-infiltrating lymphocytes may be, according to some authors, the most important characteristic in the pathological analysis of tumors, for prognosis evaluation (GALON et al., 2012). On the other hand, the induction of an immune response able to eliminate tumor cells is crucially dependent on the ability of APCs to recognize tissue disequilibrium, capture/process and present tumor antigens. However, during its establishment, the tumor microenvironment affects profoundly this recognition, thus changing the possible immune reactivity to the tumor.

1.5.1 Tumor-Associated Macrophages and Dendritic Cells

In clinical studies, the increase of TAMs in tissues has been directly correlated with tumor growth (BINGLE et al., 2002) and also with a worse clinical outcome in several types of human cancer, including ovarian, breast, non-small cell lung cancer, and Hodgkin's lymphoma (CAMPBELL et al., 2010; POLLARD, 2009; STEIDL et al., 2010). Indeed, in the tumor context, macrophages are usually associated to a range of pro-tumor actions, such as: the production of angiogenic (VEGF) and survival factors for malignant cells, the promotion of tissue remodeling and the production of immunosuppressive cytokines (e.g. IL-10, TGF-beta) that block T cell effector functions in the microenvironment (reviewed by QIAN; POLLARD, 2011). It is relevant to notice that polarization/modulation of macrophages is not exclusively due to tumor factors, but driven by reciprocal interactions with, both, malignant and

stromal cells in the microenvironment (LEWIS; POLLARD, 2006; LEWIS; HUGHES, 2007). One example of such participation of stromal cells was shown by Sharma and colleagues (2010), who demonstrated that tumor-associated fibroblasts specific molecular signatures were strongly associated with different stages of breast cancer development, and also with TAMs functional profiles.

Nevertheless, TAMs, themselves, seem to contribute for tumor growth, since their presence was associated with an increase in the tumor vasculature density in several human carcinomas, including breast (BOLAT et al., 2006; UZZAN et al., 2004). TAMs also regulate the composition and structure of extracellular matrix (ECM) through their deposition of components, which consequently may regulate tumor and stromal cell migration/invasion. As examples, we can mention the production of diverse types of collagens; the release of matrix metalloproteinases (MMPs), the production of serine proteases and cathepsins (KESSENBROCK et al., 2010). Furthermore, several studies have associated TAMs function with an increased ability of tumors to invade and metastasize, as shown in melanoma (VARNEY et al., 2005), breast (BECK et al., 2009; ROBINSON et al., 2009), ovarian (KAWAMURA et al., 2009), and colorectal (BAILEY et al., 2007) cancers.

TAMs have been detected in human tumors, mainly in retrospective studies using immunohistochemistry, by different markers. Though CD68 has been used for that, over a long period, to that purpose, CD163 has been more recently recognized as superior (HEUSINKVELD; VAN DER BURG, 2011) since subsets of dermal DCs (PETZELBAUER et al. 1993) and fibroblasts can express CD68 (RUFFELL et al., 2012). Furthermore, CD163 has been extensively associated to a M2-like profile, both for *in vitro* differentiated cells and *ex-vivo* obtained TAMs (HEUSINKVELD; VAN DER BURG, 2011), reveling a superior specificity than CD68. However, CD163 functions *per se* have not been directly associated to M2-MΦ functions. CD163 is a scavenger receptor able to capture free-hemoglobin, resulting from the rupture of red cells, as it could be expected in uncontrolled strong inflammation (FABRIEK et al., 2005) but some authors also showed its function on bacteria binding to human MΦ triggering the production of cytokines (FABRIEK et al., 2009).

Other study revealed a decrease in tumor-infiltrating DCs frequency in tumor areas when compared to normal adjacent tissues (RUFFELL et al., 2012). Besides that, the investigation of their functional status *in situ* revealed tumor-infiltrating DCs as immature in tumor bed, in contrast to activated DCs found in tumor periphery

(BALEEIRO et al., 2008; BELL et al., 1999; TREILLEUX et al., 2004). Interestingly, a recent study published by Goc and collaborators (2014) correlated a lower risk of death in lung cancer patients with the presence of mature DCs and Th1 lymphocytes in peritumoral tertiary lymphoid structures.

Even though the phenotypic and functional characterization of APCs in tumors is well established in murine models as a prognostic factor, in humans, this characterization and its relevance still represent a challenge. Thus, here, we will analyze the phenotypic and functional features of MΦ recovered from human tumors and attempt to correlate their frequency to other infiltrating immune cells and patients' survival, as comparing their characteristics with the "typical" MΦ differentiated *in vitro* from monocyte precursors.

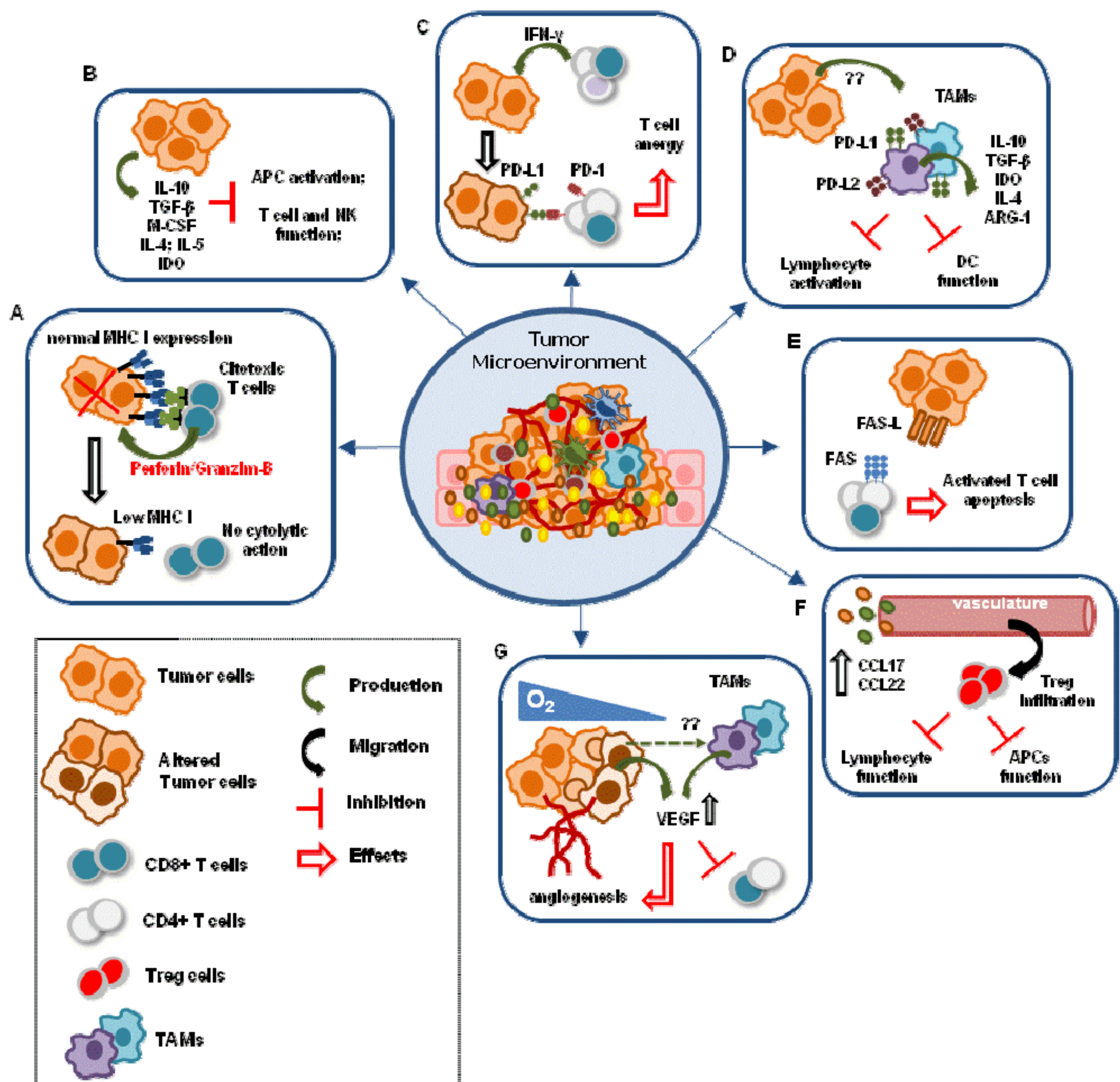
1.6 Mechanisms of tumor escape from the Immune System

It is important to consider that, as mentioned before, tumor cells are able not just to grow, invade and generate metastasis, but also present "smart strategies" to escape from the immune system (Scheme 2). The exact time point where the fine tuned adjustment, where anti-tumor immunity can avoid cancer growth, fails is difficult to define in humans. Contributes to that, surely, the genetic instability that can, eventually generate tumor cells able to evade immunity. This might occur because tumor cells reduce their "visibility", inhibit the immune cells in their environment or recruit specific cell populations.

Indeed, numerous tumor escape mechanisms have been described among which we can highlight: MHC class I down-regulation (SELIGER et al., 1996) (Scheme 2A); the production of anti-inflammatory cytokines as IL-10, IL-4 and IL-5 (YAMAMURA et al., 1993) or TGF-β (TADA et al., 1991) (Scheme 2B); the expression of negative co-stimulatory molecules as PD-L1/PD-L2 by tumor or infiltrating myeloid cells (BLANK; MACKENSEN, 2007; KUANG et al., 2009) (Scheme 2C and D), and apoptosis inducer Fas-L (GORDON; KLEINERMAN, 2009) (Scheme 2E). Actually, signals derived from tumors, not only act directly upon immune effector cells but also induce the conversion and/or the recruitment of cells with suppressive functions to the tissues, as CCL22/CCL17 do, recruiting regulatory T cells to tumor sites (GOBERT et al., 2009) (Scheme 2F). Additionally, VEGF, a well-known

angiogenesis factor, produced in the tumor microenvironment by malignant cells and/or TAMs, thus increasing nutrients's access to tumors cells, can also act as a potent inhibitor of T cell function (VORON et al., 2015) (Scheme 2G).

Scheme 2 – Tumor escape Mechanisms from the Immune System



Despite intense investigation, the precise mechanisms that lead to tumor escape are still poorly defined, but it is clear that, among these mechanisms, the functional modification of APCs should play a relevant role. For the investigation of this issue, it is relevant to note that myeloid DCs and MΦ can be differentiated from the same precursor, the blood monocytes. In inflammatory conditions or in well-defined *in vitro* conditions, this has been well established, but very few studies have investigated monocyte differentiation under the pressure of the tumor microenvironment in human systems. In fact, tumors may generate an anti-inflammatory *milieu*, rich in cytokines secreted by malignant cells, like IL-4, IL-6, VEGF, TGF-β and IL-10, which are able to promote monocytes/macrophages re-education towards an anti-inflammatory M2 profile and to block DCs functional maturation (GABRILOVICH, 2004; MANTOVANI et al., 2002; RABINOVICH et al., 2007).

Data obtained by Ménétrier-Caux and collaborators in 1998, revealed that breast tumor cell lines were able to skew healthy monocytes differentiation into macrophages through combined IL-6 and M-CSF signaling. Additionally, Thomachot and colleagues (2004) also showed that breast carcinoma cell lines were able to block DC maturation. However, the effects of the “complete” tumor microenvironment, as found *in vivo*, upon monocytes have not been explored yet. Nevertheless, we can hypothesize that, indeed, MΦ and DCs found in tumors may derive from “newly arrived” blood monocytes that, receiving the anti-inflammatory signals from the microenvironment during their differentiation, become skewed cells that favor tumor escape and growth.

1.7 Immunotherapy as a way to treat cancer patients

It is clear the crucial role of immune system in the surveillance of tissues and organs in the maintaining of homeostasis, avoiding the success of pathogens, infections and tumors. Conversely, it has become more acceptable for scientists that cancer modulates immunity in a singular way, and, thus, therapeutic interventions need to consider not only the cancer cells *per se* but also their ability to “cheat” immune control mechanisms as well. For example, one of such phenomena is the increase in PD-L1 expression by tumor cells in response to IFN-γ produced by

infiltrating T lymphocytes (BLANK et al., 2004). As a consequence, the newly expanded PD-L1+ tumors cells can inhibit infiltrating lymphocytes via PD-1, and escape from immune elimination.

Hence, it's now clear that cancer and the immune system are in close relationship whose fine-tuning may bring benefit for patients. This understanding led to several studies, pre-clinical and clinical, investigating the potential of immunotherapy against cancer in the last years (MCNUTT, 2013). Among these, the success of anti-CTLA-4 and PD-L1 monoclonal antibodies in cancer treatment clearly reinforces the point (HERBST et al., 2014; HODI et al., 2010; ROBERT et al., 2011).

1.8 Systemic effects on immune cells during tumor development

Certainly, several characteristics of malignant and stromal cells acquired during carcinogenesis can add to the establishment of a very complex microenvironment, able to support cancer growth and metastasis, and to promote its escape from the immune system. In this context, our present study will focus on the tumor microenvironment and its potential ability to affect the immune infiltrate, mainly investigating the effects of soluble factors from the microenvironment in the modulation of blood monocytes' differentiation and function. Though the direct effects of tumor derived-factors in the local inhibition of immune cells have been addressed by other studies, very few have called attention to the distant effects of tumors upon monocytes, MΦ, and DC derived from them. Such systemic effects may have profound effects upon the anti-tumor immune responses and have been reported previously in thesis and dissertations from our group, showing that circulating monocytes obtained from breast cancer patients fail to differentiate into functional DCs (Mo-DCs) *in vitro* (AZEVEDO-SANTOS, 2010; RAMOS, 2011). In these studies we described that Mo-DCs derived from breast cancer patients present an altered phenotype, produce high levels of IL-10 and fail to induce T lymphocyte proliferation. Here we will explore additional functional aspects of patients' Mo-DC, investigate the potential of patients' monocytes to differentiate into functional MΦ and we elucidate what are the characteristics of blood monocytes freshly isolated from breast cancer patients.

We expect that the characterization of the unique microenvironment generated by tumor development in humans, able to modulate the immune system and more particularly the monocytes and MΦ axis, at local and systemic levels, may provide insights for the improvement of current immunotherapeutic approaches against cancer, and, possibly, help design new ones targeting monocytes/MΦ.

2 OBJECTIVES

Our main objective is to characterize the effects of tumor microenvironment on human monocytes differentiation, exploring its impact on phenotype and functions of derived APCs and its direct versus systemic actions. More specifically we intend to:

- Analyze the frequency, phenotype, and functional competences of TAM subsets in breast and ovarian cancers, also exploring their correlation with other immune infiltrating cells and their impact in cancer patient's survival;

- Evaluate the effects of tumor microenvironment from freshly obtained tumors in the differentiation of healthy monocytes *in vitro,* investigating their phenotype and ability to stimulate T lymphocytes;

- Study the potential of blood monocytes from cancer patients to differentiate into MΦ and DCs *in vitro*, investigating the phenotype and suppressive functions of these derived APCs, but also exploring the basal status of cytokine production in blood monocytes from breast cancer patients.

3 METHODS

3.1 Human blood samples

Healthy human blood obtained anonymously from the ‘‘Etablissement Française du Sang’’ (Lyon, France) was collected in sterile bags containing CTAD and processed as described below.

Blood samples from cancer patients were obtained from Pérola Byington Hospital in São Paulo-SP-Brazil and from Centre Léon Bérard Hospital in Lyon-France and processed as described below. All patients and healthy volunteers gave written, informed consent and to all the study were considered individual older than 18 years old and with 50kg pounds. All breast cancer patients studied here were recent diagnosed and were not pre-treated with none of therapies.

3.2 Differentiation of monocyte-derived MΦ and monocyte-derived-DCs *in vitro*

Peripheral blood mononuclear cells (PBMCs) were isolated by double centrifugation on Ficoll (Dominique Dutscher) and Percoll 51% (GE), and total $CD14^+$ cells were isolated by magnetic beads (CD14 isolation kit; Miltenyi Biotec, Germany). To obtain monocyte-derived macrophages, $CD14^+$ monocytes were cultivated by 7 days in presence of GM-CSF (50 ng/ml) with the addition of IFN-gamma (20 ng/ml) in the 5th day (to M1-macrophage) or M-CSF (50 ng/ml) with addition of IL-4 (20 ng/ml) in the 5th day (to M2-macrophage) in complete medium (cRPMI): RPMI-1640 culture medium (Gibco, Grand Island, NY, USA), supplemented with 10% FCS (Gibco) plus antibiotic-antimycotic (100 U/ml penicillin, 100g/ml streptomycin, and 25 g/ml amphotericin; Gibco). To obtain monocyte-derived DCs, CD14+ monocytes were cultivated with GM-CSF (50 ng/ml; R&D Systems, Minneapolis, MN, USA) and IL-4 (50 ng/ml; R&D Systems) by 7 days.

To activate the cells, LPS was added for the last 24hrs at 100 ng/ml (*Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO, USA),

In experiments with patient’s Mo-DCs, cells were activated with sCD40L (1 mg/mL;Invitrogen, Carlsbad, CA, USA); a cytokine cocktail containing IL-1β (10ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), and TNF-α(10 ng/ml; R&D Systems); only TNF-α (50 ng/ml; R&D Systems); or LPS (500 ng/ml; Escherichia coli 0111:B4; Sigma-Aldrich, St. Louis, MO, USA).

3.3 Tumor obtaining, processing and supernatant preparation *ex-vivo*

Breast and ovarian tumor tissues were obtained from the Centre Léon Bérard Hospital (Lyon, France) after patient informed consent. In this study were used primary ductal invasive breast tumors (tumors #1 to #13) and ovarian tumors (tumors #14 to #17) samples with between 2 to 5 cm^2 from patients without any previous therapeutic interventions (chemotherapy or radiotherapy).

After collected, those tumors were mechanically dilacerated, the estromal content was filtered in 0.22µm and adjusted as each 500mg of tumor to 1ml of RPMI 1640 supplemented with 10% FCS with antibiotics (cRPMI), were we obtained the "supernatant from tumors dilacerations" (here called SN-Dil).The dilacerated tissues from tumors were then enzymatically digested for 45min at 37 ºC with collagenase Ia (1 Ag/mL) and DNase I (50 kilounits/mL; Sigma) in RPMI 1640 medium with antibiotics (penicillin 100 IU/mL and streptomycin 100 Ag/L, Invitrogen), washed in cRPMI and ressupended. Total obtained cells than submitted to flow cytometry analysis or to Facs-sorting. For some samples, part of ressupended cells was plated for 48hrs and the supernatants collected to obtained “supernatant from cultured tumors” (here called SN-Tum).

3.4 Monocytes conditioned in presence of primary tumor supernatant dilacerated or tumor cell lines supernatants

$CD14^+$ monocytes were isolated from healthy donors PBMCs’ and incubated with 25% of "supernatant from dilacerated tumors" (SN-Dil) or “supernatant of cultured tumors” (SN-Tum) by 7 days in cRPMI. Similarly, supernatant from four breast tumor cell lines (SKBR3, BT474, CAL51 and MCF-7) were collected after 48hours of have been plated and 25% those were added in $CD14^+$ monocytes isolated from healthy donors by 7 days. At day 6, LPS (100 ng/ml) was added to activate the cells for all experiments. Surface markers were investigated by flow cytometry and cytokine production was measured by ELISA.

3.5 Co-culture assays

For proliferation experiments:

CD4^{+}CD45RA^{+} T cells were isolated by magnetic beads from allogeneic healthy PBMCs, labeled with 5μM of Cell-Trace Violet (Life-technologies) and cultivated in 96 well plates ("U" bottom) with LPS-activated monocytes, macrophages, DCs or SN-Dil-conditioned monocytes by 5 days. At the end of the culture, cells were recovered from the plates and stained with viability marker (Live&Dead) and anti-CD3, and Cell-Trace dilution was determined by flow cytometry analysis. Beads anti-CD3 and anti-CD28 (ratio 1 bead: 1 T cell) were used as a polyclonal-positive stimulus.

For suppression experiments:

CD4^{+}CD45RA^{+} T cells were isolated by magnetic beads from allogeneic healthy PBMCs, labeled with 5μM of Cell-Trace Violet (Life-technologies) and cultivated in 96 well plates ("U" bottom) with LPS-activated monocytes, macrophages, DCs or SN-Dil-conditioned monocytes by 4 days in the presence of beads anti-CD3 and anti-CD28 (Dynabeads) (ratio 1 bead: 4 T cells). At the end of the culture, cells were recovered from the plates and stained with viability marker (Live&Dead) and anti-CD3, and Cell-Trace dilution was determined by flow cytometry analysis.

For co-cultures with patient's Mo-DCs:

CD4^{+}CD45RA^{+} T cells were isolated by magnetic beads from allogeneic healthy PBMCs and co-cultivated in 96 well plates ("U" bottom) with Mo-DCs (ratio 1 Mo-DC: 10 T cells) from breast cancer patients by 6 days in the presence or not of transwell or specific blocking antibodies. At the end of the culture, cells were recovered from the plates and stained with fluorescent antibodies anti-CD4, anti-CD25, anti-CD127 to surface markers and, for transcriptional factors, anti-Foxp3, anti-Gata-3 and anti-T-Bet were used.

3.6 Blocking antibodies

To the M2-macrophage *in vitro*, anti-IL-10R (1mg/ml- R&D Systems) was used to verify its role in the monocyte to macrophage differentiation.

In the suppressive assay, blocking antibodies anti-IL10 (R&D Systems), anti-IL-10R (R&D Systems), anti-PD-L1 (Biolegend) and IgG controls (same companies) were used to verify the role of those molecules in the system.

To patient's Mo-DCs co-cultures, blocking antibodies from eBiosciences (San Diego) were used: anti-CD80 (clone 2D10.4), anti-CD86 (clone IT2.2), anti-PD-L1 (clone MIH1) e anti-PDL2 (clone MIH18).

3.7 Flow cytometry

Monocytes, Macrophages, DCs and SN-Dil-conditioned-monocytes:
At least $5x10^5$ cells were labeled with each of the various specific fluorescent antibodies (CD11b, CD14, CD64, CD86, CD163, PD-L1, BDCA-1, HLA-DR) or isotype controls. (relative-CD163= CD163 MFI SN-Dil conditioned monocytes/ CD163 MFI control monocytes)

TAMs and TA-myeloid DCs:
At least $1x10^6$ cells were labeled with each of the various specific fluorescent antibodies (CD45, CD11b, CD14, CD64, CD86, CD163, PD-L1, BDCA1, HLA-DR) or isotype controls.

Legend-Screen assay:
LPS-activated monocytes, monocyte-derived macrophages or monocyte-derived DCs from the same donor were incubated with 342 PE-conjugated pre-titrated antibodies in different wells (specific and control isotypes), washed and submitted to cytometry analysis according to the manufacturers' instructions (Biolegend).

T lymphocytes co-cultured with patient's Mo-DCs
After 6 days of co-culture lymphocytes were harvested and labeled with surface specific antibodies (CD4, CD25) and, further, fixed and permeabilized (according to manufacturer's instructions – eBiosciences), and stained with intracellular specific antibodies or isotype controls to investigate transcriptional factors (T-Bet; Gata-3; Foxp3) or intracellular cytokines (TNF-α, IL-10, IL-4 and IFN-γ)
All data were acquired in the FACS-Canto cytometer or FACS-Fortessa cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

3.8 Isolation of cells by FACS

TAMs or TA-DCs were isolated by FACS using FACS-Aria-II with specific fluorescent anti-human antibodies.

- *Tumor Associated Macrophages:* $DAPI^{neg}$, $CD45^+$, $CD11b^+$, $HLADR^+$, $CD14^+$, $CD64^+$, $CD163^{high}$ or $CD163^{low}$
- *Tumor- Associated myeloid DCs:* $DAPI^{neg}$, $CD45^+$, $CD11b^+$, $HLADR^+$, $CD14^{neg}$, $CD64^{neg}$, $BDCA1^+$

(Control antibody isotypes were used to define the gates)

3.9 Morphology characterization by Cytospin

Facs-sorted TAMs and *in vitro* differentiated APCs (M0-MΦ, M1-MΦ, M2-MΦ and Mo-DCs), were obtained and submitted to Cytospin centrifugation at 600 rpm by 5 minutes to adherence in glass blades. Morphology of cytoplasm and nuclei from cells were revealed using Hematoxilyn-Eosin or May-Grunwald-Giemsa techiniques of staining depending of the experiments.

3.10 Cytokine detection in supernatants

- IL-10 and TNF-alpha levels were quantified in cell supernatants using ELISA assay from eBiosciences and R&D Systems kits, respectively.
- For patient's Mo-DCs assays: IFN-γ (BD PharMingen, San Diego, CA, USA), IL-10 (BD PharMingen), and bioactive TGF-β1 (eBioscience) were quantified by sandwich ELISA according to the manufacturers' instructions.

ELISA values were converted to pg/ml using SoftMax Pro software.

- For $CD14^+$ patient monocytes', after LPS activation, supernatant was collected and frozen. Those samples were submitted to Multiplex cytokine assay according to manufacturer (customized 48-plex; eBiosciences).

3.11 Multiplex analysis of primary supernatants from tumor dilacerations

After collected, breast and ovarian tumors were mechanically dilacerated, and the estromal content was filtered in 0.22µm and adjusted as each 500mg of tumor to 1ml of cRPMI. Obtained "supernatant from tumor dilacerations" (SN-Dil), were immediately submitted to freezing at -20ºC and subsequently conditioned at -80ºC.

Samples were then submitted to multiplex analysis for 48 cytokines (machine Bio200, Biorad), according to manufacturer instructions (eBiosciences, BioRad and Milipore).

3.12 Immunohistochemistry

Expression of CD163 on paraffin-embedded sections of breast tumor or peritumoral tissue was analyzed using a mouse IgG anti-human CD163 antibody (Menarini Diagnostics). Samples were obtained in the patient's bank of slides from the Centre Léon Bérard Hospital, Lyon – France. For negative control slides, primary antibodies were replaced by a non-immune serum.

3.13 Statistical analysis

For phenotype and cytokine production of monocytes, macrophages, DCs, SNDil-MΦ and tumors analysis as well, were used the one-way ANOVA test with the Bonferroni post-test.

For tests between patients and healthy donors, results were analyzed for normality by the Kolmogorov-Smirnov test, and comparisons were performed by Mann Whitney test.

Effects of neutralizing antibodies were compared using the paired t-test for the same donor/experiment.

For all tests consider: *$p<0.05$; **$p<0.01$; ***$p<0.0001$.

4 RESULTS

4.1 *Ex-vivo* characterization of Tumor-Associated Macrophages and myeloid Dendritic Cells in human tissues

Since decades, the description of myeloid cells infiltration in murine models of cancer allowed the investigation of TAMs and DCs contribution during the carcinogenesis process. However, for some types of cancer in humans, still unclear: 1) how TAMs could be phenotypically defined; 2) their particular functional skills; 3) their involvement in lymphocytes stimulation or inhibition; and 4) their direct correlation with patients' survival. In this section, we aim to investigate those aspects in samples from breast cancer patients.

4.1.1 Breast and ovarian tumors present high frequency of Tumor-Associated Macrophages with mixed infiltration of CD163^{high} and CD163^{low} subsets

Breast tumors were obtained from patients with consent, submitted to mechanical disruption and enzymatic digestion, and obtained cells were stained with specific antibodies. Using multicolor flow cytometry we investigated the presence of tumor infiltrating macrophages and myeloid DCs as shown in the representative cytometry analysis in Figure 1, as following: First gate was done considering SSC-A vs FCS-A; doublet were excluded (I); live cells were selected based on low Live & Dead expression (II); total CD45$^+$ immune cells were gated (III); total CD11b$^+$HLADR$^+$ cells were selected and, then, three sub-populations were defined based on CD14 *vs* BDCA-1 markers (IV): monocyte/macrophage (CD14$^+$BDCA1^{neg}), myeloid BCDA1$^+$ DCs (CD14^{neg}BDCA1$^+$) and inflammatory-like DCs (CD14$^+$BDCA1$^+$). Subsequently, CD14$^+$ gated cells were evaluated for CD64 and CD163 markers (VI), and segregated as CD163 low or high subset, and PD-L1 and CD86 molecules were also investigated (VII).

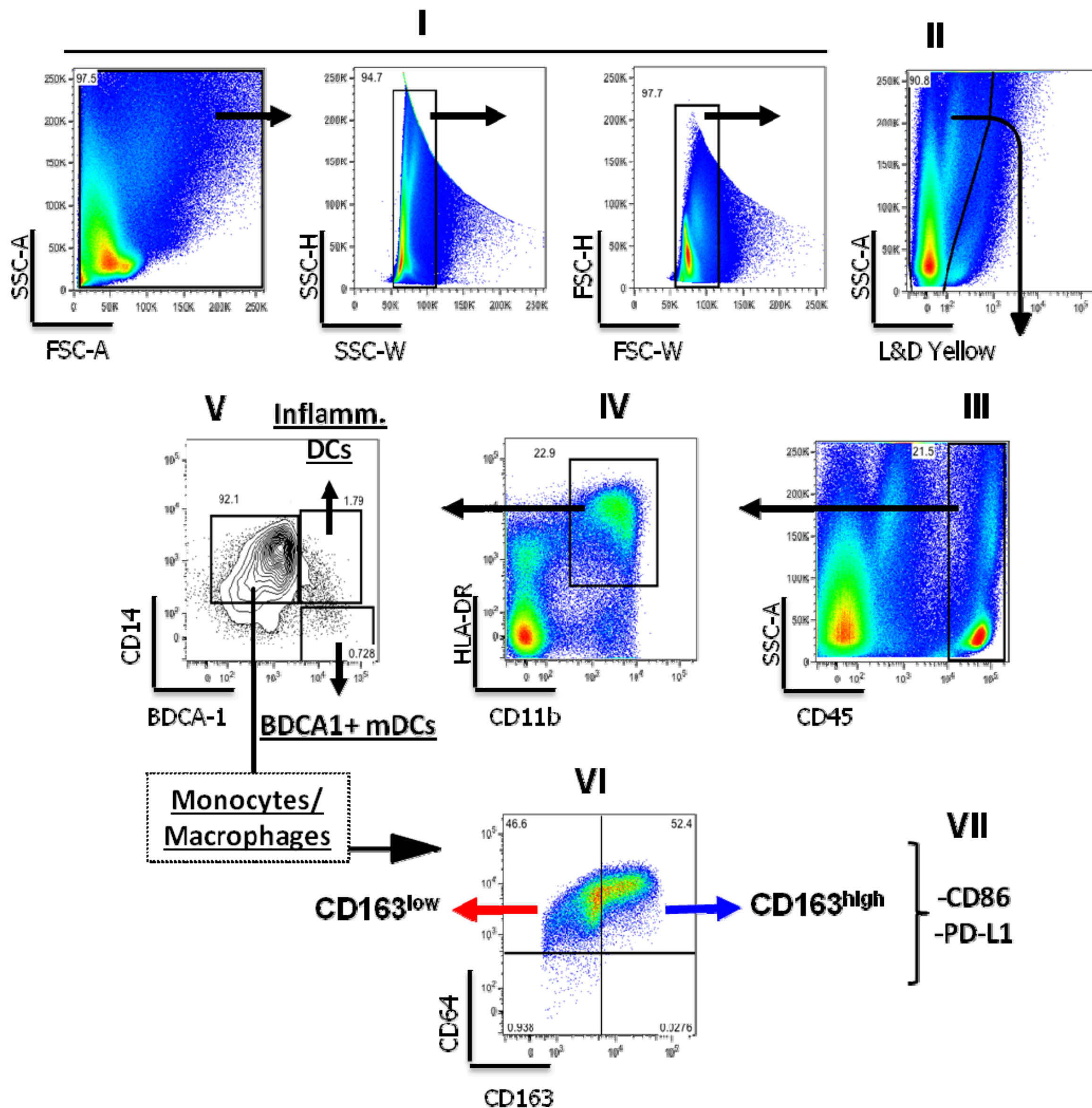


Figure 1. Representative gating strategy for flow cytometry analysis to characterize Tumor-Associated Macrophages (TAMs) and Tumor-Associated DCs (TA-DCs) in human tissues. After mechanical and enzymatic disruption of tumors, cells were resuspended and submitted to flow cytometry analysis after specific antibodies staining. The sequence of analysis was: (I) Primary gate and exclusion of doublets; (II) gate in live cells within Live and Dead low; (III) gate in total CD45^{+} cells; (IV) gate in CD11b^{+}HLADR^{+} myeloid cells; (V) gates defining 3 subpopulations: CD14^{+}BDCA1^{neg} (monocytes/macrophages), CD14^{+}BDCA1^{+} (inflammatory DCs), CD14^{neg}BDCA1^{+} (myeloid DCs). In VI, analysis of CD64^{+}CD163^{low} and CD64^{+}CD163^{high} macrophages within CD14^{+}BDCA1^{neg} gate, with subsequent investigation of co-stimulatory molecules (VII). At least 1,000,000 events were acquired for each sample.

Analyzing breast cancer tissues, we found a high infiltration of monocyte/macrophage ($CD14^{+}BDCA1^{neg}$) population in the myeloid compartment, representing a mean of 14.3% (± 2.2 SEM) of total viable $CD45^{+}$ leukocytes, while myeloid $BDCA1^{+}$ DCs and $CD14^{+}BDCA1^{+}$ inflammatory-like DCs represent about 0.45% (± 0.1 SEM) and 0.84% (± 0.2 SEM), respectively (Figure 2A). Indeed, using our panel of antibodies was not possible to confirm whether all cells expressing $CD14^{+}BDCA1^{+}$ markers were inflammatory DCs, and was not possible to go further in details here. Thus, gated TAMs ($CD14^{+}BDCA1^{neg}CD64^{+}$) were characterized by different levels of CD163 expression, depending on each patient analysis (Figure 2B and C). Based on the isotype control, TAMs were defined as $CD163^{Low}$ and $CD163^{High}$ subsets, resulting in a very heterogeneous landscape. The analysis of TAMs in the same patients, now reporting their frequency among viable $CD45^{+}$, revealed the same heterogenic picture: $CD163^{Low}$ represent 3% to 22.5%; whereas the $CD163^{High}$ subset corresponds from 0.8% to 11.6% (Figure 2D). In addition, FACs-Sorted $CD163^{Low}$ and $CD163^{High}$ subsets presented differences in size and morphology. While $CD163^{Low}$ TAMs are smaller and present a homogeneous Giemsa staining, $CD163^{High}$ subset show a bigger cytoplasm with the presence of numerous vacuoles and/or granules (Figure 2E and F).

Using the same strategy, four samples from ovarian tumors were evaluated and data were quite similar to found for breast cancer. Considering viable $CD45^{+}$ leukocytes, TAMs ($CD14^{+}CD64^{+}$) are also characterized by a heterogeneous frequency of $CD163^{low}$ (1.8% to 26.8%) and $CD163^{high}$ (0.5% to 8.4%) subsets (data not show).

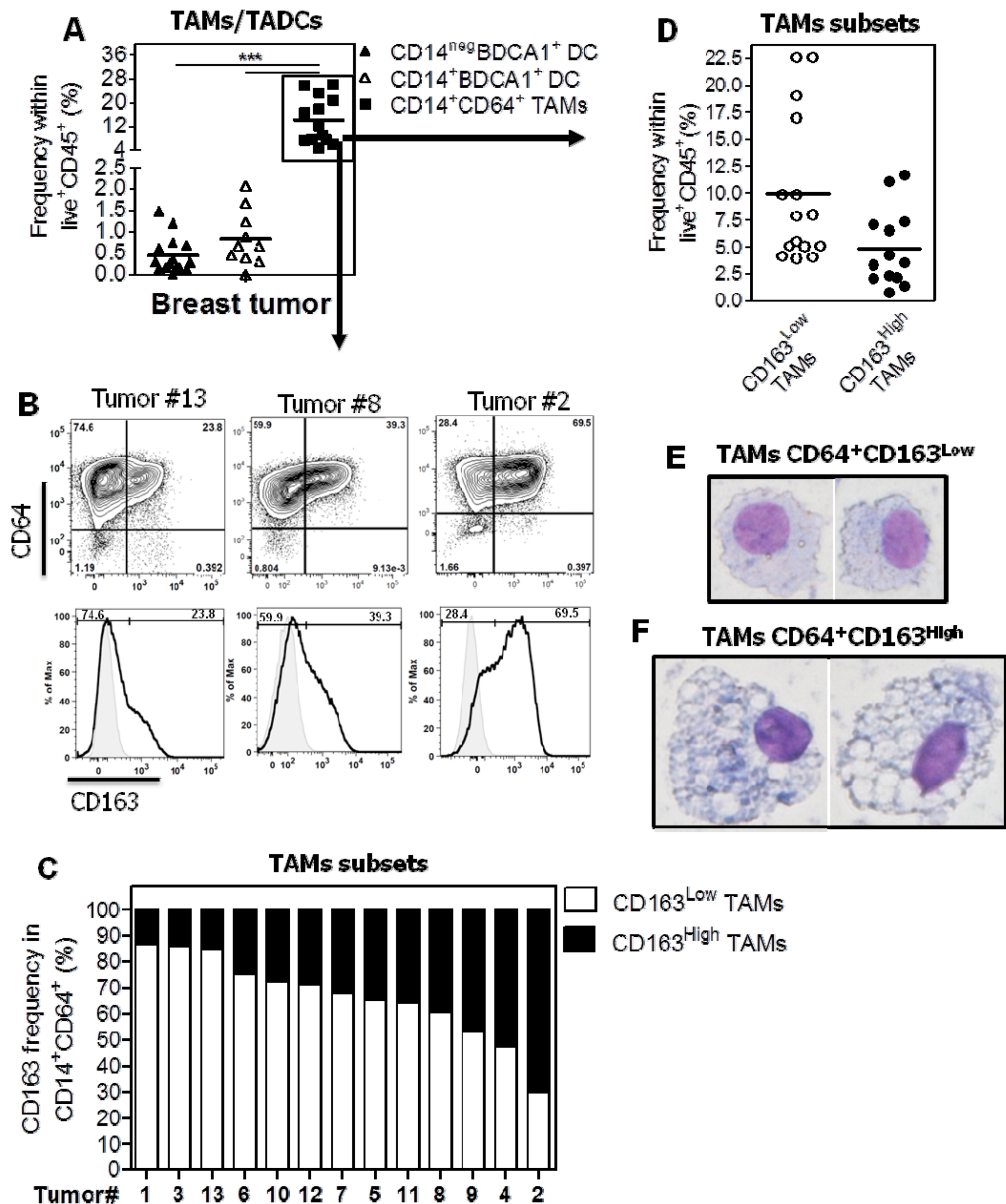


Figure 2. Breast tumors are infiltrated by TAMs with heterogeneous phenotype. After mechanical and enzymatic disruption of tumor tissues, immune cells were obtained and analyzed by flow cytometry. In A and B, frequency of TAMs ($CD64^{+}CD14^{+}$), TA-DCs (myeloid subsets) and $CD163^{low}$ and $CD163^{high}$ TAMs within total $CD45^{+}$ cells (n=13; ***p<0.0001). C and D, respectively, three representative contour-plots/histograms and graphics of CD163 expression within gated TAMs ($CD64^{+}CD14^{+}$) for each analyzed sample from breast cancer patients (gray histograms are isotype control). E and F, cytospin images from Facs-sorted $CD163^{low}$ and $CD163^{high}$ TAMs after staining by May-Grunwald-Giemsa method.

Additionally, to better characterize the TAMs in breast cancer samples, we investigated the expression of the co-stimulatory molecules CD86 and PD-L1, involved in the stimulation and inhibition of T lymphocytes, respectively. Even heterogeneously expressed among patients, PD-L1 and CD86 molecules were significantly expressed at higher levels in CD163^{high} subset in comparison with CD163^{low} TAMs (Figure 3). Indeed, CD163^{low} TAMs expressed lower levels of CD86 and lack PD-L1 expression, while CD163^{high} TAMs showed about 2.4x more expression of PD-L1 MFI than its relative control isotype (Figures 3B-C).

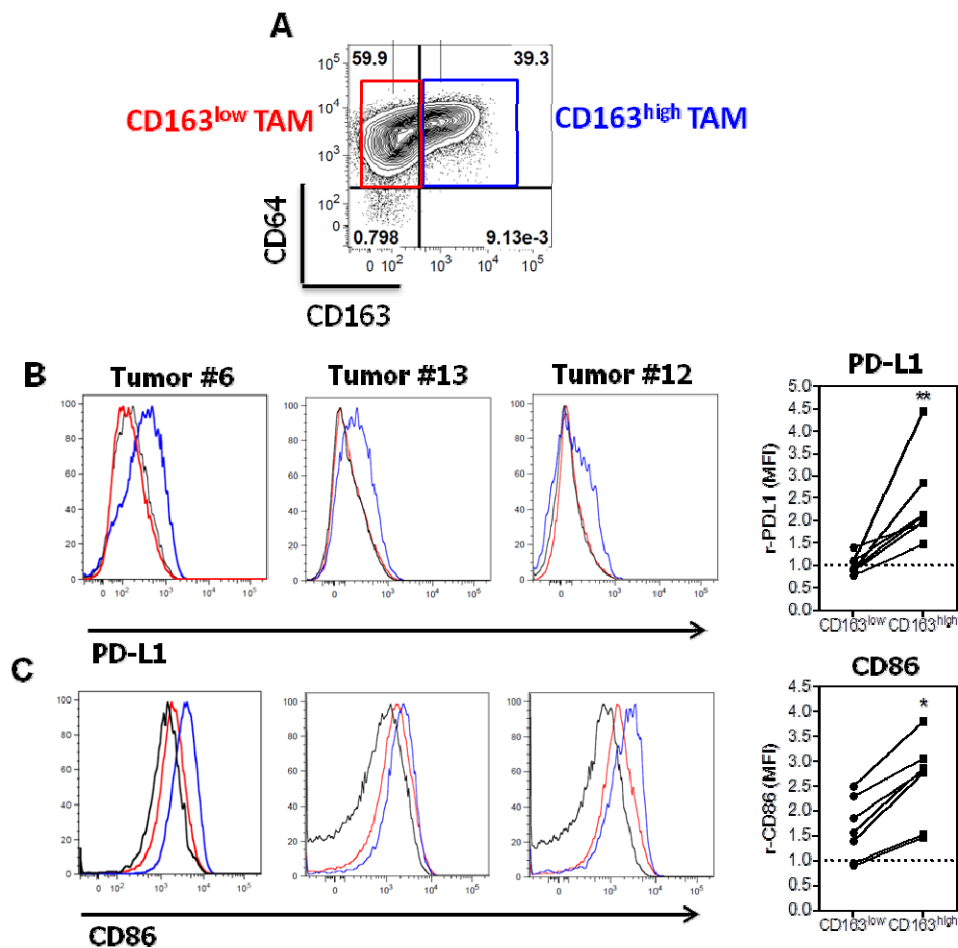


Figure 3. CD163^{high} TAMs from breast cancer express high levels of PD-L1 and CD86. After mechanical and enzymatic disruption of tumor tissues, immune cells were obtained and analyzed by flow cytometry without any stimulus. In A, representative contour-plot showing TAMs CD64^{+}CD163^{low} (red) and CD64^{+}CD163^{high} (blue). Below, representative histograms from three patients and graphics showing PD-L1 (B) and CD86 (C) expression within TAMs subsets (IgG isotype in black). r-PD-L1 or r-CD86 (relative MFI = MFI of specific stained antibody/MFI of IgG isotype); (n=7; *p<0.05, **p<0.01).

4.1.2 TAMs show increased IL-10 and PD-L1 expression and fail to activate T cell after LPS activation

To address the functional abilities of TAMs, we investigated three isolated subpopulations from ovarian cancer samples: total $CD14^{+}$, $CD14^{+}CD64^{+}CD163^{low}$ and $CD14^{+}CD64^{+}CD163^{high}$ subsets. Those cells were evaluated for the production of cytokines (IL-10 and TNF-alpha) and the modulation of surface molecules (CD86 and PD-L1) under LPS activation *ex-vivo*. Our data showed that total $CD14^{+}$ TAMs increased the production of IL-10 but not TNF-alpha after 24hrs of LPS activation (Figure 4A). That same phenomenon was observed by both isolated TAMs subsets ($CD163^{high}$ and $CD163^{low}$) with a more substantial IL-10 production by the $CD163^{high}$ compartment (Figure 4B). Additionally, we observed an increased expression of PD-L1 but not of CD86 on both TAMs subsets after LPS exposure (Figure 4C). Those findings inferred that LPS exposure stimulate TAMs to enhance IL-10 production and PD-L1 expression, with a more evident effect on $CD163^{high}$ subset. Moreover, we tested other TLR-ligands, as Poly-IC and R848, but results indicated minor effects when compared to LPS stimulation (data not shown).

To verify if these phenotypic characteristics may also impact the functions, $CD14^{+}CD64^{+}CD163^{high}$ TAMs and myeloid $BDCA1^{+}$DCs were isolated by FACs-sorting from ovarian cancer tissues and co-cultured with allogeneic $CD4^{+}CD45RA^{+}$ T cells in the presence of LPS by five days. Our findings showed that $CD163^{high}$ TAMs failed to induce T cells expansion at all ratios APC:Tcells tested, contrary to myeloid $BDCA1^{+}$DCs that induced T cell stimulation and IFN-gamma production (Figures 5A-B). Also, a preliminary experiment of suppression showed that activated $CD4^{+}$ T cells (anti-CD3/CD28 beads) were partially inhibited when co-cultured with LPS-activated $CD14^{+}CD163^{high}$ TAMs by 5 days (Figure 5C).

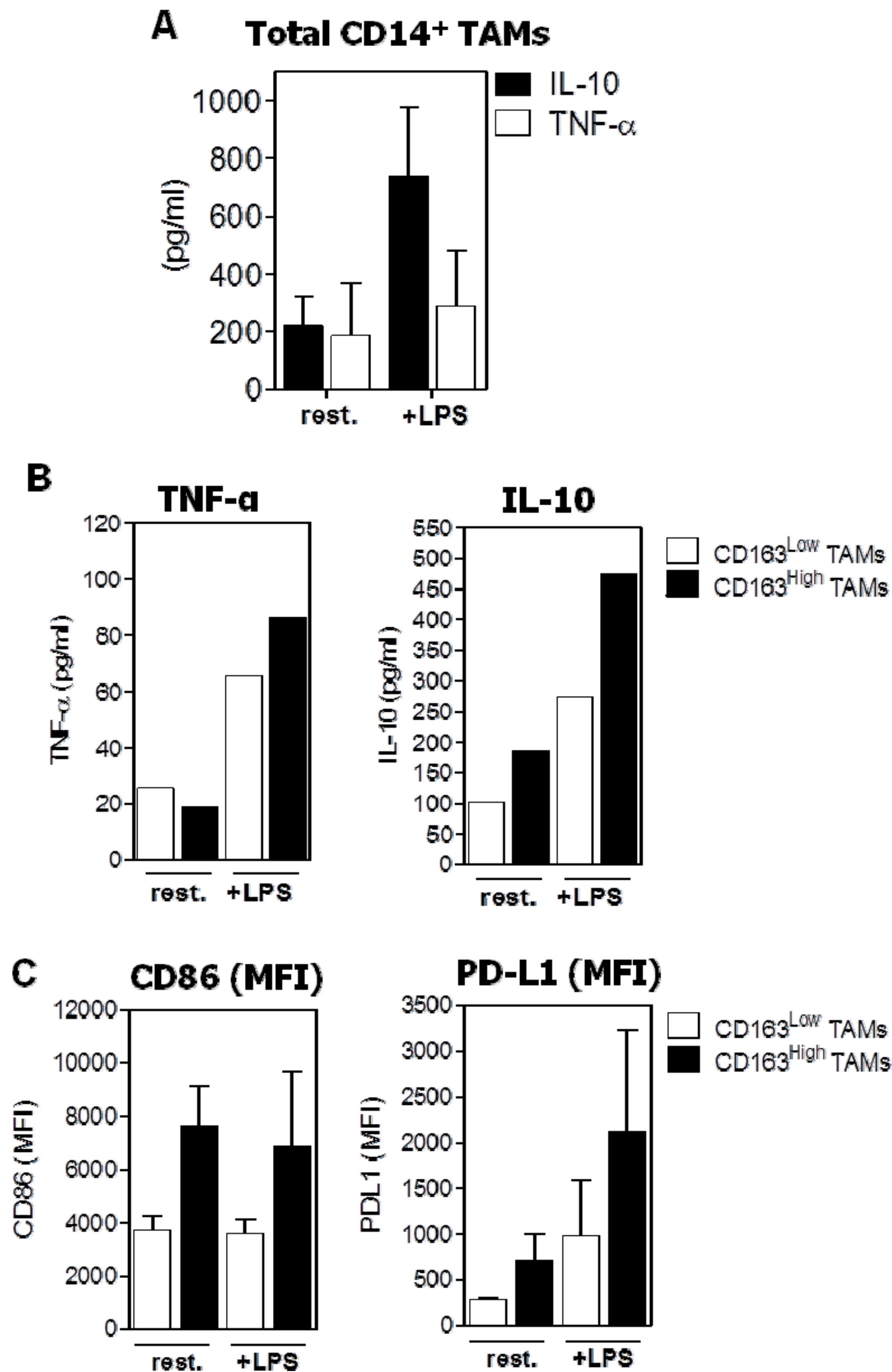
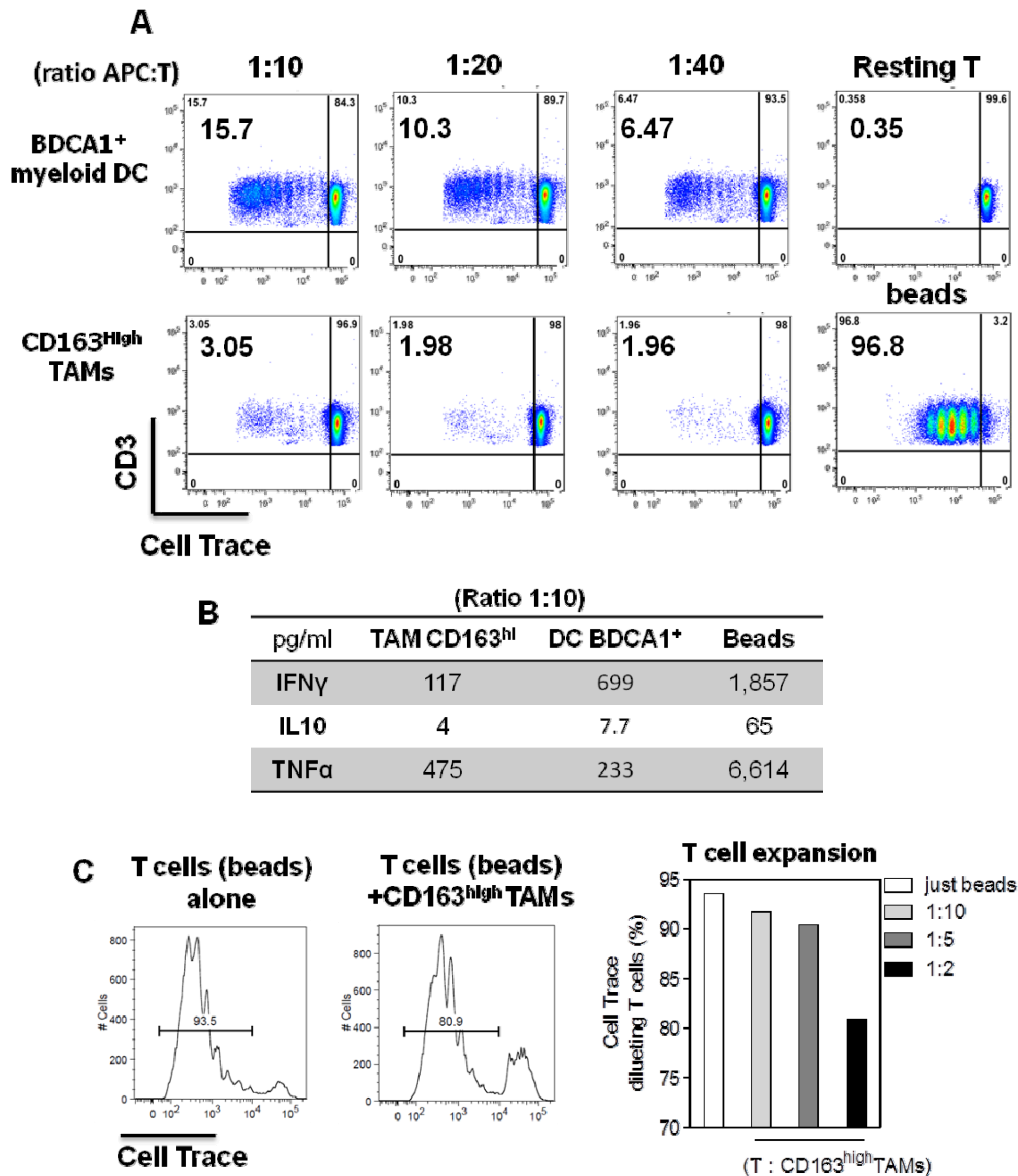


Figure 4. TAMs showed increased IL-10 production and PD-L1 expression under LPS stimulation *ex-vivo*. TAMs were isolated from ovarian tumors and stimulated by LPS (100ng/ml) for 24hrs. IL-10 and TNF-alpha were measured by ELISA in cell culture supernatants from $CD14^{+}$ total TAMs (A; rest n=02, LPS n=03) or in $CD163^{low}$ and $CD163^{high}$ Facs-sorted TAMs (B, n=01). C, graphics representing CD86 and PD-L1 MFI levels in isolated $CD163^{low}$ and $CD163^{high}$ TAMs with (+LPS) or without (rest.) LPS stimulation (n=3).



pg/ml	TAM CD163hi	DC BDCA1+	Beads
IFNγ	117	699	1,857
IL10	4	7.7	65
TNFα	475	233	6,614

Figure 5. CD163^{high} TAMs failed to stimulate naïve CD4^{+} T cells proliferation. BDCA1^{+} TADCs and CD163^{high} TAMs were isolated from ovarian tumors by FACS and co-cultured in different ratios with allogeneic naïve CD4^{+} T cell by 5 days in the presence of LPS (anti-CD3/CD28 beads were used as positive control). Representative pseudo-color plots of Cell Trace dilution (A) and cytokine production (B) by stimulated T cells in co-cultures. In C, isolated CD163^{high} TAMs were co-cultured by 5 days with allogeneic naïve CD4^{+} T cells pre-activated with anti-CD3/CD28 beads. Representative histograms show Cell Trace dilution for the ratio 1:2 (T:APC) and graphic with all tested ratios. (For both experiments, analyses were done on viable CD3^{+} gated cells).

4.1.3 High frequency of CD163^{High} TAMs is correlated to low tumor-infiltrating CD3^{+} T lymphocytes and has a negative impact on the survival of breast cancer patients

Reanalyzing data from (Figure 2) we confirm CD163 molecule was specifically expressed by TAMs, where 100% of them express CD45^{+} and about 90% were also defined as CD14^{+}BDCA1^{neg} (Figure 6). Considering it, we integrated a routine analysis in the laboratory to evaluate TAMs and T cells simultaneously in tumor samples.

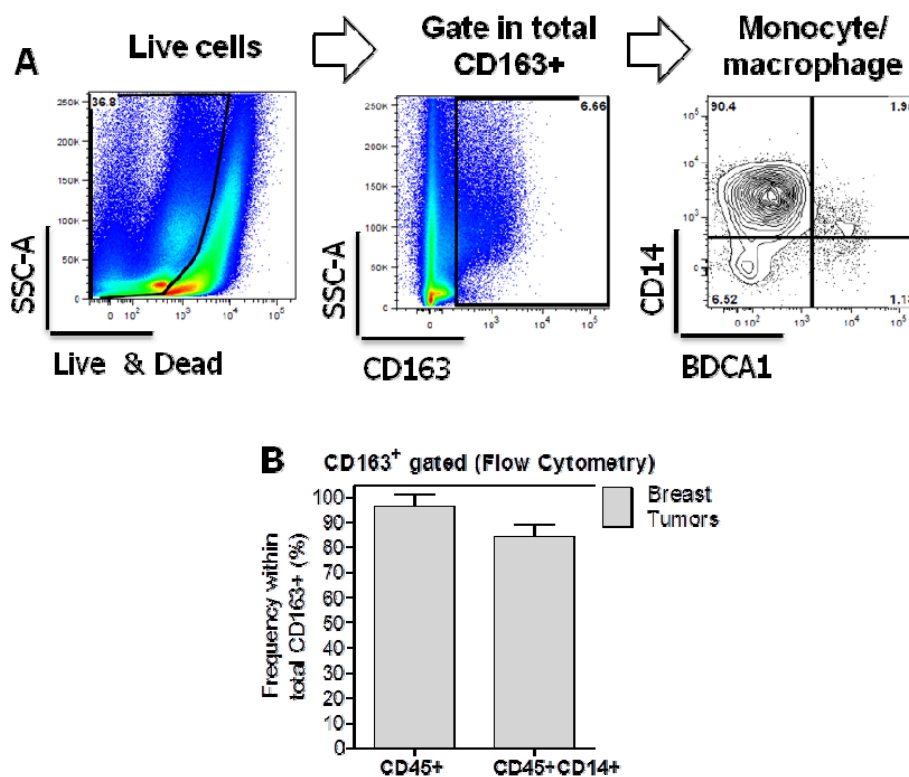


Figure 6. CD163 marker is exclusively expressed by monocytes/macrophages in tumors. Breast tumors samples already analyzed in Fig. 2 were re-analyzed considering firstly viable CD163+ cells and, subsequently, the expression of CD14 and BDCA1, as shown in A. In B, graphic of CD45^{+} and CD45^{+}CD14^{+}BDCA1^{neg} frequencies within total viable CD163^{+} cells.

In a bigger cohort of patients, breast and ovarian tumors were evaluated bu flow cytometry as following: gate in viable CD45^{+} cells, then gate on CD14^{+} TAMs and analysis of CD163^{Low} versus CD163^{High} frequency. Concomitantly, CD3 expression was evaluated in the same samples, and the correlation between TAMs

and T lymphocytes infiltration was investigated. Our data revealed that $CD45^+$ leukocytes represent about 31% (± 28.3% SD) of all viable cells found in breast tumors (Figure 7A) encompassing 52% (±18.96% SD) of $CD3^+$ T lymphocytes and 22% (±16.06% SD) of $CD14^+$ TAMs (Figure 7B). Considering TAMs subsets, $CD163^{Low}$ TAMs represent around 15% (±12.6% SD), while $CD163^{High}$ TAMs correspond approximately to 7% (±6.8% SD) of all viable $CD45^+$ leukocytes (Figure 7B).

For ovarian cancer, despite a lower number of analyzed samples, the composition of immune infiltrate was quite different. In these samples, $CD45^+$ leukocytes represent about 21% (± 19.6% SD) of all viable cells (Figure 8A) encompassing 33% (±20.63% SD) of $CD3^+$ T lymphocytes and 26% (±15.4% SD) of $CD14^+$ TAMs (Figure 8B). Considering TAMs subsets, $CD163^{Low}$ TAMs represent around 14% (±8.7% SD), while $CD163^{High}$ TAMs correspond approximately to 11% (±12% SD) of all viable $CD45^+$ leukocytes (Figure 8B). For both group of patients, it is remarkable to underline the heterogeneity among immune populations of interest, suggesting that additional points are needed for the global comprehension.

Therefore, we investigated whether the presence of TAMs could be correlated to the presence of other immune cells in tumors. Our findings showed a tendency between the presence of $CD14^+$ or $CD163^{High}$ TAMs and a decrease in $CD45^+$ infiltration for breast (Figure 7C) but not for ovarian cancer (Figure 8C). More interestingly, the increased frequency of total $CD14^+$ TAMs in breast tumors was negatively correlated with $CD3^+$ T cells infiltration, however with a stronger coefficient (r^2) when correlated with $CD163^{High}$ TAMs subset (Figure 7D). These data suggest $CD163^{High}$ TAMs can be potent inhibitors of T lymphocytes in breast cancer tissues. However, no correlation was observed in ovarian cancer patients, but more samples are needed to confirm this result (Figure 8D).

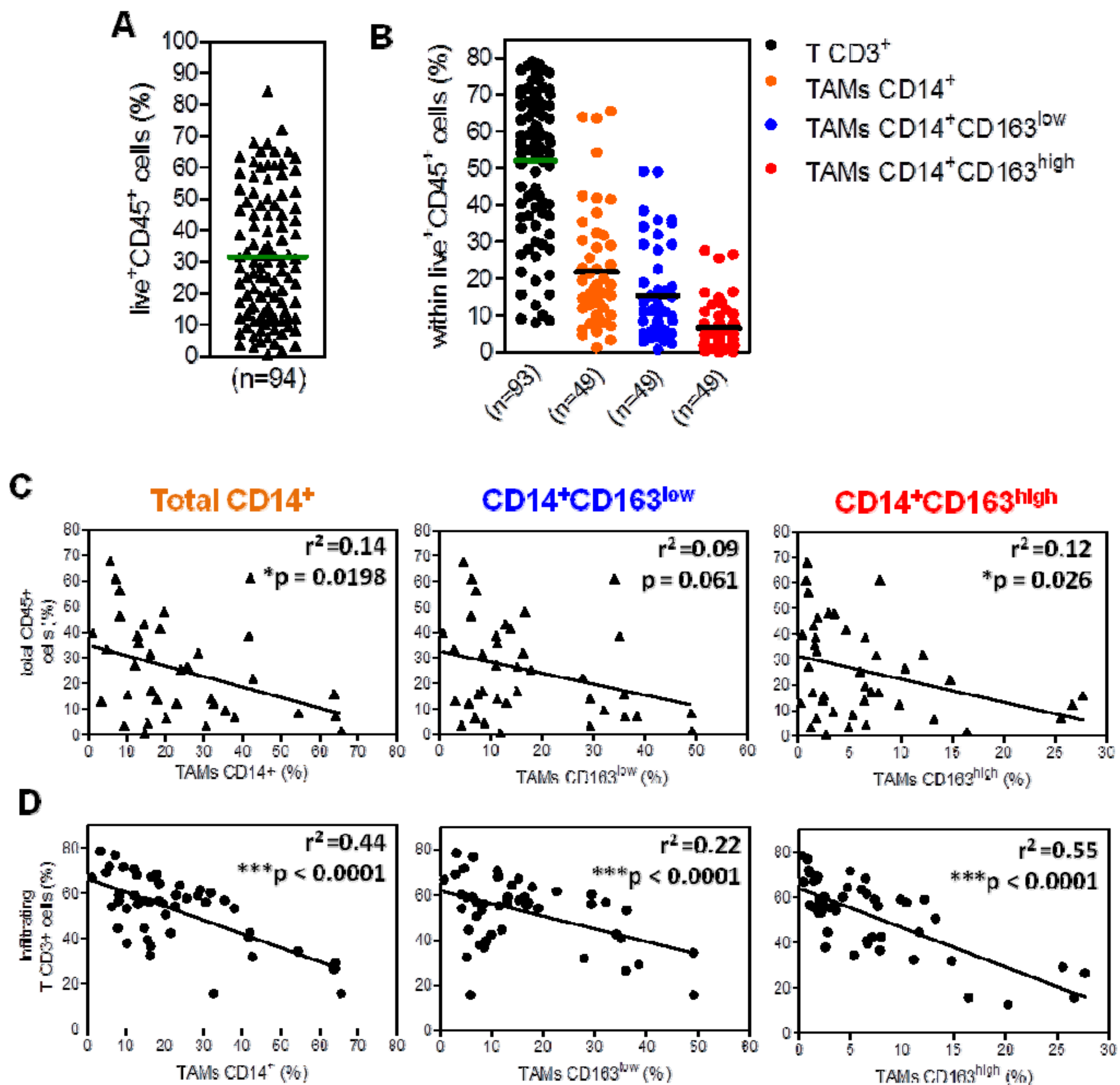


Figure 7. Increased proportion of $CD163^{high}$ TAMs is correlated to low $CD3^{+}$ T lymphocyte infiltration in breast tumors. After mechanical and enzymatic disaggregation of breast tumor tissues, immune cells were obtained and analyzed by flow cytometry. Graphics are showing the frequency of total viable $CD45^{+}$ cells (A); and of $CD3^{+}$ T cells, $CD14^{+}$ TAMs, $CD163^{low}$ and $CD163^{high}$ TAM subsets within viable $CD45^{+}$ leukocytes (B). Correlation of total $CD45^{+}$ cells (C) and $CD3^{+}$ T lymphocytes (D) with $CD14^{+}$ TAMs, $CD163^{low}$ and $CD163^{high}$ TAM subsets are shown (n=49).

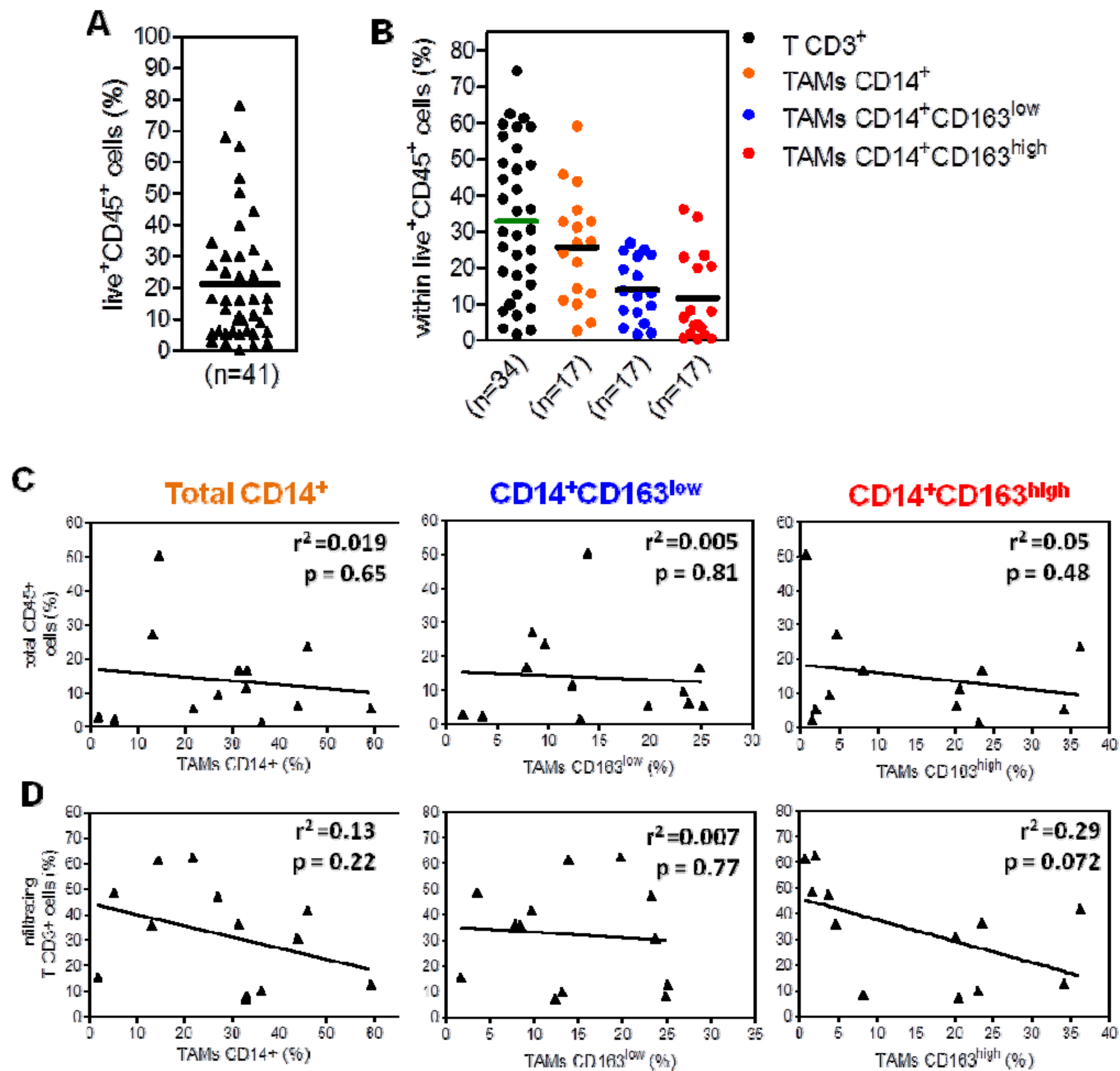


Figure 8. TAMs and T lymphocytes are heterogeneously distributed in ovarian tumors, but no correlation was found among them. After mechanical and enzymatic disaggregation of ovarian tumor tissues, immune cells were obtained and analyzed by flow cytometry. Graphics showing the frequency of total viable $CD45^+$ cells (A); and $CD3^+$ T cells, $CD14^+$ TAMs, $CD163^{low}$ and $CD163^{high}$ subsets within viable $CD45^+$ leukocytes (B). Correlation of total $CD45^+$ cells (C) and $CD3^+$ T lymphocytes (D) with $CD14^+$ TAMs, $CD163^{low}$ and $CD163^{high}$ TAM subsets are shown (n=13).

Considering the importance of CD163 molecule as a marker for TAM subsets, even heterogeneously distributed in breast cancer patients, we investigated whether $CD163^+$ TAMs could have an impact in patients' survival. For that, the expression of CD163 was investigated by immunohistochemistry (IHC) in 238 paraffin-embedded breast cancer samples in a retrospective analysis with 12.5 years of follow-up. In collaboration with the Biopathology department of the *Centre Léon Bérard Hospital*, the IHC analyses were performed considering the immune-infiltration areas and revealed two distinct profiles of CD163 frequency: CD163low= level 0 (Figure 9A) and CD163high= level 1-2 (Figure 9B). Progression Free-survival analysis indicated that patients presenting a high infiltrate of $CD163^+$ TAMs showed a higher risk of relapse when compared to patients presenting low proportion of $CD163^+$ TAMs (Figure 9C). Interestingly, considering the molecular classification (PEROU et al., 2000) of breast tumors, Luminal B, Her2+, and Triple-negative (or basal-like), the most aggressive subtypes, presented high infiltration of $CD163^+$ TAMs (Figure 9D). Added, the same IHC analysis was done for the same cohort of patients in the tumor bed sides, but no statistical correlation with survival was found (data not shown).

Together, our findings suggest a central role of $CD163^+$ TAMs in the modulation of tumor development, since the accumulation of these cells may results in the inhibition of T cell infiltration and/or activation, which probably has a deleterious impact on breast cancer patients' survival.

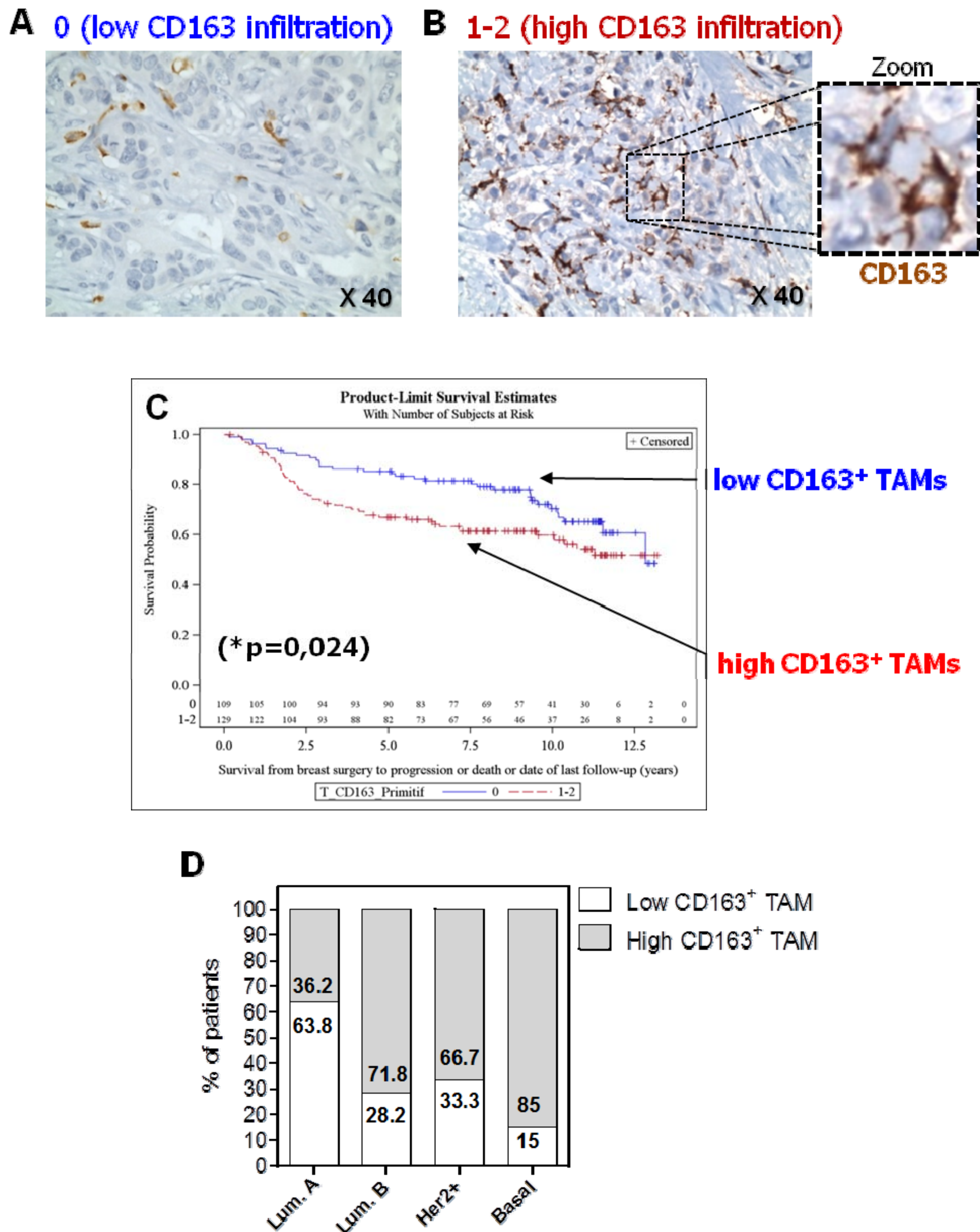


Figure 9. High infiltration with CD163^{+} TAMs is correlated to high risk of relapse in breast cancer patients. Paraffin-embedded breast cancer samples were submitted to immunohistochemistry (IHC) staining against CD163 molecule. (A-B) Representative IHC images considering low and high CD163 infiltration, respectively. In C, Progression Free Survival analysis (PFS) considering CD163 expression in the immune-infiltrating area of breast cancer patients (log-rank analysis *p<0.05, n=238). In D, analysis of CD163 expression according to the different molecular profiles of breast cancers.

4.2 Tumor microenvironment effects on human monocyte differentiation *in vitro*

Accumulated studies in literature have described that blood monocytes are the major progenitors of tissue macrophages and certain subsets of DCs. Considering the important role of TAMs in the modulation of immune system and their involvement in tumor development described above, we went further to investigate, how the tumor microenvironment could modulate the differentiation of monocytes. More specifically, we investigated here the phenotype and the functional skills of monocytes exposed to primary tumor supernatants.

4.2.1 Primary Tumor microenvironment can skew monocytes into CD163^{high}PD-L1^{high}IL-10^{high} phenotype

Taking into consideration that breast and ovarian cancers are highly infiltrated by TAMs, we hypothesized whether the tumor microenvironment may induce a spontaneous differentiation of monocytes *in vitro*. For that, CD14^{+} blood monocytes from healthy donors were isolated by magnetic beads and cultivated for 7 days in the presence of 25% of supernatants from dilacerated primary tumors (SNDil-MΦ) as described in methods. As positive controls of differentiation, CD14^{+} blood monocytes were submitted to well-defined conditions to obtain M1-MΦ (GM-CSF+IFN-γ) and M2-MΦ (M-CSF+IL-4) and Mo-DCs (GM-CSF+IL-4). Additionally, as a basal control of differentiation, monocytes were cultivated just in the presence of cRPMI medium (M0-MΦ). At day 6, LPS (100ng/ml) was added to all cultures and, 24hrs later, the expression of surface molecules and cytokine production were investigated. Moreover, for the first group of experiments, supernatants from tumor cell lines or from cultured tumors (SNTum) were used at 25% as a comparative for the tumor effects.

Representative images from cells were obtained from plates or after Cytospin technique at the last day of cultures (Figures 10 and 11). We observed that monocytes cultivated with SNDil were quite similar to M0-MΦ and M2-MΦ, but did not assume a big size as M1-MΦ (Figure 10). In fact, the density of cells in M0-MΦ and SNDil-MΦ was quite similar, but differed from positive controls of differentiation. By cytospin assays, we observed an interesting difference in size and cytoplasm morphology among cultured cells. M1-MΦ presented big nuclei and cytoplasm area

with few vacuoles, whereas SN-Dil-MΦ, displaying similar size, presented reduced nuclei dimension due to the high numbers of cytoplasmic vacuoles/granules (Figure 11). Even presenting similarities in the H&E staining and nuclei, M2-MΦ and SNDil-MΦ differed in cell size and content of cytoplasm (Figure 11). However, further experiments are needed to better characterize those morphologic differences and the identification of intracellular contents.

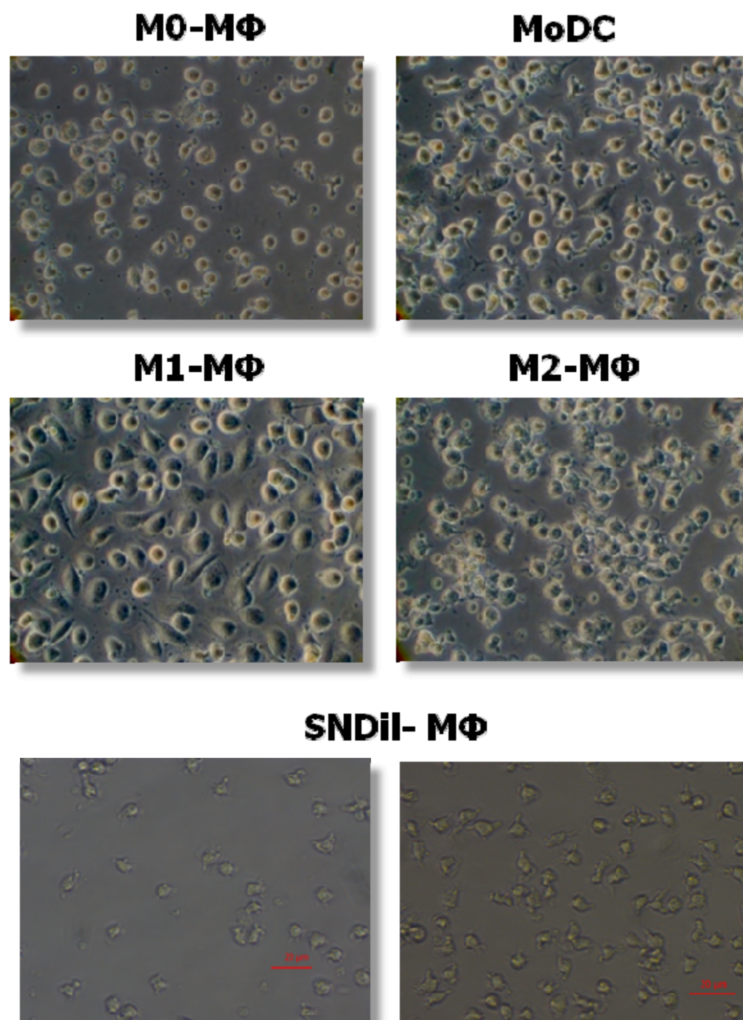


Figure 10. Representative images from cultures of differentiated APCs *in vitro.* CD14+ blood monocytes were differentiated *in vitro* in well-defined conditions (cRPMI (M0-MΦ); GM-CSF+IL-4 (Mo-DC); GM-CSF+IFN-γ (M1-MΦ), M-CSF+IL-4 (M2-MΦ)) or conditioned by supernatants from dilacerated primary tumors (SNDil-MΦ) during 7 days (LPS was added at day 6 in all conditions).

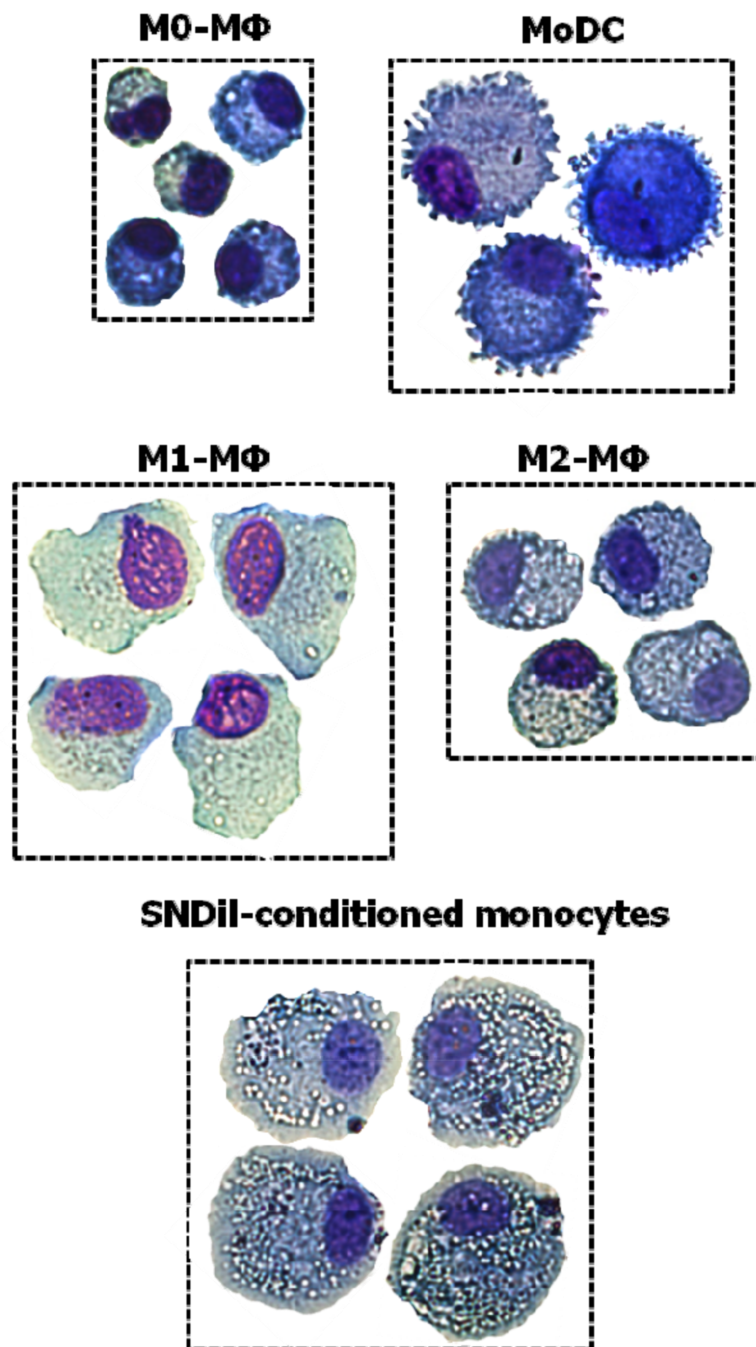


Figure 11. Representative images of Cytospin from differentiated APCs *in vitro.* CD14+ blood monocytes were differentiated *in vitro* in well-defined conditions (cRPMI (M0-MΦ); GM-CSF+IL-4 (Mo-DC); GM-CSF+IFN-γ (M1-MΦ), M-CSF+IL-4 (M2-MΦ)) or conditioned by supernatants from dilacerated primary tumor (SNDil-MΦ) during 7 days(LPS was added for the last 24hs) and then were analyzed by microscopy after Giemsa/May-Gruenwald staining.

Thus, we performed flow cytometry analysis to better characterize the cells we differentiated in all conditions, as shown in gating strategy presented in figure 12 as following: a first gating based on size versus granulosity and exclusion of doublets (I), Live&Dead low viable cells were gated (II) and, among them double CD11b^{+}HLADR^{+} cells (III) (Figure 12). Subsequently, APCs from all culture conditions were evaluated for CD14 versus BDCA1 expression and CD64 versus CD163 expression (Figures 13 and 14). We observed that all SN-Dil and SN-Tum conditioned monocytes presented a CD14^{+}BDCA1^{neg} phenotype, similarly to M0, M1, and M2 macrophages (Figure 13) and did not show a phenotype like Mo-DCs that lost CD14 and increased BDCA1 expression.

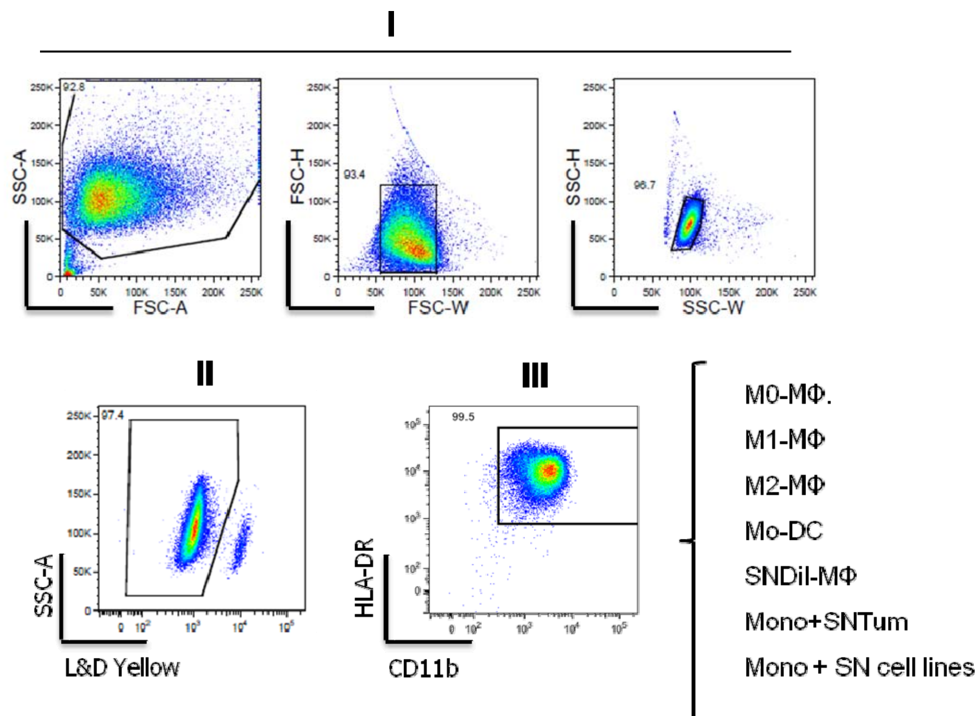


Figure 12. Representative flow cytometry strategy to characterize *in vitro* differentiated APCs. CD14^{+} blood monocytes were differentiated *in vitro* in well-defined conditions or in the presence of 25% supernatants from dilacerated primary tumors for 7 days. Cells were activated by LPS for the last 24hrs, were removed from the plates and submitted to staining with specific antibodies. Here we show the sequence of gate analysis: I) Primary gate and exclusion of doublets; II) gate in live cells within Live and Dead low; III) gate in total CD11b^{+}HLADR^{+} myeloid cells. This strategy was used to analyze all *in vitro* differentiated cells.

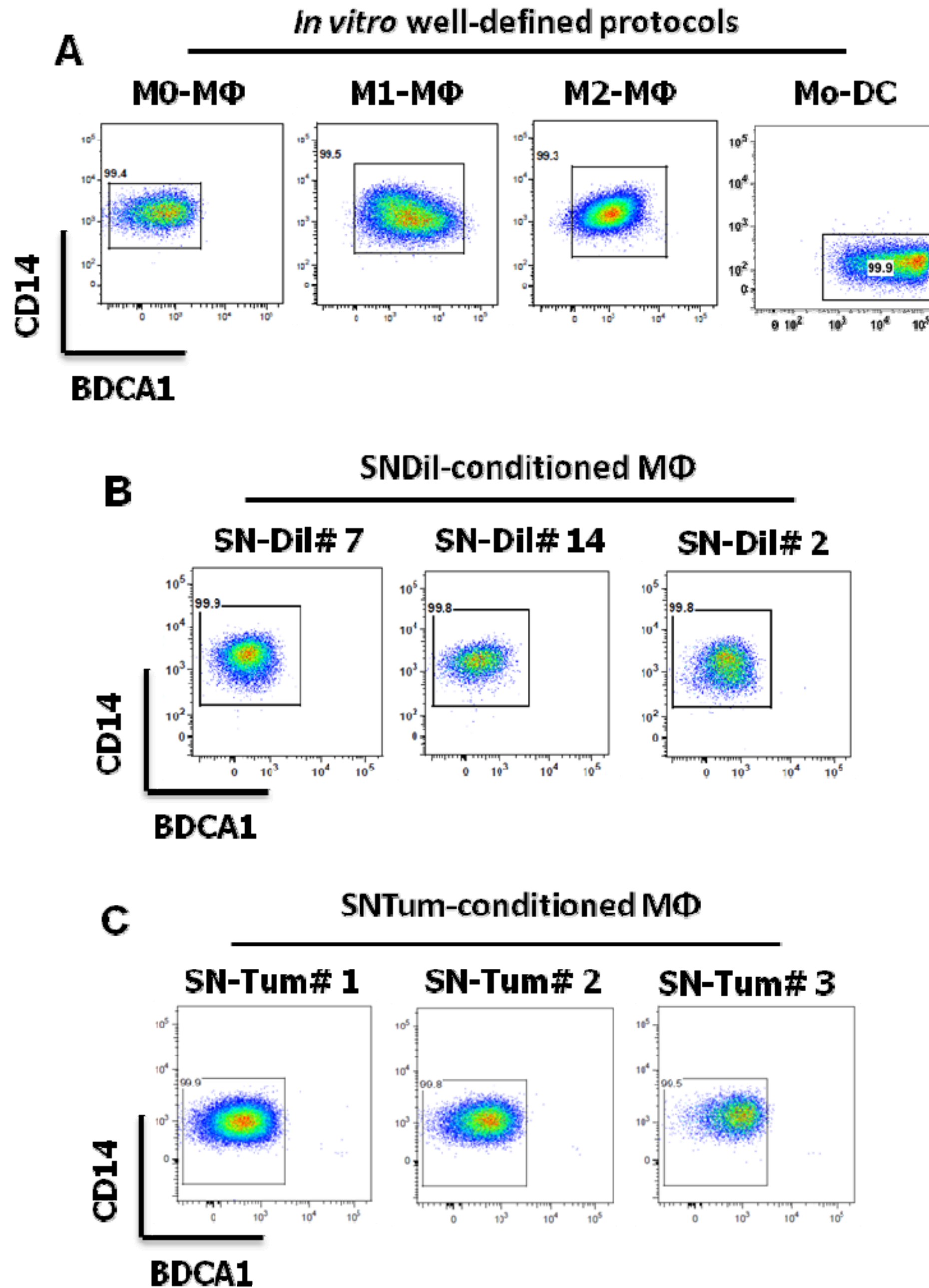


Figure 13. Tumor-conditioned monocytes display a $CD14^{+}BDCA1^{neg}$ phenotype after 7 days of *in vitro* culture. Healthy $CD14^{+}$ blood monocytes were differentiated *in vitro* in well-defined conditions or in the presence of 25% of supernatants primary tumor by 7 days. Cells were activated by LPS for the last 24hrs, were removed from the plates and submitted to staining with specific antibodies. Representative pseudo-plots in gated $CD11b^{+}HLADR^{+}$ cells showing CD14 vs. BDCA1 staining from well-defined APCs (A), SNDil-conditioned MΦ (B) and SNTum-conditioned MΦ (C).

Further, we next investigated the expression of CD64 and CD163 molecules in the $CD14^{+}BDCA1^{neg}$ or $CD14^{neg}BDCA1^{+}$ gated cells (the last for Mo-DCs), and we found SNDil-MΦ showed heterogeneous frequency of $CD64^{+}CD163^{+}$ cells, as observed for M0-MΦ (Figures 14 and 15A). Interestingly, SNTum-MΦ, M2-MΦ, and monocytes conditioned by supernatants from breast tumor cell lines (SKBR-3, BT474, CAL51 or MCF-7) showed a more homogeneous frequency of $CD64^{+}CD163^{+}$ cells. Contrastingly, M1-MΦ lost CD163 expression, whereas Mo-DCs lost both CD64 and CD163 markers after differentiation (Figures 14A and 15A). These data strongly suggested both SN-Dil and SN-Tum conditioned MΦ are not acquiring a Mo-DC neither a M1-MΦ phenotype, because they maintained CD14 and CD64 expression and presented high frequency of $CD163^{+}$ cells.

Moreover, even the frequency of $CD163^{+}$ cells was comparable among SNDil-MΦ, SNTum-MΦ, M0-MΦ and M2-MΦ, we noted that the Median Intensity of Fluorescence (MFI) to CD163 was variable. To investigate whether the supernatants from tumor microenvironment may induce CD163 levels similarly to M0-MΦ or M2-MΦ, we evaluated the relative-CD163 MFI (r-CD163), calculated as (CD163 MFI samples/CD163 MFI M0-MΦ for each group of experiment). As expected, M1-MΦ and Mo-DCs showed very low r-CD163 values, contrasting with M2-MΦ that strongly up-regulated r-CD163 in comparison to M0-MΦ (Figure 15B). Interestingly, SNDil-MΦ showed a heterogeneous distribution of r-CD163 MFI with a large range of values, but, in the other hand, SNTum-MΦ showed a homogeneous intensity of r-CD163 MFI with values close to control M0-MΦ (Figure 15B). Additionally, supernatants from breast tumor cell lines also acted differently in monocytes, inducing heterogeneous expression of r-CD163 MFI depending on the cell line used (Figures 15B).

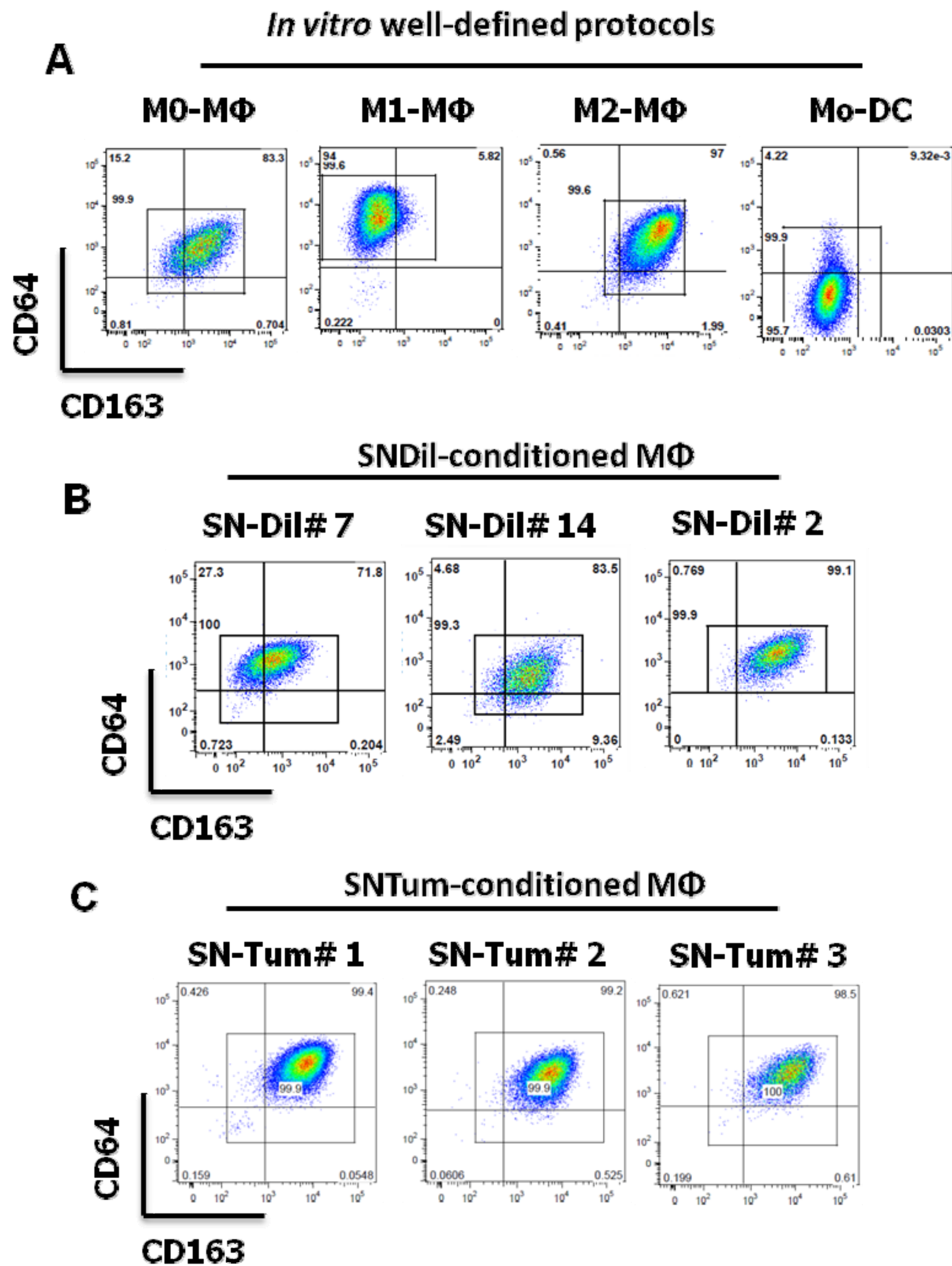


Figure 14. Tumor conditioned-monocytes express differential levels of CD163 7 days after *in vitro* culture. $CD14^{+}$ blood monocytes were differentiated *in vitro* in well-defined conditions or in the presence of 25% of primary tumor supernatant by 7 days. Cells were activated by LPS for the last 24hrs, were removed from the plates and submitted to staining with specific antibodies. Representative pseudo-color plots showing CD64 vs. CD163 staining within gated $CD14^{+}BDCA1^{neg}$ for M0-MΦ, M1-MΦ, M2-MΦ and SNDil-MΦ and SNTum-MΦ; or within $CD14^{neg}BDCA1^{+}$ for Mo-DCs. In A, B, and C, CD64 vs. CD163 expression for well-defined APCs, SNDil-MΦ and SNTum-MΦ, respectively.

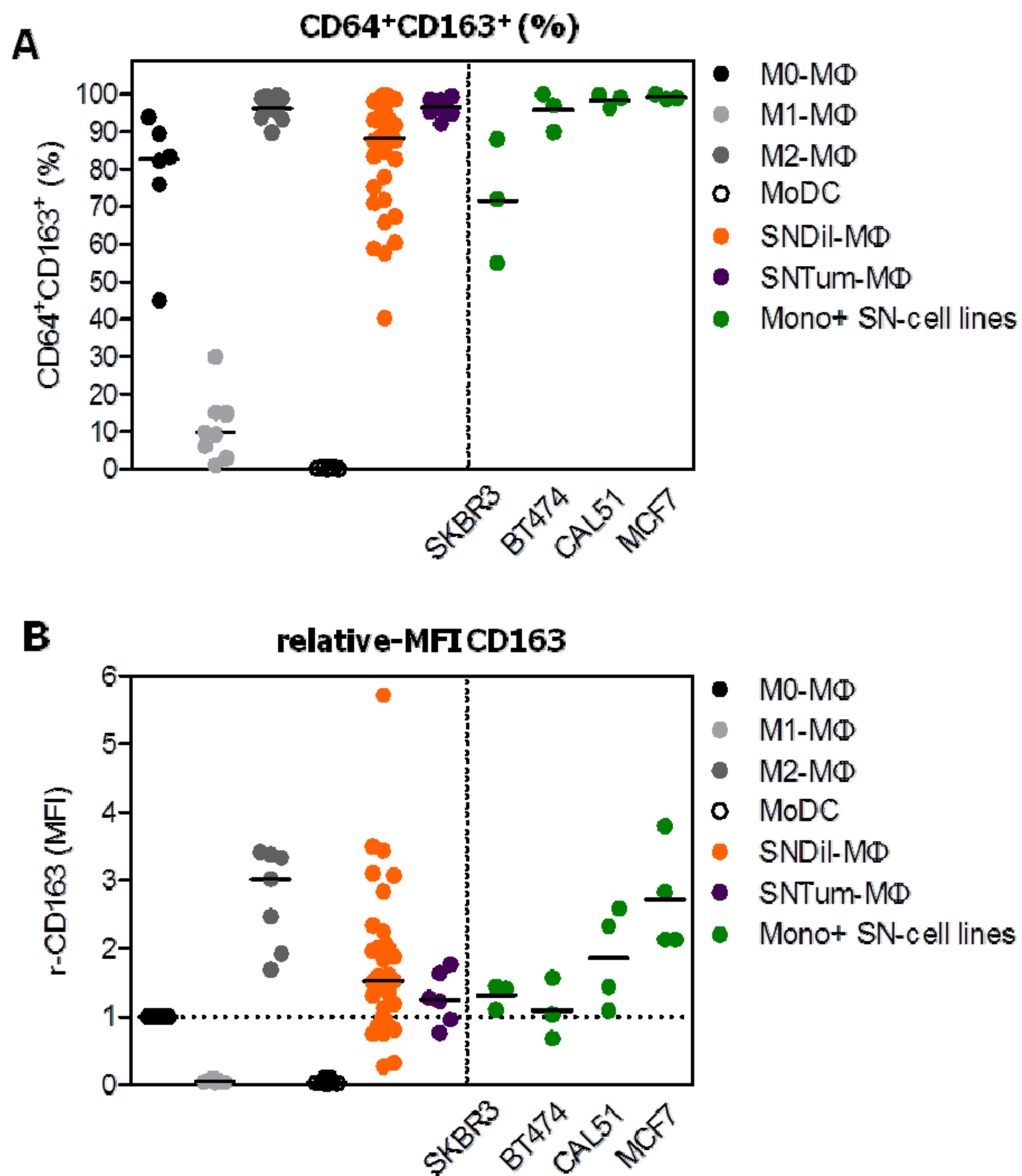


Figure 15. SNDil-conditioned MΦ present a very heterogeneous levels of CD163 expression. CD14⁺ monocytes were isolated from healthy donors and cultivated in well-defined condition or in the presence of 25% of supernatants from dilacerated primary tumors (SNDil); cultured tumors' medium (SNTum) or tumor cells line supernatants by 7 days (LPS addition by the last 24hs). Then cells were submitted to staining and analyzed by flow cytometry. The median of $CD64^{+}CD163^{+}$ frequency (A) and median of relative-CD163 MFI (B) were analyzed within $CD14^{+}BDCA1^{neg}$ gates in all groups, except MoDC, analyzed within $CD14^{neg}BDCA1^{+}$ gate. (M0-MΦ black dots; M1-MΦ light grey dots; M2-MΦ dark grey dots; MoDC white dots; SNDil-MΦ orange dots; SNTum-MΦ purple dots; Mono-SN-cell lines green dots). The relative-CD163 MFI (r-CD163) was calculated considering M0-MΦ from each group of experiment as the reference (value= 1), and all other r-CD163 MFI values were calculated as CD163 MFI samples/CD163 MFI M0-MΦ.

Collectively, the analysis of CD163 levels (frequency and MFI) on SNDil-MΦ generated an interesting variability among all tested samples (Table 1) that could be compared to the heterogeneity found for CD163 expression on TAMs isolated from breast tumors (Figures 1 to 9). Indeed, SN-Dil generation may represent the most confident "microenvironment picture", reproducing the complex interactions between tumor cells, stromal cells, and infiltrating immune cells. For these reasons, we decided to focus on the effects of SN-Dil on monocytes differentiation, investigating other phenotypic and functional characteristics acquired by these cells.

For APCs differentiated in well-defined conditions, M2-MΦ showed high r-CD163 MFI expression; whereas M1-MΦ and Mo-DCs showed significantly lower r-CD163 MFI compared to M0-MΦ. Importantly, some of the tested SN-Dils induced a remarkable up-regulation of CD163 levels on conditioned MΦ, while other SN-Dils induce similar levels to control M0-MΦ (Figure 16A). The diversity of CD163 expression allowed us to classify SNDil-MΦ in two different sub-groups: I) SNDil-MΦ that presented high CD163 levels, similarly to M2-MΦ, when compared to M0-MΦ (called SNDil-MΦ $CD163^{high}$); and II) MΦ that did not up-regulate CD163 and expressed CD163 at lower levels than M2-MΦ (called SNDil-MΦ $CD163^{low}$) (Figures 16B-C). Interestingly, about 50% of all tested SN-Dils induce a SNDil-MΦ $CD163^{high}$ phenotype, which does not seem to be due to the monocyte donor' variability (Figure 16C). Indeed, signals derived from tumor microenvironment can modulate CD163 expression in conditioned-monocytes, a phenomenon possibly occurring in monocytes that arrive in tumor tissues and became $CD163^{+/high}$TAMs.

Table 1 – List of breast cancer patients and SNDils

Tumor	SNDil	Cod. Pat	Age at surgery	Morphological classification	Molecular classification
#1	not	8801790	44	IDC	Her2+
#2	#2	135297	NA	IDC	Luminal A
#3	#3	1213598	64	IDC	Luminal A
#4	not	137463	71	**not available**	**not available**
#5	#5	1311244	79	ILC	Luminal A
#6	not	1315245	47	IDC	Luminal A
#7	#7	1209809	92	IDC	triple-negative
#8	#8	1304700	52	IDC	Triple-negative
#9	#9	1315353	45	IDC	Luminal A
#10	#10	1318307	60	ILC	Luminal A
#11	not	1315984	78	ILC	Luminal A
#12	not	1321472	81	infiltrating adenocarcin.	Triple-negative
#13	not	1317148	52	IDC	Triple-negative
#14	#14	1201592	88	ILC	Triple-negative
#15	#15	401813	50	IDC	Lum. A or Triple-neg.
#16	#16	511520	60	IDC	Luminal A
#17	#17	1201917	17	fibroadenom	**not available**
#18	#18	1203551	77	IDC	Luminal A
#19	#19	1205207	47	ILC	Luminal A
#20	#20	1209677	33	IDC	Triple-negative
#21	#21	1302499	50	ILC	Luminal A
#22	#22	1301226	54	ILC	Luminal A
#23	#23	1307390	86	IDC	Lum. A or Triple-neg.
#24	#24	1303247	84	IDC	Luminal A
#25	#25	905488	52	IDC	Triple-negative
#26	#26	1211398	38	IDC	Luminal B
#27	#27	1317690	80	IDC	Triple-neg or Her2+
#28	#28	9309854	54	IDC	Luminal A
#29	#29	1311244	79	ILC	Luminal A
#30	#30	1310259	50	ILC	Luminal A
#31	#31	1301852	74	Metaplastic infiltrating	Triple-negative
#32	#32	1314838	79	IDC	Luminal A
#33	#33	1317184	69	ILC	Luminal A
#34	#34	105901	45	IDC	Luminal B
#35	#35	1312607	59	IDC	Triple-negative
#36	#36	1214542	54	Mucinous	Luminal A

IDC= Invasive Ductal Carcinoma; ILC= Invasive Lobular Carcinoma.

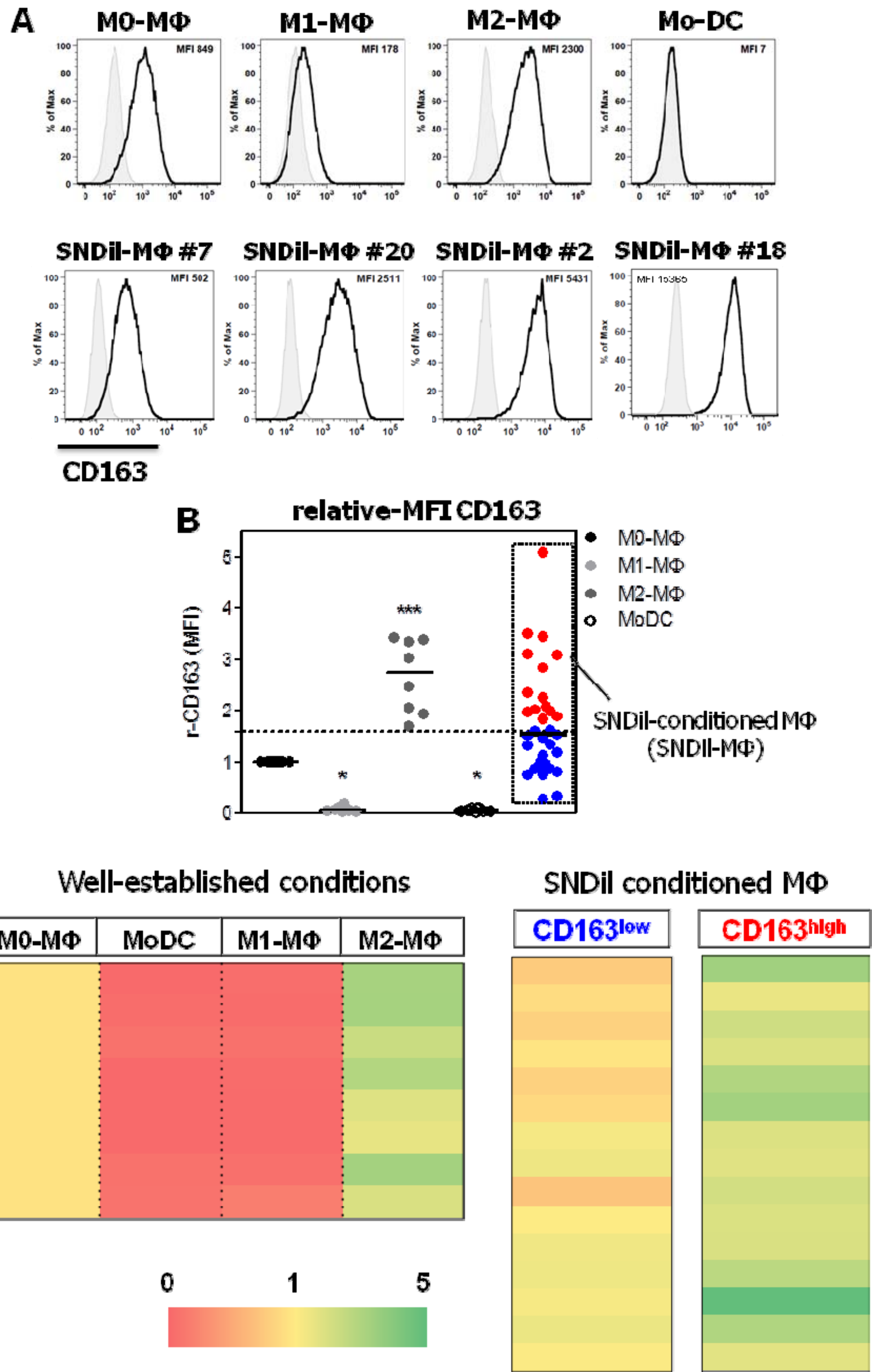


Figure 16. SNDils induced diverse levels of CD163 in conditioned-MΦ. $CD14^+$ monocytes were isolated from healthy donors and cultivated in well-defined conditions or in the presence of 25% of supernatants from dilacerated primary tumors (SN-Dil) by 7 days and activated by LPS for the last 24hrs. Then, cells were submitted to staining with specific antibodies and analyzed by flow cytometry. In A, representative histograms of CD163 levels for all groups (in grey control isotype). B and C, graphic and detailed heat-map respectively, showing the relative-CD163 MFI expression to define conditioned monocytes as $CD163^{low}$ and $CD163^{high}$ subtypes (*p<0.05, ***p<0.0001).

To further characterize the effects of tumor microenvironment on monocyte differentiation and function, we investigated the expression of some surface molecules and the production of cytokines by SNDil-MΦ and differentiated APCs. The molecules HLA-DR, CD80, CD86, PD-L1 and PD-L2 were analyzed within the $CD14^{+}BDCA1^{neg}CD64^{+}$ gated cells, except for MoDC, analyzed in $CD14^{neg}BDCA1^{+}CD64^{neg}$ gate.

Both SNDil-MΦ $CD163^{low}$ and $CD163^{high}$ showed reduced MFI of CD86 molecule when compared to M0-MΦ, while M1-MΦ and Mo-DCs significantly expressed high levels of CD86 (Figure 17). For PD-L1 MFI, we observed a heterogeneous expression in the SNDil-MΦ $CD163^{high}$ sub-population, but a significantly higher expression by M2-MΦ compared to M0-MΦ (Figure 17). The analysis of HLA-DR, CD80, and PD-L2 MFI revealed no significant differences of expression among control APCs and SNDil-MΦ (data not shown). Investigating the production of cytokines, TNF-alpha production was very heterogeneous, with a little tendency of increase in both SNDil-MΦ subpopulations (Figure 17), while M1-MΦ and Mo-DCs produced higher levels than M0-MΦ. For IL-10, SNDil-MΦ $CD163^{high}$ produced higher levels when compared to SNDil-MΦ $CD163^{low}$ group, but only M2-MΦ produced significantly more than M0-MΦ (Figure 17). Regarding VEGF-A production, only M2-MΦ produced significantly higher levels than all other APCs (not shown). Moreover, we also verified other cytokines in supernatants, like: CXCL9, CCL22, CCL3, CCL5 and IL12-p70, but a strong variability among the different monocyte donors' was found (data not shown). These data confirmed the polarization of in *vitro* differentiated macrophages, defined by surface expression and cytokine production: M1-MΦ as $CD163^{low}CD86^{high}TNF\text{-}alpha^{high}IL\text{-}10^{low}$ versus M2-MΦ as $CD163^{high}PD\text{-}L1^{high}VEGF^{high}IL\text{-}10^{high}$. Altogether, the comparison of SNDil-MΦ subgroups revealed that the levels of CD163 and IL-10 may be useful to distinguish them, but functional experiments were used for a better characterization.

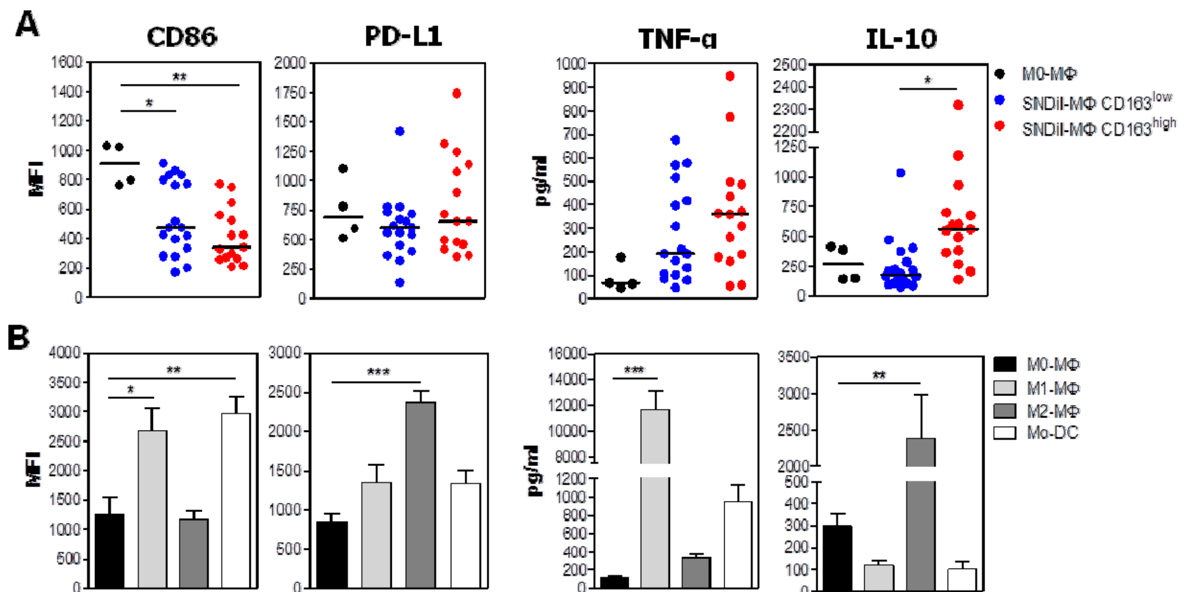


Figure 17. CD163^{high} SNDil-MΦ showed reduced levels of CD86 and produced high amounts of IL-10. CD14^{+} monocytes were isolated from healthy donors and cultivated in well-defined conditions or in the presence of 25% of primary dilacerated tumors (SNDil) by 7 days with LPS stimulation for the last 24hrs. Cells were submitted to flow cytometry analysis for surface molecules while supernatant were collected and cytokines were evaluated by ELISA. CD86 and PD-L1 MFI, and TNF-alpha and IL-10 levels were evaluated in SNDil-MΦ (A) and in control differentiated APCs (B). CD163^{low} (blue dots) and CD163^{high} (red dots) SNDil-MΦ; M0-MΦ (black dots/bars); M1-MΦ (light grey bar, n=8); M2-MΦ (dark gray bar, n=8); Mo-DC (white bar, n=8); (*p<0.05, **p<0.01, ***p<0.0001).

Trying to explore other surface markers that might help to associate SNDil-MΦ subgroups with well-polarized APCs and to define M1-MΦ, we performed a flow cytometry screen (Legend-Screen - Biolegend). The analysis of about 330 surface molecules in LPS-activated control APCs (M0-MΦ, M1-MΦ, M2-MΦ and Mo-DC) revealed that 121 lymphocyte-markers were not expressed by none of these APCs (not shown) and that the 210 other markers were at least expressed by one cell type as represented by a heat-map in figure 18A. Even so, none of the tested molecules were exclusively expressed by M1-MΦ. Interestingly, we identified CD200R molecule as a great candidate to discriminate M0-MΦ from M1- and M2-MΦ or Mo-DCs (Figure 18B). In additional experiments, we confirmed that CD200R was significantly up-regulated in M1-MΦ, M2-MΦ and Mo-DCs when compared to M0-MΦ (Figures 19A-B). Interestingly, M0-MΦ and both SNDil-MΦ CD163^{low} and CD163^{high} showed similar expression of CD200R (Figures 19A and C).

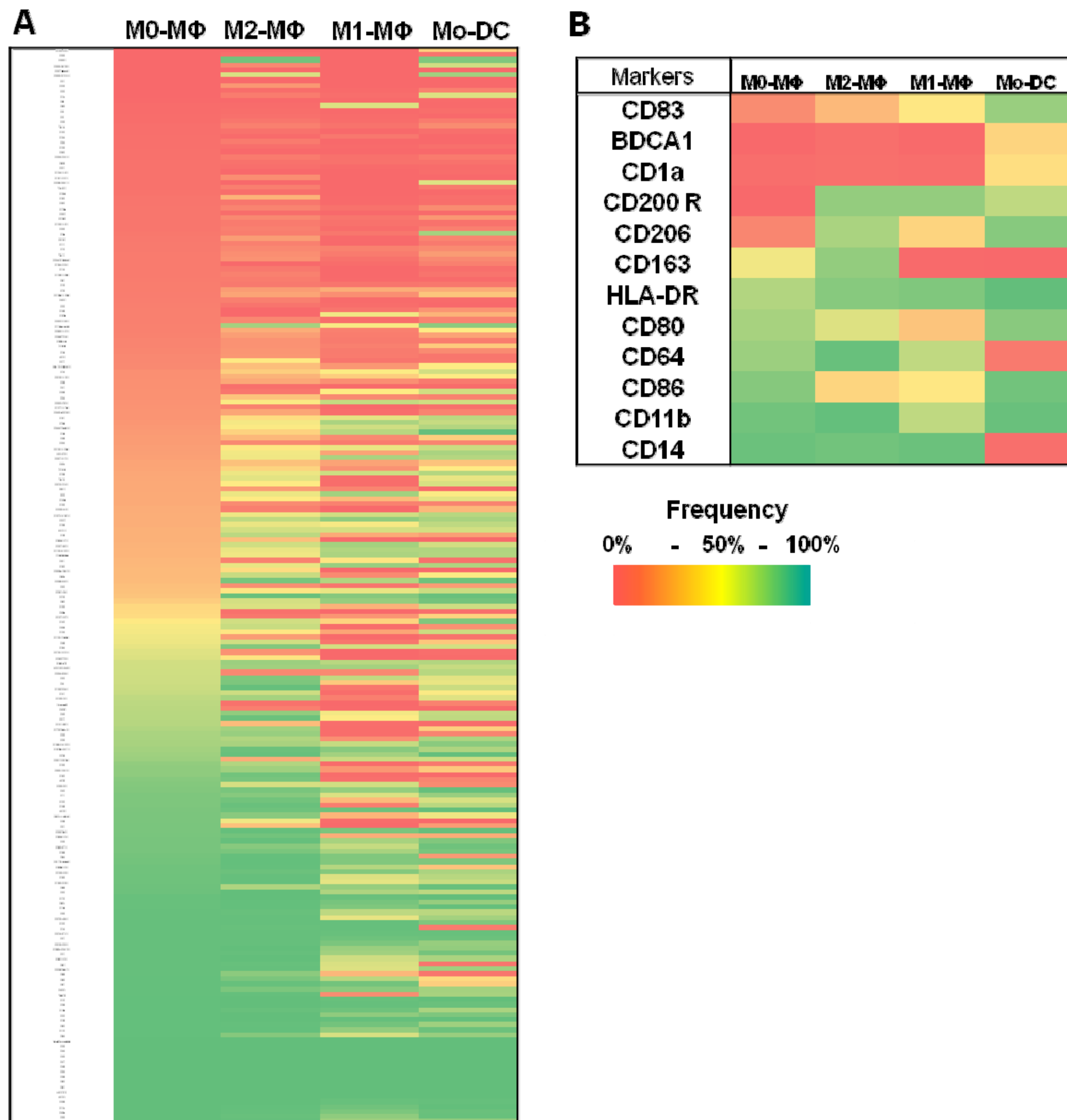


Figure 18. Flow Cytometry Screen analysis of cell surface markers on differentiated APCs. $CD14^+$ monocytes were differentiated with defined protocols *in vitro* by 7 days (methods). Later, using flow cytometry screen, we evaluated the surface expression of more than 330 molecules. In A, representative heat-map panel showing 210 markers at least expressed by one cell type. In B, heat map showing frequency of selected markers that may define the studied APCs.

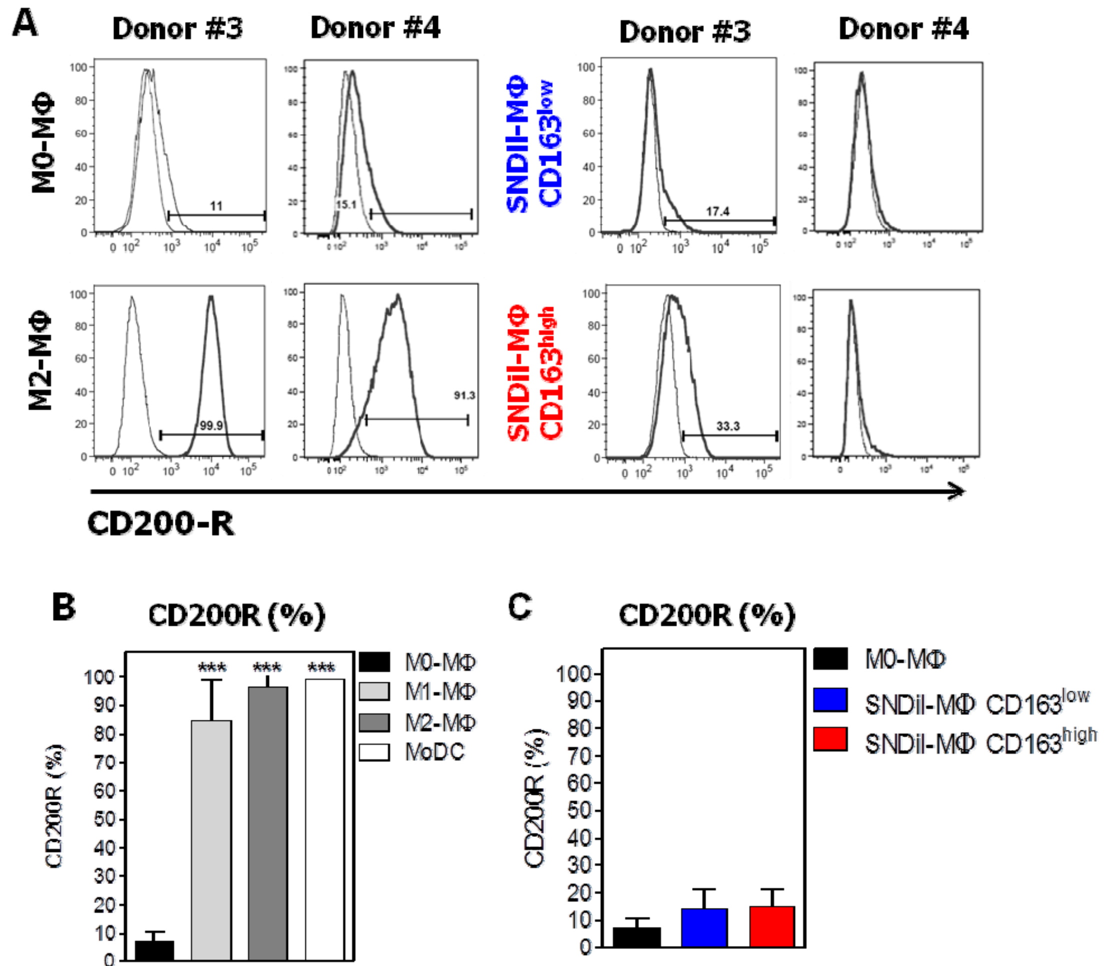


Figure 19. CD200R is up-regulated in differentiated APCs but not in M0-MΦ neither in SNDil-MΦ. CD14$^+$ monocytes were isolated from healthy donors and cultivated in well-defined conditions or in the presence of 25% of primary dilacerated tumors (SNDil) by 7 days with LPS stimulation by the last 24hrs. Cells were removed from the plates, submitted to CD200R staining and analyzed by flow cytometry. Representative histograms of two different donors (A) and graphics (B-C) showing CD200R expression in differentiated APCs and SNDil-MΦ. M0-MΦ (black bar, n=4); M1-MΦ (light grey bar, n=4); M2-MΦ (dark gray bar, n=4); Mo-DC (white bar, n=4); SNDil-MΦ CD163^{low} (blue bar, n=3) and SNDil-MΦ CD163^{high} (red bar, n=4); (dotted line = control isotype, black line= specific staining; ***p<0.0001 in relation to M0-MΦ).

Collectively, our findings suggest an interesting correlation between surface molecule and cytokine production, where cells that acquired a CD163^{high} phenotype also showed high production of IL-10. We noted that CD163^{high} SNDil-MΦ and M2-MΦ presented this feature after LPS activation (Figures 20A-B), suggesting that IL-10 may be responsible for CD163 up-regulation. To verify this hypothesis, M2-MΦ were differentiated as previously described (methods) in the presence of anti-IL-10R monoclonal antibodies that were added 30min before LPS stimulus. Interestingly, IL-10R blockage significantly inhibited the up-regulation of CD163 and PD-L1, but not of CD80 and CD86 (Figures 20C-D). Similar results were obtained when anti-IL-10R was added at day zero of differentiation (data not shown). These findings suggest that LPS activation may induce the increase of IL-10 production as a first step that subsequently, may act in an autocrine/paracrine way, up-regulating CD163 and PD-L1 molecules and conferring a final suppressive phenotype to M2-MΦ.

Altogether, our findings suggested that SNDil-conditioned MΦ that acquired a CD163^{high} phenotype, also may share additional skills as like “M2-phenotype”. IL-10 and CD163 up-regulation and CD86 down-regulation may represent the most important findings in relation to phenotype, but the functional abilities will be further explored.

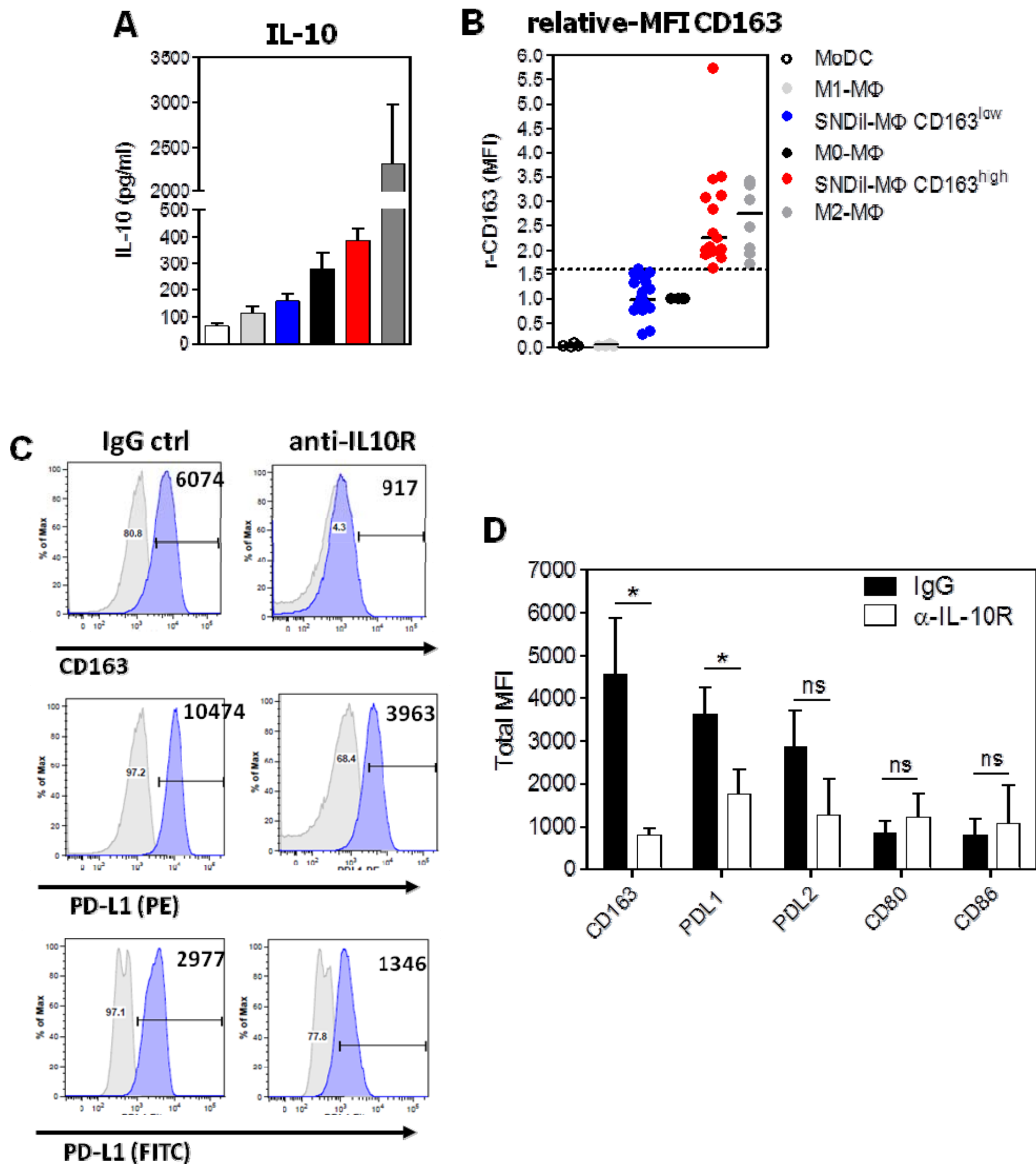


Figure 20. IL-10 production promotes autocrine/paracrine CD163 and PD-L1 up-regulation in M2-MΦ. $CD14^+$ monocytes were isolated from healthy donors and submitted to M2-MΦ differentiation. At day 6, blocking mAb anti-IL-10R were added 30 minutes before LPS activation and 24 hrs later cells were submitted to staining and flow cytometry analysis. In A and B, graphics showing the production of IL-10 and the expression of r-CD163 on SNDil-MΦ and APCs. In C, representative histograms showing CD163 and PD-L1 MFI in M2-MΦ surface in the presence of control IgG (left) or blocking anti-IL-10R (right) (control isotype histograms in grey and specific antibody staining in blue; values of MFI were obtained by subtraction from IgG background). In D, graphic showing all analyzed surface molecules (n=3; *p<0.05).

4.2.3 CD163^{high} SNDil-MΦ have suppressive abilities through IL-10/PD-L1 dependent mechanisms

To investigate whether the phenotypic differences described above may have an impact in the functional ability of SNDil-MΦ, we co-cultivated those cells or control APCs with allogeneic naïve CD4^{+} T cells for 5 days to evaluate their capacity to induce T cell proliferation. Preliminary experiments using control APCs cocultured at different ratios with T lymphocytes (APC:T= 1:10; 1:20; 1:40) identified the ratio 1:10 as the best (Figure 21A), and was chosen for the next experiments.

Concerning control APCs, Mo-DCs and M1-MΦ induced strong naïve T cell proliferation (65% (±4.3% SEM) and 43% (±7.7% SEM), respectively) (Figures 21A-B). Contrastingly, M0-MΦ and M2-MΦ induced a weak T cell proliferation (25% (±5.8% SEM) and 9% (±3.5% SEM), respectively), which suggest their defective function as T lymphocyte stimulators (Figures 21A-B). Considering SNDil-MΦ, the CD163^{low} subgroup is a weak activator of T cells (18% ±1% SEM), close to M0-MΦ, whereas CD163^{high} SNDil-MΦ were almost incapable of stimulating T lymphocytes (7% ±1.1% SEM) (Figures 21B-C). These findings highlight the similarities between M2-MΦ and CD163^{high} SNDil-MΦ, supporting the phenotypic features mentioned above.

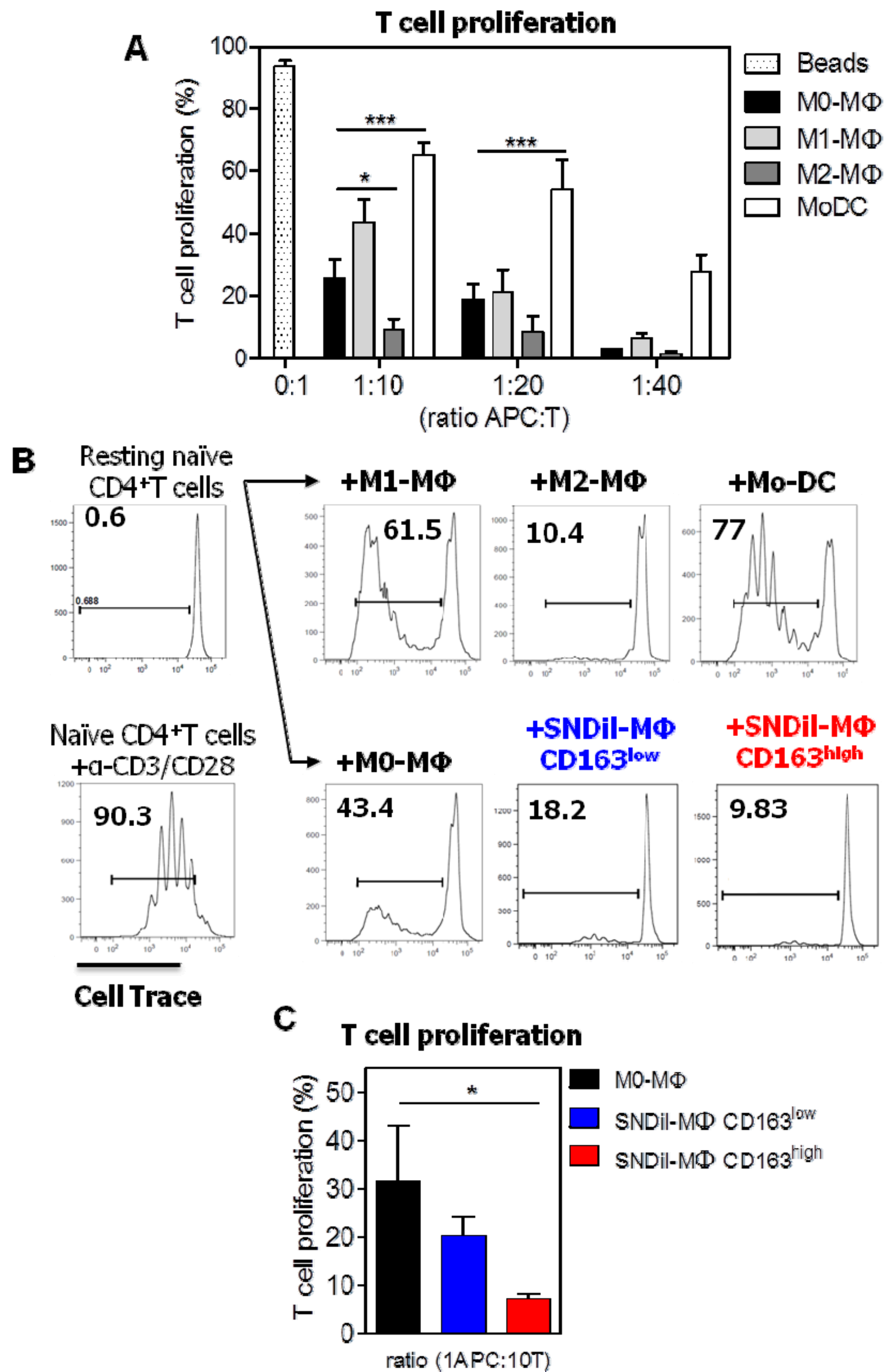


Figure 21. CD163^{high} SNDil-MΦ failed to induce T cell proliferation. SNDil-MΦ and control APCs were co-cultured with allogeneic naïve CD4^{+} T cell by 5 days in the presence of LPS. T cell proliferation was assessed by flow cytometry considering cell trace dilution in live CD3^{+} cells. In A, graphic showing frequency of T cell proliferation stimulated by different APCs at distinct ratios (at least n=4). In B and C, representative histograms and graphic showing frequency of T cell proliferation stimulated by control APCs, CD163^{low} SNDil-MΦ (blue bar, n=3) and CD163^{high} SNDil-MΦ (red bar, n=5) at the ratio 1 APC:10 T; (*$p<0.05$, ***$p<0.0001$).

Considering the particular weak ability of $CD163^{high}$ SNDil-MΦ and M2-MΦ to stimulate naïve T cells, we hypothesized that those cells also have the capacity to actively suppress lymphocytes. To test this hypothesis, we performed suppressive assays, where allogeneic naïve $CD4^{+}CD45RA^{+}$ T cells were pre-incubated with anti-CD3/anti-CD28 microbeads for 30-40 minutes and after, different APCs or SNDil-MΦ were added for additional 4 days. Firstly, to choose the best protocol, control APCs were co-cultured at different ratios with T cells (APC:T= 1:10; 1:5; 1:2), and Cell-Trace dilution was evaluated by flow cytometry after 4 days. We designated the ratio 1:2 as the most distinguishable, where M2-MΦ significantly suppressed anti-CD3/CD28 activated naïve T cell expansion, while M0-MΦ just partially reduced the basal level of beads stimulation (Figure 22A). In addition, no reduction in T cell expansion was observed when M1-MΦ or Mo-DCs were co-cultivated with activated T cells for none of ratios tested (Figure 22A). Furthermore, both $CD163^{low}$ and $CD163^{high}$ SNDil-MΦ were able to significantly suppress the expansion of anti-CD3/CD28-activated T cells, with a stronger effect for $CD163^{high}$ SNDil-MΦ (Figures 22B-D). Interestingly, $CD163^{high}$ SNDil-MΦ displayed a suppressive ability similar to M2-MΦ, while $CD163^{low}$ SNDil-MΦ showed an intermediate effect, with values near from M0-MΦ.

We also investigated the presence of different cytokines by multiplex in the supernatant of suppressive assays at day 4. We noted that $CD163^{low}$ and $CD163^{high}$ SNDil-MΦ, M0-MΦ, and M2-MΦ were able to significantly block the production of IFN-γ, IL-2, and GM-CSF by anti-CD3/CD28-activated $CD4^{+}$ T cells (Figure 23). Additionally, we detected high levels of IL-10, while IL-13 levels were decreased in the presence of $CD163^{high}$ SNDil-MΦ and M2-MΦ (Figure 23). Contrastingly, we found high amounts of IFN-γ, GM-CSF, and TNF-α in co-cultures with M1-MΦ (Figure 23B). For IL-6 and CCL22, no significant differences were noted among groups (data not shown). Together, those findings suggest that $CD163^{high}$ SNDil-MΦ may acquire similarities close to M2-MΦ in terms of phenotype and suppressive functions, whereas $CD163^{low}$ SNDil-MΦ showed an intermediate profile, sharing some characteristics with control M0-MΦ.

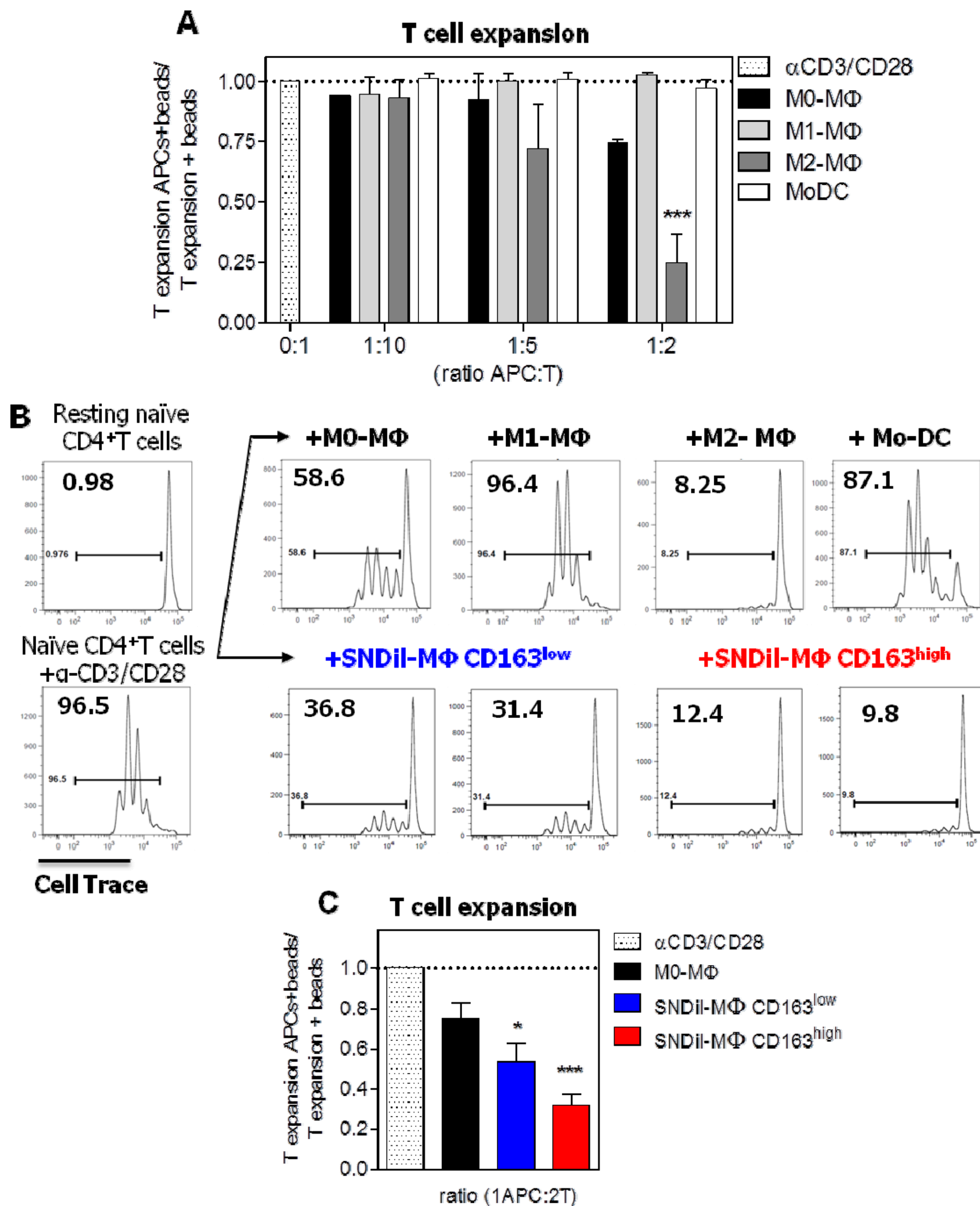


Figure 22. CD163^{high} SNDil-MΦ suppressed CD4$^+$ T cell expansion. Allogeneic naïve CD4$^+$ T cells were pre-activated with anti-CD3/CD28 beads and then co-cultured with SNDil-MΦ or control APCs for 4 days in the presence of LPS. T cell proliferation was assessed by flow cytometry considering cell trace dilution in live CD3$^+$ cells. In A, graphic showing relative T cell expansion in the presence of different APCs at distinct ratios (at least n=3). In B and C, representative histograms and graphic showing relative T cell expansion stimulated by control APCs, CD163^{low} SNDil-MΦ (blue bar, n=3) and CD163^{high} SNDil-MΦ (red bar, n=5) at the ratio 1APC:2Tcells, (*$p<0.05$, ***$p<0.0001$) (relative T cell expansion = % of cell trace dilution of beads-activated T cells in the presence of APCs / % of cell trace dilution of beads-activated T cells alone).

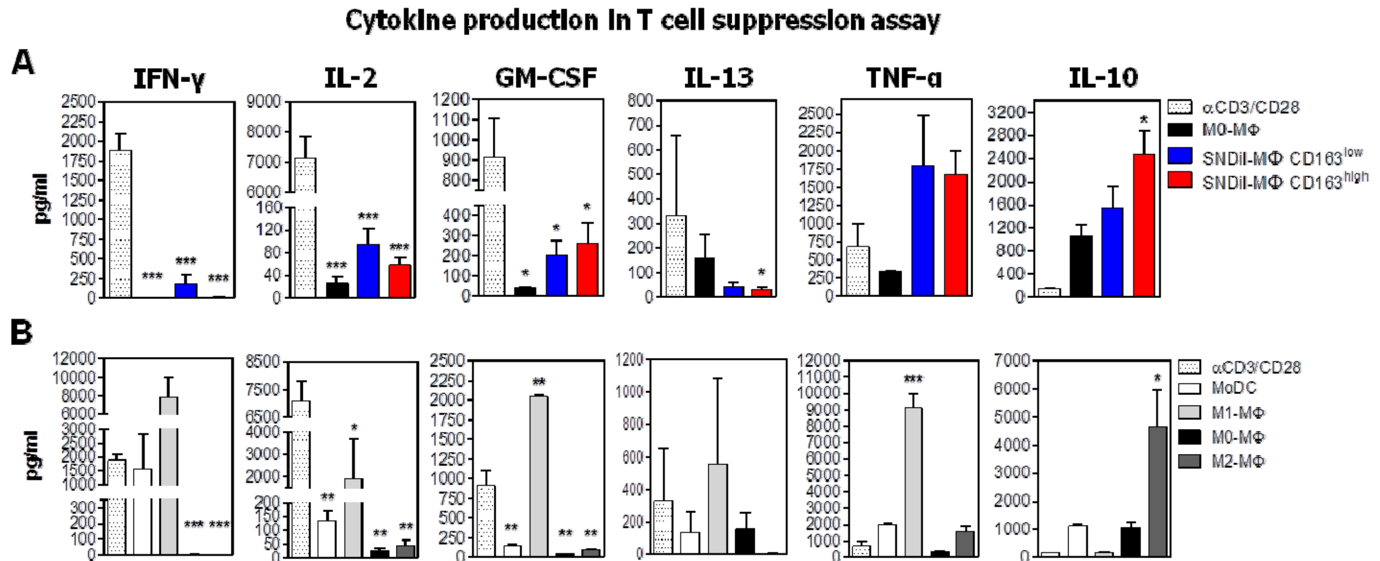


Figure 23. CD163^{high} SNDil-MΦ blocked IFN-γ and IL-13 production by activated T CD4^{+} lymphocytes. Allogeneic naïve CD4^{+} T cells were pre-activated with anti-CD3/CD28 beads and then co-cultured with SNDil-MΦ or control APCs for 4 days (ratio 1APC:2T cells) in the presence of LPS. Supernatants from co-cultures were recovered and submitted to multiplex analysis of cytokines. A and B, respectively, graphics showing the presence of different cytokines in co-cultures where SNDil-MΦ or control APCs were added (*p<0.05; **p<0.01 ***p<0.0001).

Collectively, CD163^{high} SNDil-MΦ showed suppressive abilities, probably associated to their phenotype, acquired following exposure of monocytes to tumor microenvironment. We found that these cells produce high amounts of IL-10, that induced PD-L1 and PD-L2 up-regulation, probably involved in their suppressive capability. For these reasons, we performed the suppressive assay experiments using one chosen CD163^{high} SNDil-MΦ or M2-MΦ in the presence of specific monoclonal antibodies to block TNF-alpha, IL-10, PD-L1, and PD-L2 molecules. Our data revealed that the combined blockage of IL-10/IL-10R and PD-L1 resulted in an important recovery in T cell expansion. Indeed, even partially reversed, we found about 20% of CD4^{+} T cell expansion recovery in the presence of anti-IL-10/IL-10R + anti-PD-L1 blocking antibodies (Figure 24). Noteworthy, anti-PD-L1 antibodies alone did not substantially recover T cell expansion, while anti-IL-10/IL-10R antibodies alone indicated a tendency of T cell recovery for both CD163^{high} SNDil-MΦ and M2-MΦ co-cultures (Figure 24).

In a second round of experiments, we also evaluated PD-L2 participation in suppression assays. The presence of anti-PD-L2 blocking antibodies alone or in combination did not reverted T lymphocyte suppression (Figure 25). However, the combination of anti-IL-10/IL-10R + anti-PD-L1 resulted in about 12 to 17% of recovery of T cell expansion for both CD163^{high} SNDil-MΦ and M2-MΦ co-cultures (Figure 25). It's noteworthy in both rounds of experiments, the blockage of IL-10/IL-10R represented a key factor for T cell recovery in the co-cultures with CD163^{high} SNDil-MΦ, indicating that IL-10 signalization may be the starter point for the PD-L1 and PD-L2 up-regulation, as well as may direct suppress T cell proliferation.

To further characterize the effects of IL-10 and PD-L1 blockage in T cell suppression assays, we collected the supernatants from the co-cultures from the first experiment (from Figure 24), and submitted them to cytokine analysis by multiplex. The presence of anti-IL-10/IL-10R blocking mAb induced a partial recovery of IFN-γ, IL-13, and GM-CSF production by activated CD4+ T cells (Figure 26). Surprisingly, we noted that blocking IL-10/IL-10R axis increased the production of IL-12p40, TNF-alpha, IL-6, and CCL22, most of them being produced by APCs (Figure 26). In those experiments, we cannot assert which cells are the source of IL-10, GM-CSF, and TNF-alpha, but it is not negligible that IL-10/IL-10R blocking has a broad spectrum of activity, also modulating the anti-inflammatory properties of APCs. Still, the presence of anti-PD-L1 mAb alone or combined with anti-IL-10/IL-10R in co-cultures did not represent great gain in the recovery of cytokine production by T cells (Figure 26).

Although IL-10/PD-L1 axis emerged as possible mechanism by which APCs suppress T cells in our study, most of the suppressive effects rely on the ability of APCs to produce IL-10. In fact, autocrine and/or paracrine effects of IL-10 confer a high CD163 and PD-L1 expression on APCs, and concomitantly inhibit APCs activation, as assessed by the down-regulated expression of CD86 expression, and the decreased production of TNF-α and IL-12p40 .

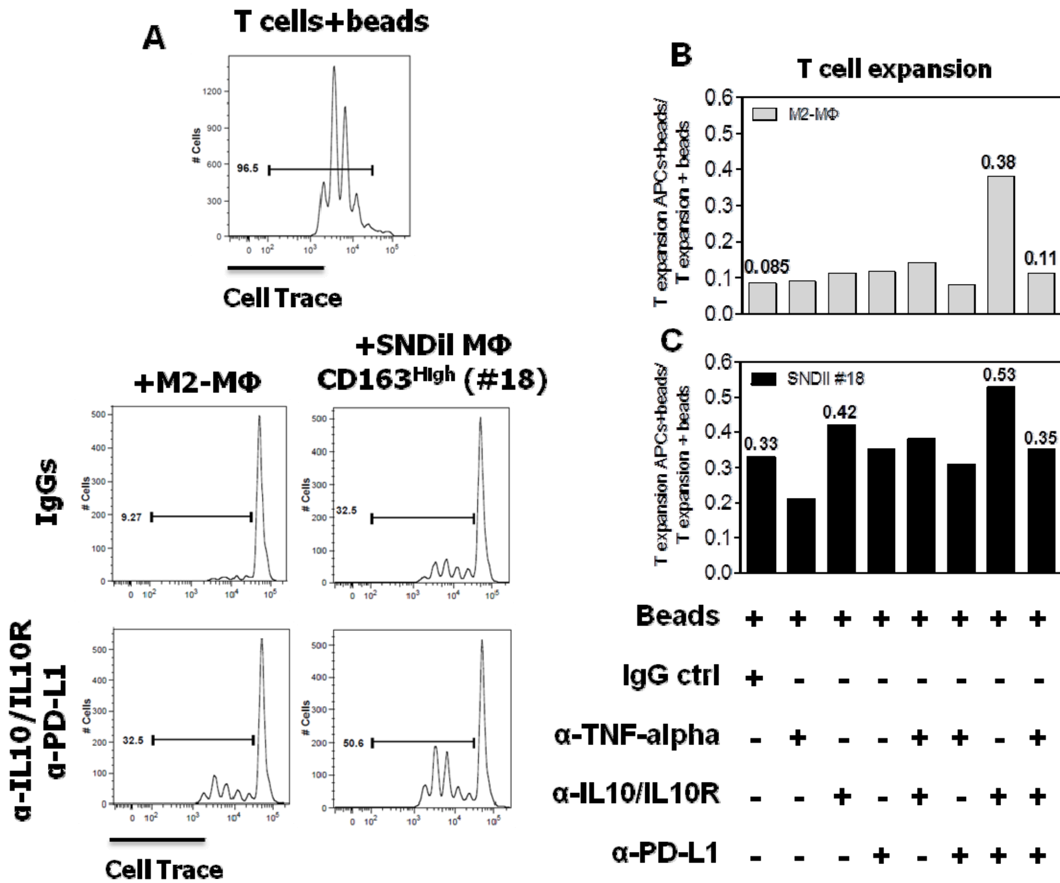


Figure 24. IL-10 and PD-L1 participate in T cell suppression promoted by CD163^{high} SNDil-MΦ and M2-MΦ. Allogeneic naïve CD4^{+} T cells were pre-activated with anti-CD3/CD28 beads and then co-cultured with CD163^{high} SNDil-MΦ or M2-MΦ for 4 days in the presence of LPS and monoclonal antibodies against IL-10, IL-10R, PD-L1, and TNF-α. Representative histogram of cell trace dilution (A) and graphics (B-C) showing the relative T cell expansion of beads-activated T cells co-cultured with M2-MΦ or CD163^{high} SNDil-MΦ in the presence of blocking mAb (one representative monocyte donor out of two performed).

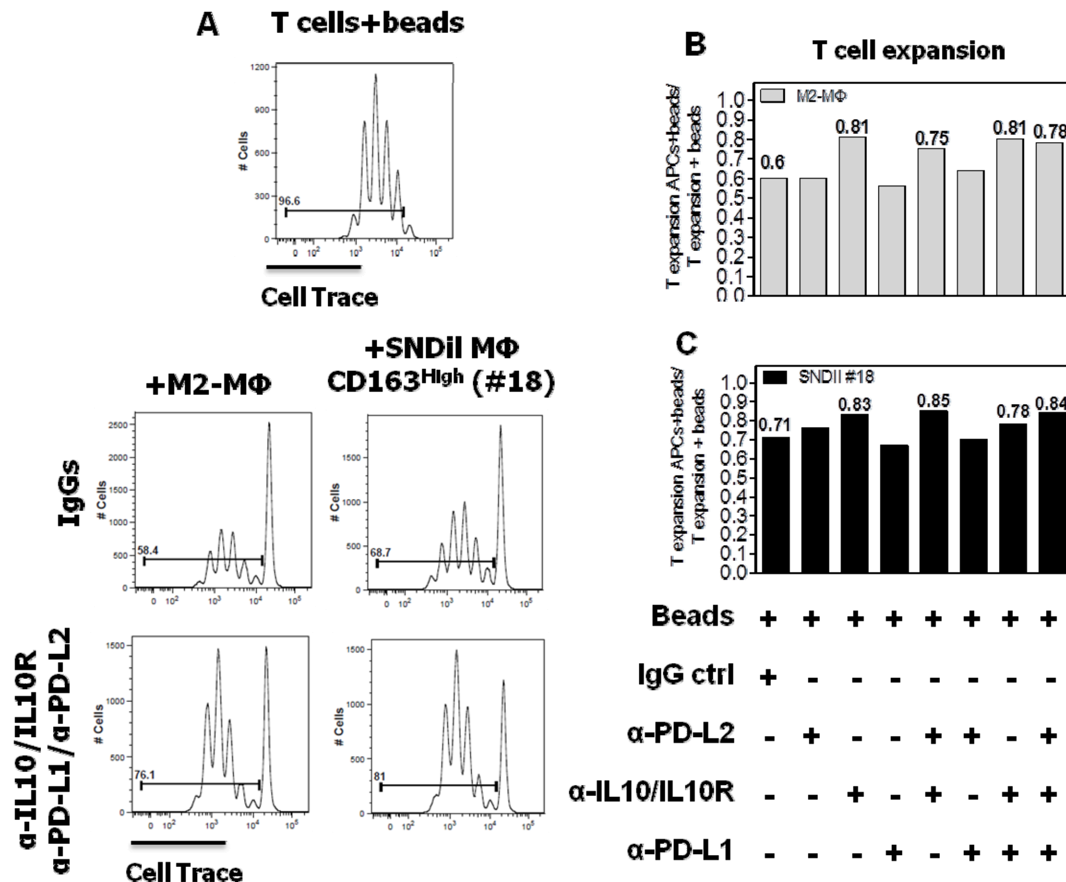


Figure 25. IL-10 and PD-L1 but not PD-L2 participate in T cell suppression promoted by CD163^{high} SNDil-MΦ and M2-MΦ. Allogeneic naïve CD4^{+} T cells were pre-activated with anti-CD3/CD28 beads and then co-cultured with CD163^{high} SNDil-MΦ or M2-MΦ for 4 days in the presence of LPS and monoclonal antibodies against IL-10, IL-10R, PD-L1, and PD-L2. Representative histogram of cell trace dilution (A) and graphics (B-C) showing the relative T cell expansion of beads-activated T cells co-cultured with M2-MΦ or CD163^{high} SNDil-MΦ in the presence of blocking mAbs (one representative monocyte donor out of two performed).

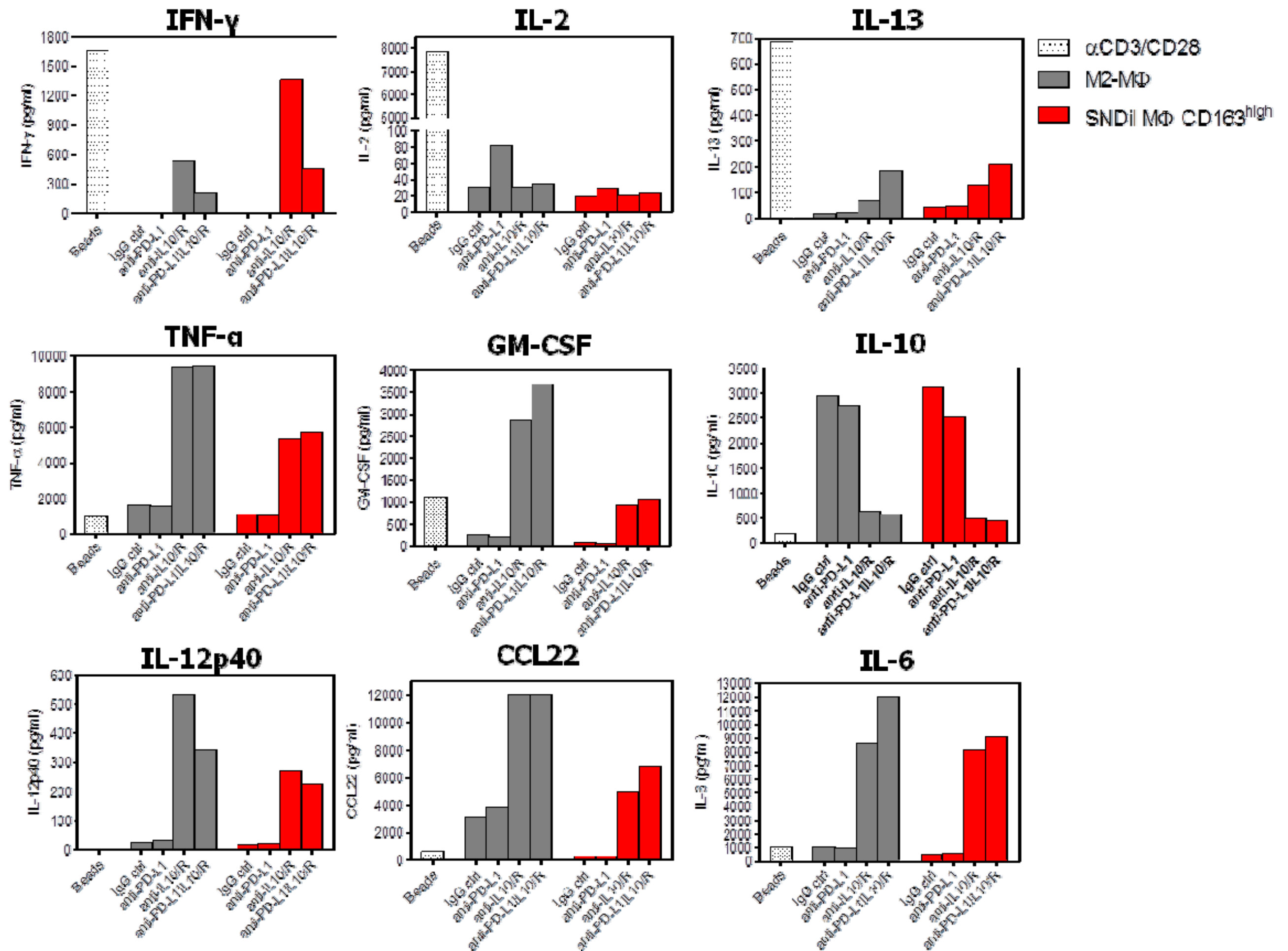


Figure 26. IL-10/IL-10R blocking induces the recovery of cytokine production by activated T cells in co-culture with CD163^{high} SNDil-MΦ or M2-MΦ. Allogeneic naïve CD4^{+} T cells were pre-activated with anti-CD3/CD28 beads and then co-cultured with CD163^{high} SNDil-MΦ or M2-MΦ for 4 days in the presence of LPS and monoclonal antibodies against IL-10, IL-10R, and PD-L1 (same donor as figure 24). Supernatants from co-cultures were collected and different cytokines were quantified by multiplex (one representative monocyte donor out of two performed).

4.2.4 The presence of TGF-β1, TGF-β3, CCL22, M-CSF, CCL21, and VEGF in the tumor microenvironment may induce a CD163^{high}IL-10^{high} SNDil-MΦ profile in conditioned monocytes

To identify the soluble factors from the breast tumor microenvironment that may possibly induce the differentiation of monocytes into suppressive cells, we performed a multiplex analysis evaluating 44 proteins (cytokines, chemokines, and factors of differentiation) in tested SNDils. An overview with all investigated proteins can be found in the table 1, showing: molecules not detected in the assay; molecules detected but not correlated with phenotypic/functional data of SNDil-MΦ; and molecules that correlated with the phenotype and/or function of SNDil-MΦ.

We analyzed the presence of cytokines and their correlation with two main characteristics acquired by suppressive SNDil-MΦ: the expression of CD163 and the production of IL-10. The global analysis revealed that the high expression of CD163 by SNDil-MΦ was significantly correlated to the presence of TGF-β1 and CCL22 in the SN-Dils (Figure 27A). Additionally, we also found a positive association between the levels of IL-10 and the presence of TGF-β3 and CCL22 in SN-Dils (Figure 27B).

Further, we also investigated which factors present in SN-Dils could be responsible for the concomitant high expression of CD163 and elevated IL-10 production. For this, we divided all samples in two groups of analysis according to the expression of these molecules: I) SNDil-MΦ that concomitantly expressed high levels of r-CD163, similar/superior to the lowest value of M2-MΦ (r-CD163 ≥ 1.6), and produce similar/superior amounts of IL-10 compared to M0-MΦ (IL-10 ≥ 415 pg/ml) (Figure 28A – called Q2); II) SNDil-MΦ that differ from Q2 group, by corresponding to only one or none of the criteria (Figure 28A – called Q1+Q3+Q4). We observed that SN-Dils which induced the Q2 profile (CD163^{high}IL-10^{high} SNDil-MΦ) contained high levels of TGF-β3 and CCL22 (Figure 28B). We also found a tendency of elevated levels of CCL19, CCL21, IL-8, VEGF, and MCSF, but lower amounts of CXCL11, in SN-Dils inducing Q2 profile in comparison to Q1+Q3+Q4 SN-Dils (Figure 28B). These findings identified new combinatory factors that could be required for the differentiation of blood monocytes into suppressive CD163^{high}IL-10^{high} SNDil-MΦ.

Table 2. Content of molecules in SN-Dils measured by Multiplex

Non-detected molecules	Molecules not correlated with SNDil-MΦ phenotype/function	Molecules associated to SNDil-MΦ phenotype/function
IL-1α, IL-2, IL-3, IL-4, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-23, IL-28, IL-29, IFN-α2, IFN-β, IFN-γ, GM-CSF, sCD40L, TNF-α, TNF-β.	CCL2, CCL17, CCL20, CXCL9, CXCL10, CXCL12, CXCL13, IL-1β, IL-1RA IL-6, IL-18, IL-33, APRIL, BAFF, sCD30, TGF-β2.	CCL19, CCL21, CCL22, CXCL11, IL-8, M-CSF, TGF-β1, TGF-β3, VEGF.

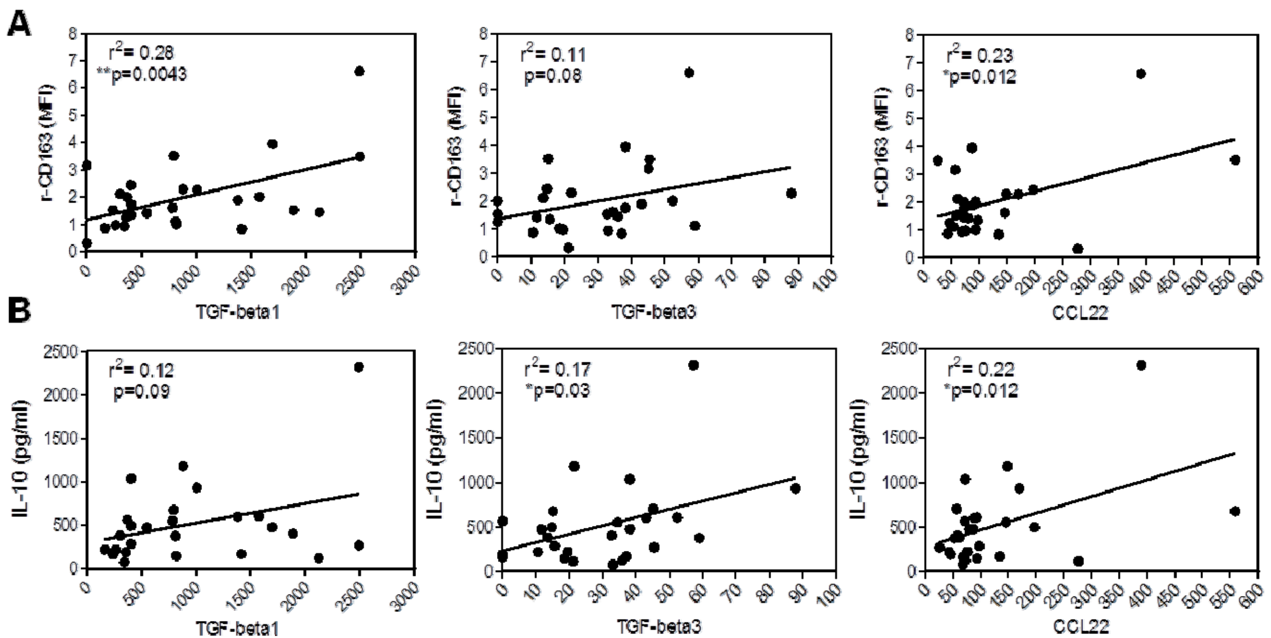


Figure 27. TGF-β and CCL22 levels in SN-Dils correlated to the CD163^high IL-10^high phenotype of SNDil-MΦ. SN-Dils were submitted to multiplex analysis of cytokines/chemokines and the correlation with SNDil-MΦ phenotype was performed. In A and B respectively, r-CD163 (MFI) and IL-10 production correlated to TGF-β1, TGF-β3, and CCL22 levels in SN-Dils (n≥ 25; *p<0.05).

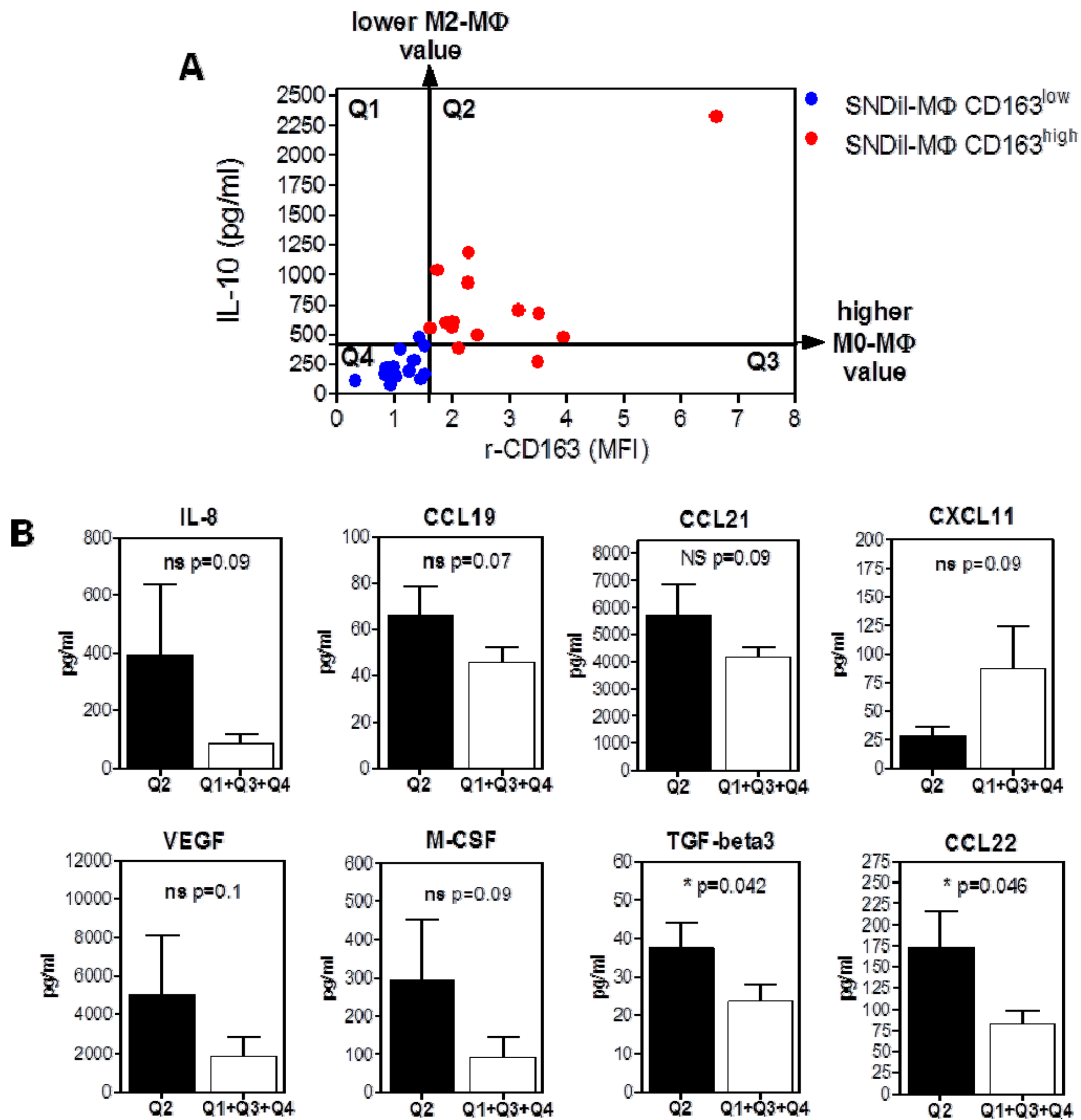


Figure 28. Levels of IL-8, CCL19, CCL21, VEGF, M-CSF, TGF-β3, and CCL22 are elevated in SN-Dils that skewed monocytes into CD163^{high}IL-10^{high} phenotype. SN-Dils were submitted to multiplex analysis of cytokines/chemokines and the correlation with SNDil-MΦ phenotype was performed. In A, IL-10 production by r-CD163 expression in SNDil-MΦ. Quadrants represent SNDil-MΦ that acquired different phenotypes. In B, presence of factors in SN-Dils that induced different phenotypes in SNDil-MΦ. Phenotypes: Q1= CD163^{low}IL-10^{high}; Q2= CD163^{high}IL-10^{high}; Q3= CD163^{high}IL-10^{low}; Q4= CD163^{low}IL-10^{low} (n≥ 25; *p<0.05).

4.2.5 High frequency of CD163^{high} TAMs is associated with high IL-10 production by the corresponding SNDil-MΦ in vitro

For 7 patients, we were able to generate SNDils and investigate the frequency of TAMs infiltrate which allowed us to correlate with the effects of the corresponding SN-Dil. Interestingly, among all characteristics evaluated, we found a significant positive correlation between high frequency of CD163^{high} TAMs and high IL-10 production by the associated SNDil-MΦ (Figures 29A-B).

A

TAM *ex-vivo*		SN-Dil effects on monocytes				
Tumor/ SN-Dil	**CD163^{high} TAM (%)**	**Relative CD163**	**IL-10 (pg/ml)**	**TNF-α (pg/ml)**	**PD-L1 (MFI)**	**CD86 (MFI)**
#3	12.3	0.93	76.2	190	557	329
#10	26.7	1.52	164.1	46.5	716	473
#7	28.4	0.32	114.2	578	652	760
#8	39.3	0.75	109.8	131.6	776	512
#9	45.3	1.34	286.1	162	621	414
#5	35.1	1.23	377	191.6	400	198
#2	67.2	2	563	485	1738	213

IL-10 production by SNDil-MΦ *vs* % of CD163^{High} TAMs

B

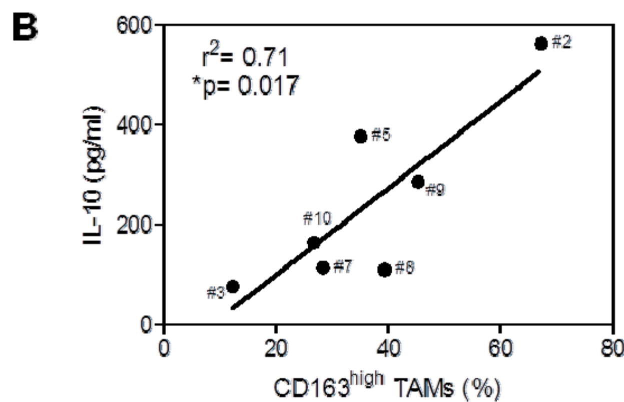


Figure 29. High frequency of CD163^{high} TAMs in tumors is associated to the capacity of SNDils to generate IL-10-producing SNDil-MΦ. In A, table correlating TAMs' frequency with cytokine production and costimulatory molecules expression by the associated SNDil-MΦ. In B, graphic correlating IL-10 production by SNDil-MΦ and frequency of infiltrated CD163^{high} TAMs by the same donor (n= 7; r^2= 0.71; *p<0.05).

4.3 Tumor systemic effects on circulating monocyte

In the previous sections of the results, we showed how complex are the tumor microenvironment and the characterization of tumor-infiltrating APCs, and the direct effects of tumor microenvironment products on healthy blood monocytes differentiation. Thereafter, we investigated the systemic effects of tumor development on blood monocytes from cancer patients. In 2010, thesis developed in our laboratory reported a defect in the ability of blood monocytes from breast cancer patients to differentiate into functional Mo-DCs *in vitro* (AZEVEDO-SANTOS, 2010). Considering that, we characterized more deeply cancer patients' monocytes, exploring additional phenotypic and functional aspects of their differentiation into Mo-DCs.

4.3.1 Mo-DCs differentiated from breast cancer patients' monocytes are biased to induce high frequency of $CD4^+CD25^+Foxp3^+$ T cells

During the period of my master degree, we observed that Mo-DCs differentiated from breast cancer patients' monocytes failed to induce T lymphocyte proliferation and produced high levels of anti-inflammatory cytokines, as IL-10 and TGF-β1. To further investigate the immunosuppressive features from these cells, during my PhD we investigated their capacity to induce T cells with regulatory abilities and the possible mechanisms governing this. We present here our recently published data exploring the immunosuppressive bias of breast cancer patients' Mo-DCs (RAMOS et al., 2012a - Appendix A; RAMOS et al., 2013 - Appendix B).

The phenotypic analysis revealed immature Mo-DCs (Mo-iDCs) differentiated from breast cancer patients monocytes expressed higher levels of CD86 and PD-L1 MFIs in comparison to healthy donors Mo-iDCs (Figures 30A-C). To investigate their functional abilities, cancer patient's Mo-iDCs were co-cultured with allogeneic non-activated $CD3^+CD25^{neg}$ T lymphocytes. Our findings showed that patient's Mo-iDCs induced a lower expression of the activation marker CD25 (Figures 30D-E), but a higher frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells when compared to healthy Mo-iDCs (Figures 30F-G). In addition, in the co-cultures where patient's Mo-iDCs were added, we noted high amounts of TGF-β1 and low quantities of IFN-γ in contrast to healthy Mo-iDCs co-cultures (Figure 30H). Also, even not significantly different, we found patients' Mo-iDCs have a tendency to produce elevated levels of

IL-10 and TGF-β1 at non-activated status (RAMOS et al., 2012a - APPENDIX A). Similar results were obtained when Mo-iDCs were generated from isolated $CD14^+$ monocytes (RAMOS et al., 2012a - APPENDIX A).

Next, we investigated whether breast cancer patients' Mo-DCs, either immature or mature (Mo-mDCs), could stimulate also $CD4^+$ T helper subsets. For that, allogeneic naïve $CD4^+CD45RA^+$ T lymphocytes were isolated and cultivated with Mo-DCs from breast cancer patients to evaluate the induced expression of the transcription factors T-bet (Th1), Gata-3 (Th2), and Foxp3 (Treg). In agreement with our previous results, patient's Mo-DCs induced weak CD25 expression on stimulated T lymphocytes, even in their mature form (Mo-mDCs - TNF-α activated) in comparison to healthy Mo-DCs (Figures 31A-B). Regarding Gata-3 and T-bet expression in stimulated $CD4^+CD25^+$ T lymphocytes, no differences were observed between Mo-iDCs or Mo-mDCs from patients or healthy donors (Figures 31C-D). However, patient's Mo-DCs, even after TNF-α maturation (Mo-mDCs), induced more $Foxp3^+$ cells among $CD4^+CD25^+$ T cells compared to healthy Mo-DCs (Figure 31E). Importantly, patient's Mo-DCs are biased to induce high frequency of Tregs than Th1 or Th2 profiles; whereas healthy Mo-DCs showed a tendency to stimulate more Th2 lymphocytes when immature and preferentially Th1 lymphocytes after TNF-α maturation (Figures 31C-E). Additionally, we evaluated the expression of intracellular cytokines in stimulated lymphocytes, but no significant differences were found between patients' and healthy Mo-DCs donors (Figure 31F). However, we noted a tendency of high IFN-γ expression in T cells stimulated by healthy Mo-mDCs when compared to their immature form or to Mo-DCs from cancer patients (Figure 31F).

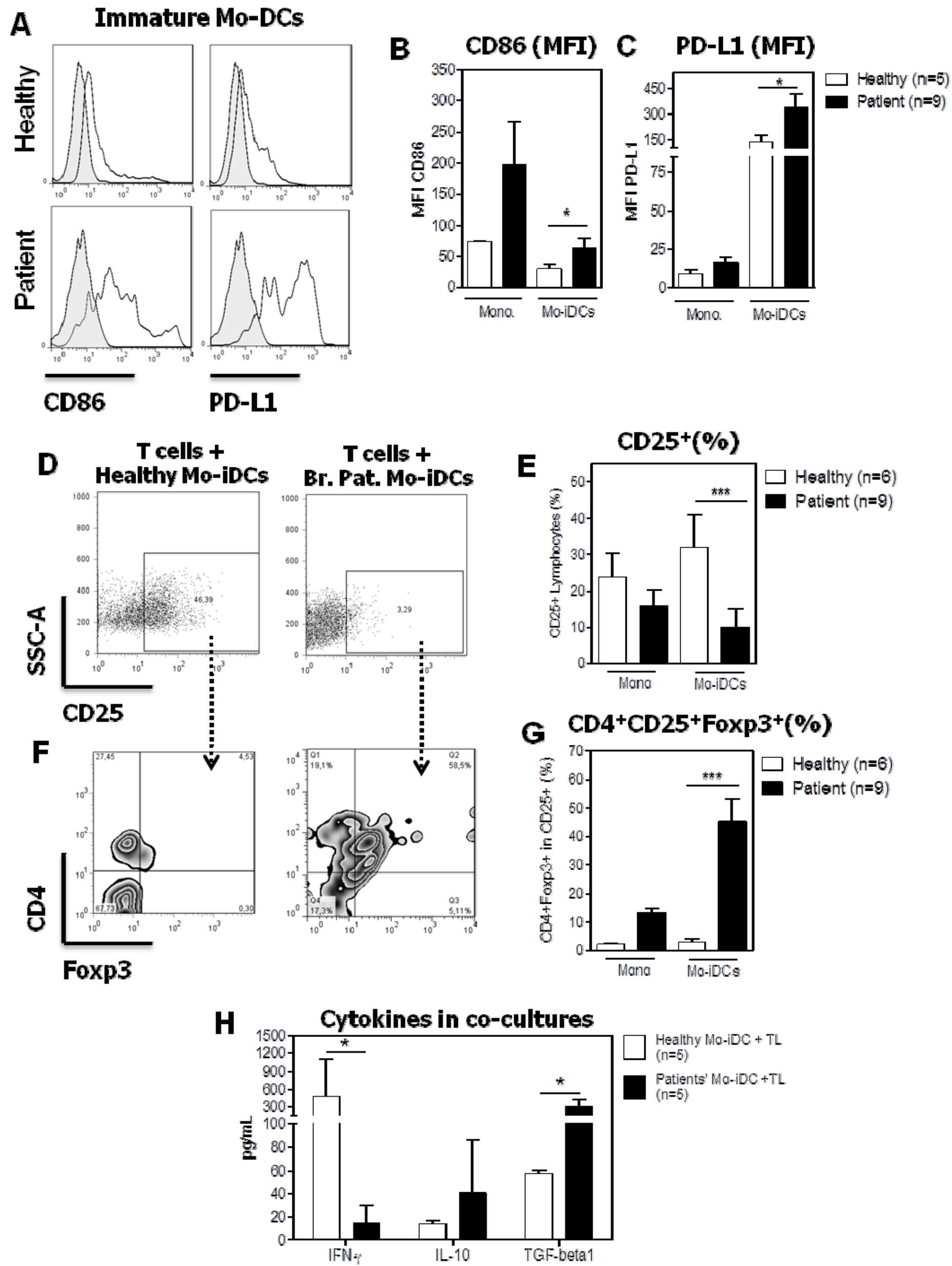


Figure 30. Patients' Mo-iDCs showed up-regulated expression of CD86 and PD-L1 and were able to induce high frequency of CD4$^+$CD25$^+$Foxp3$^+$ Tregs. Monocytes from breast cancer patients' blood (Mono) were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 by 7 days. Further, immature Mo-DC (Mo-iDCs) were characterized by flow cytometry and co-cultured with allogeneic CD3$^+$CD25^{neg} T lymphocytes for 6 days. Figures A to C, CD86 and PD-L1 MFI expression comparing healthy and patients' Mono and Mo-iDCs (grey histograms= non-stained; white= specific staining). In co-cultures, representative dot-plots and graphics of CD25 expression in CD3$^+$ T cells (D-E) and CD4/Foxp3 expression within gated CD25$^+$ T cells (F-G). In H, cytokine production in co-cultures measured by ELISA. (*p<0.05, ***p<0.0001). (Figures extracted from Ramos et al., 2012a)

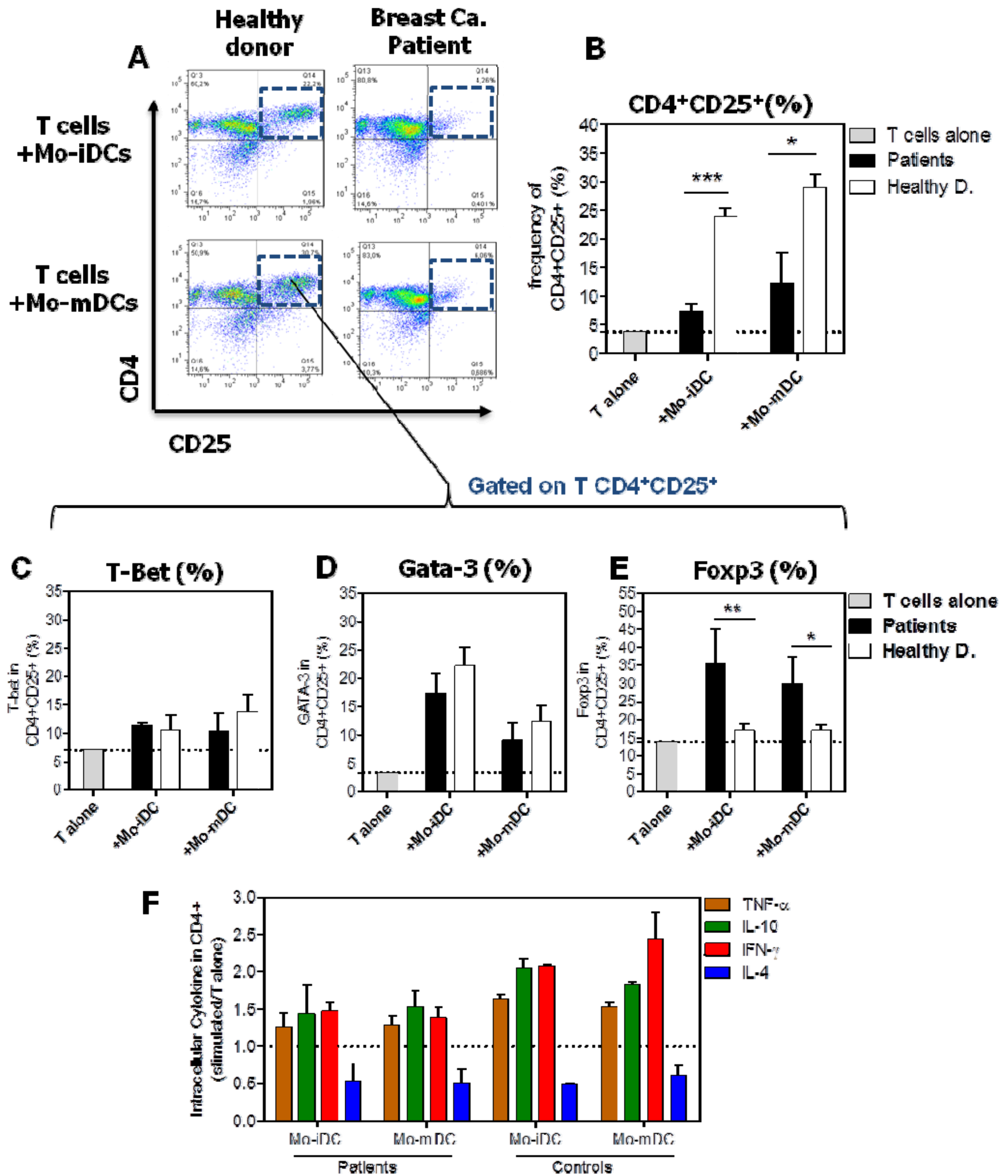


Figure 31. Patients' Mo-DCs stimulate significantly high frequency of CD4$^+$CD25$^+$Foxp3$^+$ Tregs than Th1 or Th2 subsets. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 by 7 days and were activated or not by TNF-α at day 5. Mo-DCs were removed and co-cultured with allogeneic CD4$^+$CD45RA$^+$ T lymphocytes by 6 days. Figures A and B, show representatives pseudo-color plots and graphics of CD25 frequency in stimulated lymphocytes, respectively. In C to E, evaluation of T-Bet, Gata-3 and Foxp3 frequency within CD4$^+$CD25$^+$ T lymphocytes (Mo-iDCs n=4; Mo-mDC n=3; *$p<0.05$, **$p<0.01$, ***$p<0.0001$). In F, intracellular cytokine expression in gated CD4$^+$CD25$^+$ T lymphocytes (patients Mo-iDCs and Mo-mDC n=3; healthy Mo-iDCs and Mo-mDC n=2). (for all graphics, dotted line represent the cut-off from relative values of T cells alone).

4.3.2 Activating signals modify patient's Mo-DC phenotype but do not avoid their ability to induce Treg cells

Trying to modulate patients' Mo-DCs physiology and maybe to counteract their immunosuppressive properties, patient's monocytes were differentiated into DCs and were subjected to different stimuli of activation: TNF-α alone; soluble CD40 ligand (sCD40L); cytokine cocktail (TNF-α + IL-1β + IL-6), and LPS. We observed that LPS significantly up-regulated CD86 and CD40 molecules by patients' Mo-mDCs but maintained CD80 and PD-L1 at similar levels as immature Mo-DCs (Figures 32A-D). Furthermore, under sCD40L or cytokine cocktail activation, patients' Mo-mDCs showed no differences in the modulation of the same surface molecules evaluated among groups (data not show). In the functional assays with allogeneic $CD3^{+}CD25^{neg}$ T cells, patient's Mo-mDCs significantly increase CD25 expression on T cells when activated by cytokine cocktail or LPS (Figure 32E). Additionally, the induction of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs was partially diminished when patients' Mo-mDCs were activated by sCD40L or LPS (Figure 32F). However, even altering their phenotype and "correcting" in part their bias of CD25 and Treg induction, the functional ability of patients' Mo-mDCs was yet far away from what we have observed for Mo-DCs derived from healthy donors. Even though we did not evaluate the cytokines profile, our findings suggest that a strong activator like LPS may represent an interesting way to modulate patients' Mo-DCs trying to modify/correct their immunosuppressive bias.

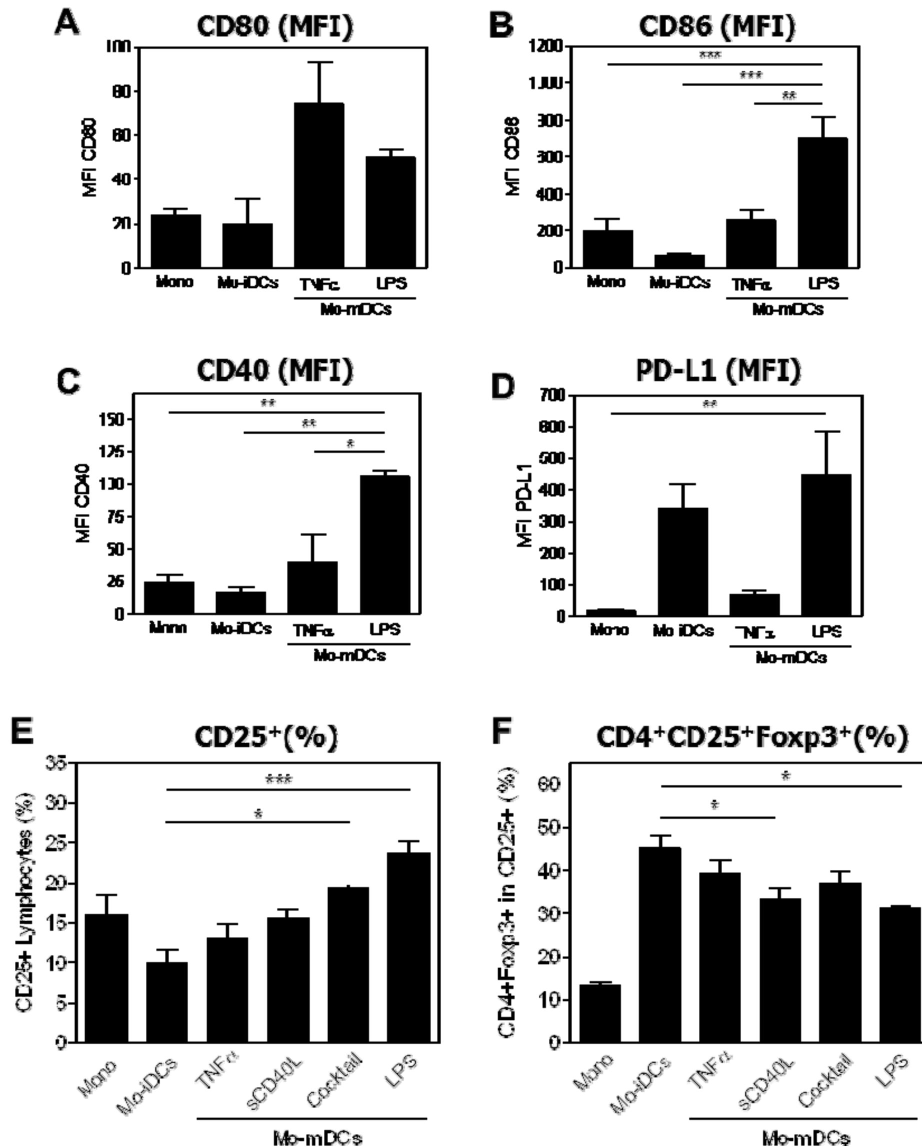


Figure 32. The phenotype and the suppressive function of patient's Mo-DCs are partially modulated after LPS activation. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 for 7 days. At day 5, Mo-DC received TNF-α, soluble CD40L, cytokine cocktail (TNF-α +IL-1β + IL-6) or LPS as stimuli. At the end of cultures Mo-mDCs were characterized by flow cytometry and co-cultured with allogeneic $CD3^{+}CD25^{neg}$ T lymphocytes for 6 days. Graphics A to D, MFI evaluation of surface molecules in patient's Mo-DCs. In E and F respectively, frequency of $CD25^{+}$ and $CD4^{+}CD25^{+}Foxp3^{+}$ induced in T lymphocytes after co-culture with patients' Mo-DCs (Monocytes (Mono) n=3; Mo-iDCs n=8; Mo-$mDC_{TNF\text{-}\alpha}$ n=4; Mo-mDC_{sCD40L} n=3; Mo-$mDC_{cocktail}$ n=3; Mo-mDC_{LPS} n=3; *p<0.05, **p<0.01, ***p<0.0001).

4.3.3 Mo-iDCs derived from breast cancer patients' monocytes induce $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs via TGF-β and PD-L1

As immature Mo-DCs derived from breast cancer patients expressed high levels of PD-L1 and induced strong production of TGF-β in co-cultures, that are two important molecules involved in anti-inflammatory or suppressive conditions, we went further to investigate the eligible mechanisms of Treg induction by patients' Mo-DCs. For that, we co-cultured allogeneic naïve $CD4^{+}CD45RA^{+}$ T lymphocytes with patients' Mo-DCs in transwell systems, avoiding the physical contact between cells. We observed a reduced expression of both CD25 and Foxp3 expression in stimulated T cells in transwell cultures, independently of the maturation status of patients' Mo-DCs (Figures 33A-D). For the induction of T-bet and Gata-3 expression, the same was noted for transwell cocultures with immature patient's Mo-DCs (Figures 33E-F). Those findings indicated a double participation of contact and cytokine signalization mainly in the induction of Foxp3 expression.

Accumulated data above led us to consider immature patient's Mo-DCs as the most potent "inducers" of Foxp3 expression on naïve T lymphocytes, a phenomenon partially dependent on cell-contact. As we know that TGF-β was highly released in Mo-iDCs patients' co-cultures, we verified the participation of that cytokine in our systems. The addition of anti-TGF-β mAb in patients' Mo-iDCs co-cultures significantly increased the total number of stimulated cells (Figure 34A), did not impactl on the CD25 expression on T cells (Figure 34B), but significantly reduced the induction of Treg by patient's Mo-iDCs (Figure 34C). However, the frequency of induced Tregs by patients' Mo-iDCs was not at the same level as found in co-cultures stimulated by healthy Mo-iDCs. Collectively, TGF-β participation emerged as an important piece of the whole puzzle for the explaining of suppressive abilities of Mo-DC differentiated from breast cancer patients' monocytes.

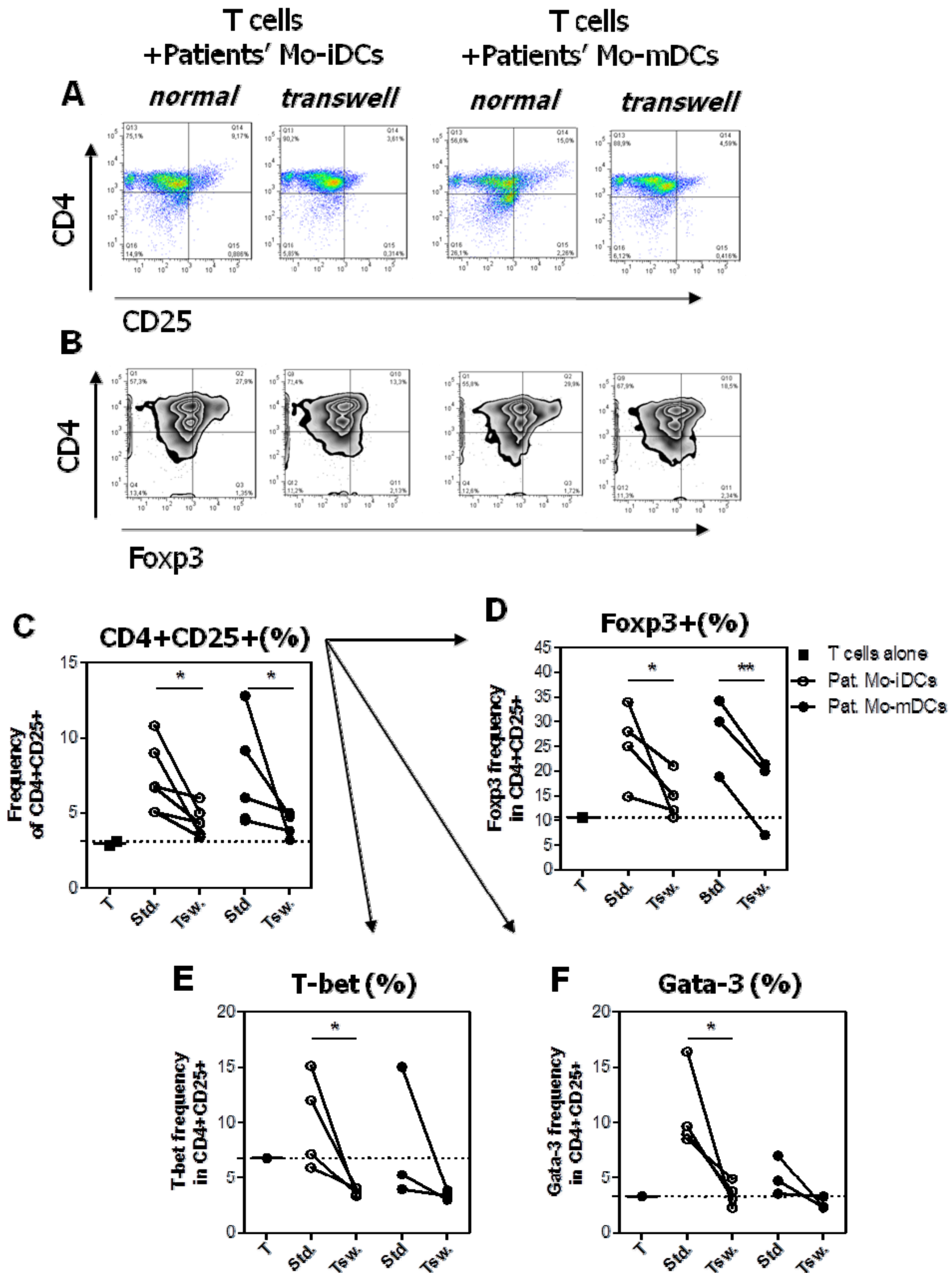


Figure 33. Patients' Mo-DCs induced CD4⁺CD25⁺Foxp3⁺ Tregs with partial dependence of cell-to-cell contact. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 for 7 days. At the end of differentiation Mo-iDC or Mo-mDCs (TNF-α) were co-cultured with allogeneic naïve $CD4^+$ T lymphocytes cells for 6 days in transwell system and the phenotype of lymphocytes was evaluated by flow cytometry. Representative dots of CD25 (A) and Foxp3 expression (B) in $CD4^+$ lymphocytes stimulated by patient's Mo-iDCs or Mo-mDCs in normal or transwell condition. C to F, graphics showing the frequency of $CD25^+$, $Foxp3^+$, T-bet$^+$ and Gata-3$^+$ induced in $CD4^+$ lymphocytes in same conditions mentioned. (Std= no transwell; Tsw= transwell system; Mo-iDCs n=4; Mo-mDC n=3; *p<0.05, **p<0.01).

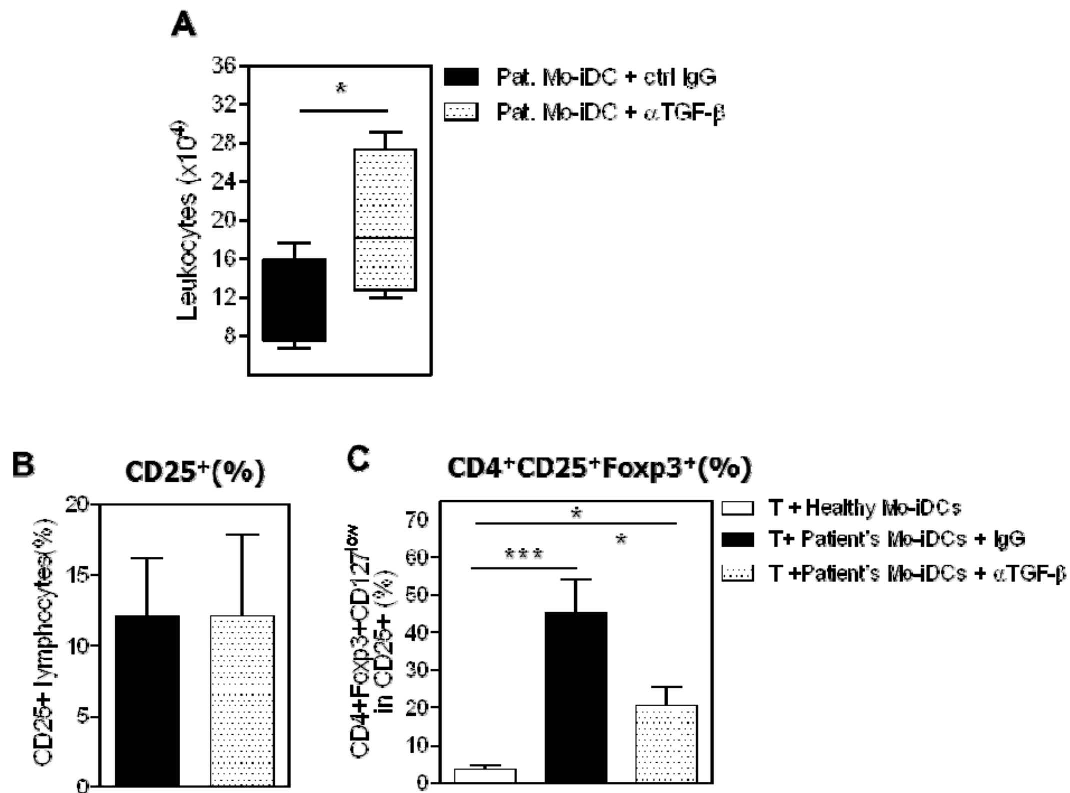


Figure 34. TGF-β blockage partially inhibits $CD4^+CD25^+Foxp3^+$ Treg induction by patients' Mo-iDCs. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 for 7 days. Patient's Mo-iDCs were co-cultured with allogeneic $CD3^+CD25^{neg}$ T lymphocytes for 6 days in the presence of neutralizing anti-TGF-β or control IgG. T cells were harvested, counted, and submitted to flow cytometry analysis. (A) Box and whisker plots showing absolute cell numbers recovered in co-cultures. Graphics showing CD25 expression (B) and $CD4^+CD25^+Foxp3^+CD127^{low}$ T cell frequency (C) after stimulation with Mo-iDCs (healthy Mo-iDCs n=4; patients Mo-iDCs + IgG, n=6; patients Mo-iDCs + anti-TGF-β, n=6; *p<0.05, **p<0.01, ***p<0.0001). (Figures extracted from Ramos et al., 2012a)

Moreover, we also investigated the role of surface molecules in the induction of Tregs by Mo-DCs using neutralizing mAbs against CD80, CD86, PD-L1, and PD-L2. Mo-iDCs from healthy donors significantly lost their ability to induce CD25 expression on stimulated naïve $CD4^+$ T cells in the presence of anti-CD80 (20µg/ml) and anti-CD86 (20µg/ml) mAbs, while anti-PD-L1 (3.5µg/ml) and anti-PD-L2 (3.5µg/ml) antibodies had no effect (Figures 35A-B). On the other side, no significant modulation was noted in the presence of all tested blocking mAb for patients' Mo-iDCs (Figures 35A and D). We also evaluated Foxp3 induction by Mo-iDCs in the presence of blocking antibodies. An increase in Treg induction by healthy Mo-iDCs was observed in the presence of anti-CD80 and anti-CD86 mAbs, while no alterations were found with patients' Mo-iDCs (Figures 35C and E).

Taking into account that the expression of CD86 and PD-L1 was higher in patients' Mo-iDCs in comparison to healthy donors' Mo-iDCs (Figures 30A-C), we hypothesized that elevated concentration of anti-PD-L1 mAb could have a more consistent impact on T cell phenotype. Another group of experiments using Mo-iDCs led us to demonstrate that CD25 induction was not altered with increased/higher doses of anti-PD-L1 mAb (data not shown). In contrast, we noted that the highest dose of anti-PD-L1 mAb (10.75µg/ml) was able to significantly reduce the expression of $Foxp3^+$ in naïve $CD4^+$ T cells stimulated by patients' Mo-iDCs (Figure 36). Even though those findings are encouraging, we need to increase the number of experiments to demonstrate that PD-L1 plays a major role in the induction of $Foxp3^+$ regulatory T cells by patients' Mo-DCs.

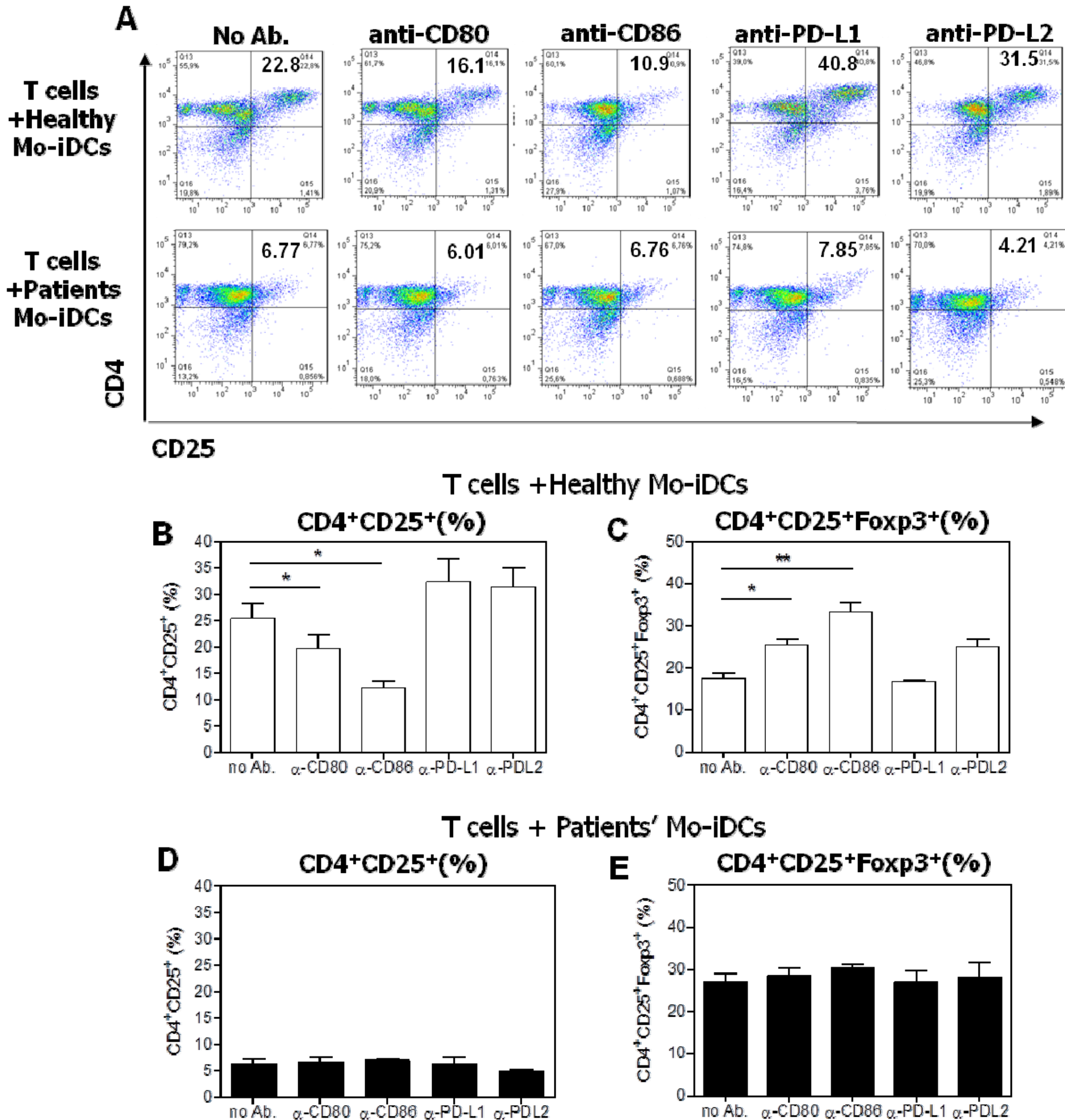


Figure 35. Anti-PD-L1 antibodies at low concentration did not alter patient's Mo-iDCs ability to induce $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 for 7 days. Mo-iDCs were co-cultured with allogeneic naïve $CD4^{+}$ T lymphocytes for 6 days in the presence of blocking antibodies against CD80, CD86, PD-L1 and PD-L2. T cells were harvested and analyzed by flow cytometry. Images show representative pseudo-color plots of CD4/CD25 expression (A) and graphics showing the frequency of $CD4^{+}CD25^{+}$ T lymphocytes stimulated by healthy (B) or by patient's Mo-iDCs (D). In C and E, frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ T cells induced by healthy and patient's Mo-iDCs, respectively (healthy Mo-iDCs n=3; patients Mo-iDCs n=4; *p<0.05, **p<0.01).

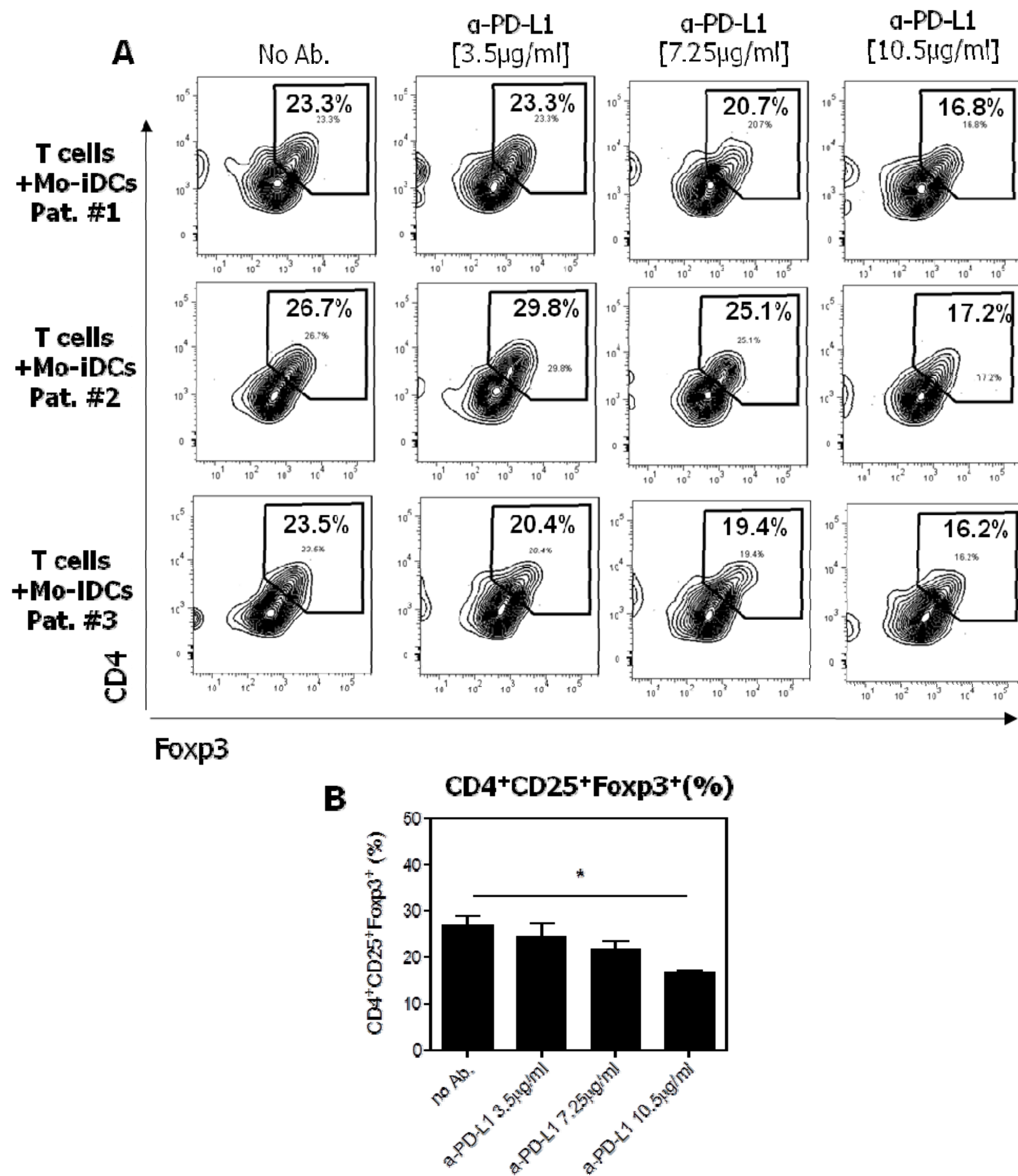


Figure 36. Increased concentration of neutralizing anti-PD-L1 mAb reduces the ability of patients' Mo-iDCs to induce $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs. Breast cancer patients' monocytes were differentiated into Mo-DCs in the presence of GM-CSF+IL-4 for 7 days. Mo-iDCs were co-cultured with allogeneic naïve $CD4^{+}$ T lymphocytes for 6 days in the presence of increasing concentrations of blocking antibodies against PD-L1. T cells were stained and analyzed by flow cytometry. A and B, respectively, show representative contour plots and graphics of frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs induced by patient's Mo-iDCs in the presence of anti-PD-L1 antibodies at 3.5μg/ml, 7.25 μg/ml, and 10.5 μg/ml (n=3; *$p<0.05$).

4.3.4 Monocytes from breast cancer patients failed to fully differentiate into M1-MΦ, by maintaining partial CD163 expression and producing high amounts of IL-10

As already showed, Mo-DCs differentiated from breast cancer patients presented a suppressive functionality. Herein, we went further to evaluate the potential of patient monocytes' to differentiate into MΦ. For that, we first investigated the basal levels of CD163 and GMCSF-R on blood monocytes by flow cytometry. Using gating strategy presented in figure 37A, we found that the frequency of $CD14^+$ monocytes and $BDCA1^+$ DCs was not altered in PBMCs obtained from breast cancer patients or healthy donors (data not shown). For the phenotype of $CD14^+$ monocytes, both CD163 and GM-CSF-R molecules showed the same profile of expression (MFI and percentages) in healthy donors and breast cancer patients (Figures 37B-C).

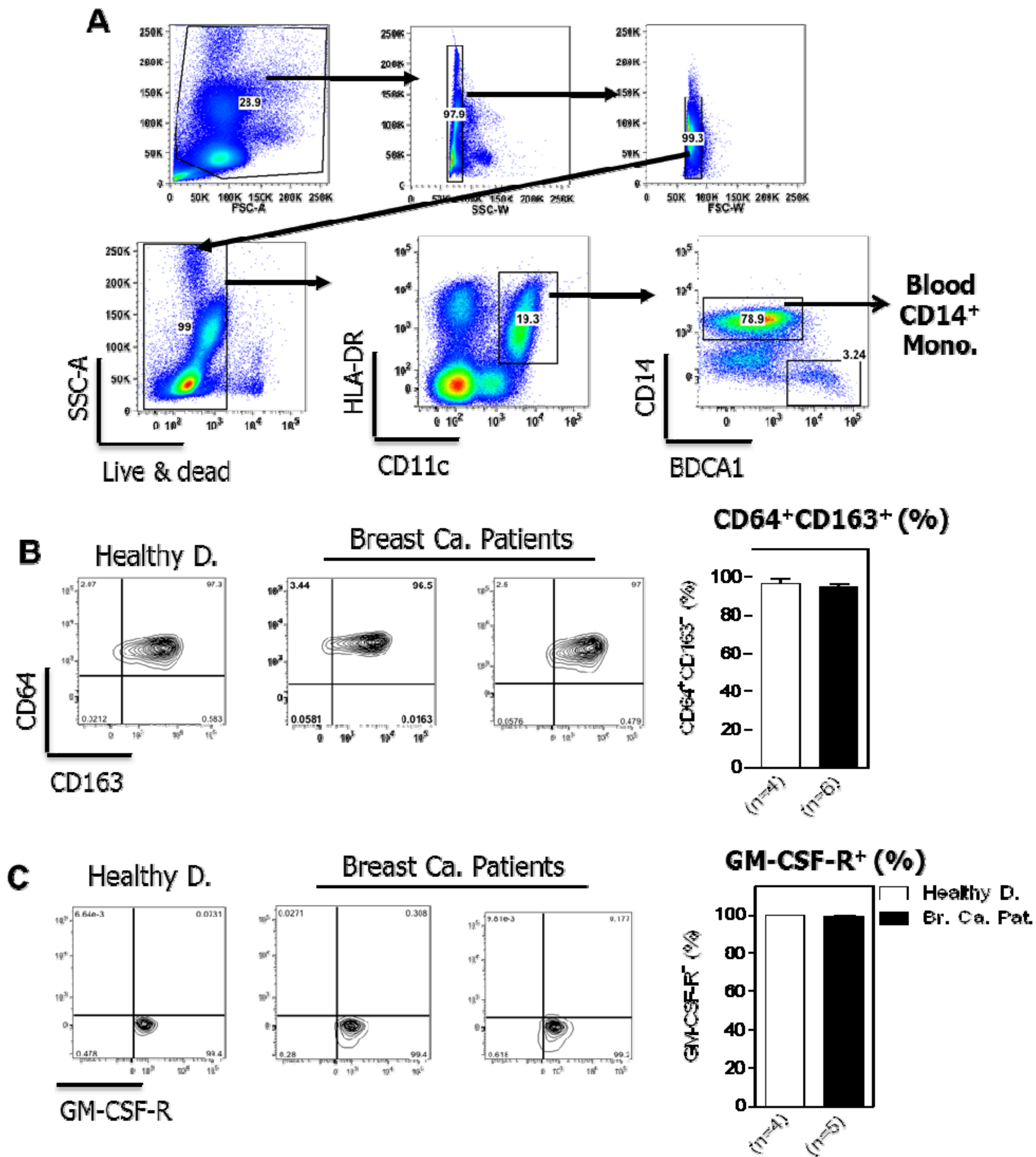


Figure 37. Blood monocytes from healthy donors and breast cancer patients display similar expression of CD163 and GMCSF-R. PBMCs obtained from healthy donors and breast cancer patients were submitted to flow cytometry analysis to evaluate the phenotype of CD14$^+$ monocyte. In A, representative pseudo-color graphics showing gate strategy to evaluate the phenotype of monocyte. Below, representative contour-plots and graphics of CD64$^+$CD163$^+$ (B) and GMCSF-R$^+$ (C) frequency of expression in gated CD14$^+$ monocytes from healthy donors and breast cancer patients.

Subsequently, we investigated the potential of $CD14^+$ blood monocytes from breast and ovarian cancer patients to differentiate *in vitro* into M1-MΦ (GM-CSF + IFN-γ) and M2-MΦ (M-CSF + IL-4), as described in methods. At day 7, the phenotype of resting or LPS-activated cells was evaluated by flow cytometry. Interestingly, 45% (10 from 22) of breast cancer patients' monocytes submitted to M1-MΦ protocol without any activation, maintained high frequency of CD163 expression, in contrast to healthy donors' M1-MΦ which strongly downregulated CD163 expression (Figures 38A-B). Similar data were found for ovarian cancer patients' monocytes that were not capable to fully differentiate into M1-MΦ in the resting status (Figure 38B). In addition, after LPS exposure, M1-MΦ derived from cancer patients' monocytes partially down-regulated CD163 expression, but part of patients maintained the same deviation and did not completely lose CD163 expression, unlike M1-MΦ from healthy donors (Figure 38). We also evaluated the expression of CD86 and PD-L1 molecules in M1-MΦ derived from patients' monocytes, but no differences were found between cancer patients and healthy donors (data not shown). In addition, we also performed the differentiation of patient's monocytes into M2-MΦ (M-CSF+IL-4), but no differences were noted (data not shown).

Moreover, we evaluated the production of cytokines by M0-MΦ and M1-MΦ derived from breast cancer patients' monocytes after 24hrs of LPS activation. We observed that differentiated M1-MΦ produced high levels of TNF-α in comparison to M0-MΦ, but no differences were found between M1-MΦ derived from patients and healthy donors' monocytes (Figure 39A). Regarding IL-10, patients' M1-MΦ produced significantly higher levels when compared to M1-MΦ from healthy donors (Figure 39B). It's interesting to note that M1-MΦ differentiated from healthy donors lost their ability to produce IL-10 in comparison to their relative M0-MΦ (non-differentiated), whereas M1-MΦ from cancer patients produced similar levels of IL-10 as M0-MΦ (Figure 39B).

To evaluate whether those phenotypic differences could impact the functions of macrophages, we performed suppression assay experiments to test if M1-MΦ derived from breast cancer patient's monocytes were able to suppress $CD4^+$ T cells (similarly to figure 22). In our preliminary data, M1-MΦ derived from cancer patient's monocytes were able to partially suppress anti-CD3/CD28-activated $CD4^+$ T

lymphocytes proliferation after 4 days of co-culture (Figure 39C). However, additional experiments are needed to confirm this interesting observation.

Altogether these results showed that monocytes from part of breast cancer patients presented a bias in the differentiation into M1-MΦ, as assessed by the maintained expression of CD163 and the high production of IL-10. Importantly, the experiments investigating the potential of monocytes to differentiate into M1-MΦ were performed with patients from CLB hospital in Lyon/France, while the experiments regarding Mo-DCs differentiation were conducted with samples from Perola Byington Hospital in Brazil. Although the study involves the differentiation of two distinct cell types (Mo-DCs and Mo-MΦ), the alterations found in monocytes from breast cancer patients did not seem to be associated with ethnic/genetic intrinsic variation.

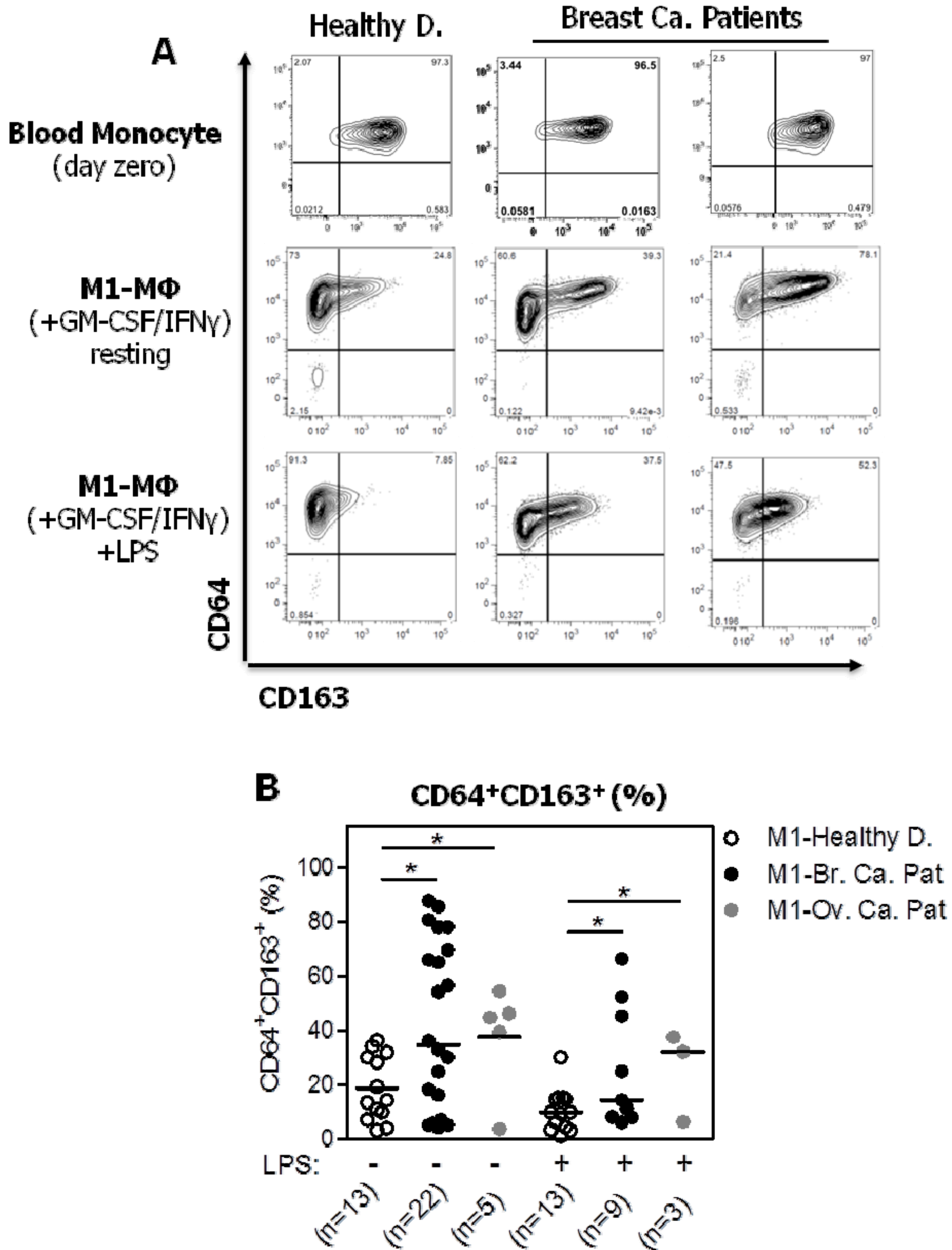


Figura 38. Blood monocytes from cancer patients fail to fully differentiate into M1-MΦ by maintaining CD163 expression. $CD14^+$ monocytes were isolated from breast and ovarian cancer patients' blood and cultivated in the presence of GM-CSF + IFN-γ (M1-MΦ settings, see methods). After 7 days, cells were stained and analyzed by flow cytometry in the gate of $live^+CD14^+$ cells. Representative contour-plots (A) and graphic (B) of $CD64^+CD163^+$ frequency of resting and LPS-activated M1-MΦ differentiated from healthy and patient's monocytes (*p<0.05).

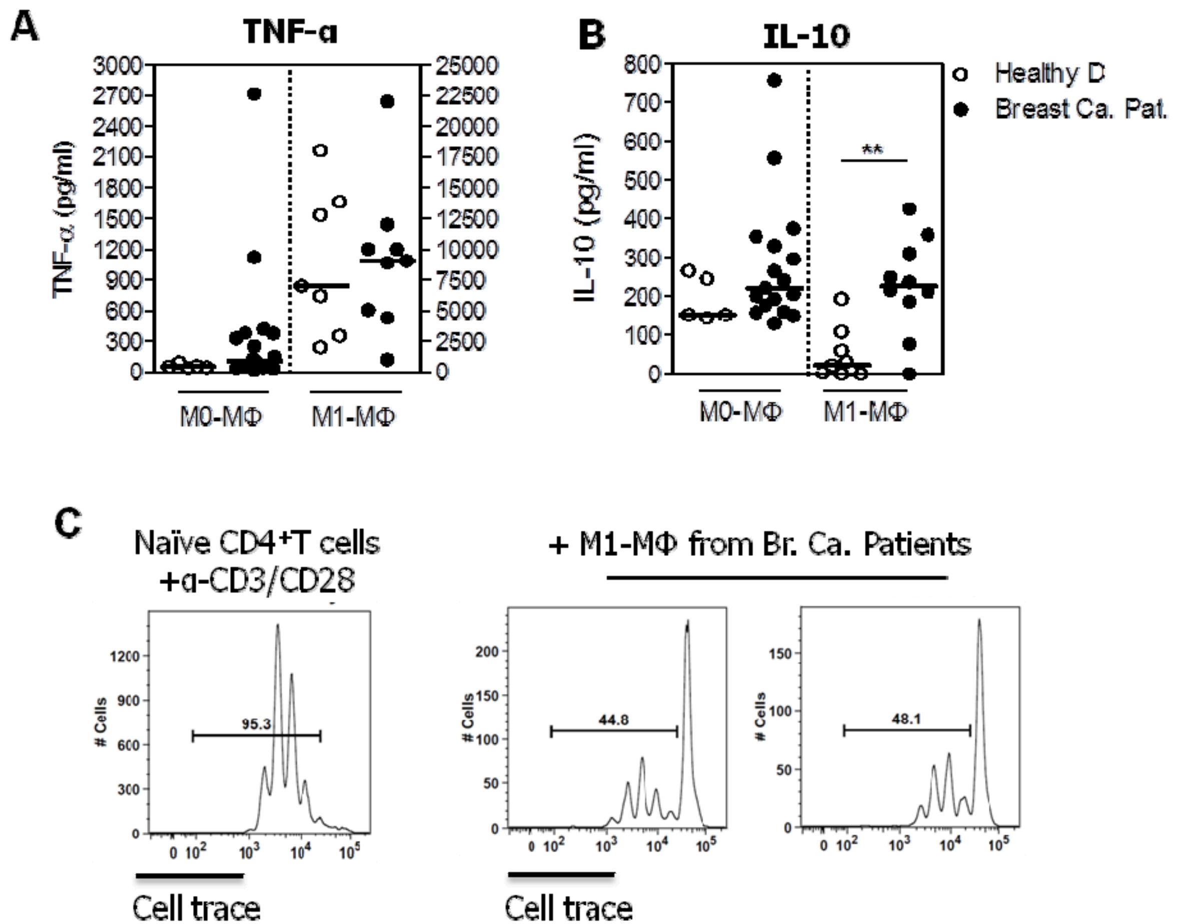


Figure 39. M1-MΦ derived from cancer patients' monocytes produce high amounts of IL-10 and partially block CD4$^+$ T cell expansion. CD14$^+$ monocytes were isolated from breast and ovarian cancer patients' blood and cultivated in cRPMI medium (M0-MΦ settings) or in the presence of GM-CSF + IFN-γ (M1-MΦ settings, see methods). After 7 days, supernatants from LPS activated cells were submitted to ELISA assays. A and B, respectively, TNF-α and IL-10 levels produced by cells derived from patients (black dots) and healthy donors (white dots) (n= 5 at least; **p<0.01). In C, allogeneic naïve CD4$^+$ T cells were pre-activated with anti-CD3/CD28 beads and co-cultured with M1-MΦ derived from breast cancer patients' monocytes; histograms represent T cell expansion after 4 days of co-culture by the assessment of cell trace dilution (n=2 representative experiments).

4.3.5 CD14$^+$ monocytes from breast cancer patients' blood display an anti-inflammatory profile of cytokine production

We have shown here that monocytes from breast cancer patients generated suppressive Mo-DCs and also failed to differentiate properly into M1-MΦ *in vitro*. Those differentiated APCs displayed suppressive characteristics concerning phenotype and functional capabilities. Herein, trying to investigate the step before differentiation, CD14$^+$ monocytes were isolated from breast cancer patients or healthy donor's blood and incubated in the presence of LPS for 24 hours. Subsequently, supernatants were collected and different cytokines were evaluated by multiplex assay.

Analyzing a total of 10 healthy donors and 20 breast cancer patients' blood samples (Table 3) in two rounds of experiments, the following molecules were not detected in the assay: CCL11, CX3CL1, CXCL10, EGF, IFN-α, IL-2; IL-5; IL-12-p70; IL-13; IL-17A; PDGF-BB; SCF, TGF-α, TNF-β, VEGF-D. Considering the detected molecules, we found that cancer patients' monocytes produced significantly higher levels of IL-10, VEGF-A, IL-27, sCD40L, IL-21, and IL-1RA compared to healthy donors' monocytes (Figures 40A-B). At the same time, cancer patients' monocytes also secreted significantly lower amounts of IL-23 and CXCL1 than healthy donors' monocytes (Figures 40A-B). For CCL5, GM-CSF, CCL7, IL-22, IL-1α, IL-1β, TNF-α, IFN-β, MIF, CXCL12, TRAIL, IL-15 and IL-9, even some tendencies was noted, no significant differences were noted between monocytes from cancer patients and healthy donors (Figure 40A). Additionally, for M-CSF, we analyzed data in two rounds of experiments we found that monocytes from patients produced elevated levels of M-CSF in comparison to healthy donors' monocytes, even just the first round showed statistical differences (Figure 40C).

Our analysis of correlation also suggests that monocytes from cancer patients have a differential pattern of cytokine secretion. We noted for patients' monocytes, and not from healthy donors, an interestingly positive correlation in concomitant production of several molecules (Figure 41) as: IL-10 x sCD40L (A); CCL5 x sCD40L (B); IL-27 x IL-10 (C); IL-21 x IL-1RA (D); and a negative correlation in IL-27 x CXCL1 (E). Moreover, M-CSF x IL-1RA and IL-6 x IL1-RA were also correlated, but this phenomenon was observed similarly in patients and healthy donors' monocytes (data not shown).

It is important to note certain variability among breast cancer patients' monocytes. Indeed, about 30% of patients produced similar levels of cytokine as healthy monocytes. This heterogeneity was also found for the differentiation of breast cancer patients' monocytes into M1-MΦ (Figures 38-39), where about 45% of patients' monocytes presented the alteration.

More interestingly, we noted that patients' monocytes that were not fully differentiated into M1-MΦ were the same as those producing higher levels of CCL5, sCD40L, VEGF-A, and IL-10 after 24hrs of LPS activation, in comparison to those that acquired a classical M1-MΦ phenotype (Figure 42). These findings need to be confirmed by increasing the number of patients and performing a systematic analysis of the basal characteristics of fresh collected monocytes and of their potential to differentiate into Mo-DC and/or M1-MΦ. Of note, we observed a tendency for monocytes from patients with lobular invasive carcinomas (data not shown) to produce more IL-1β, IL-10, and IL-21, but the differences found for the production of cytokines by monocytes or for Mo-DC and M1-MΦ differentiation were not statistically associated with the molecular or morphological characterization of breast tumors for the evaluated cohort of patients with breast cancer.

Table 3 – List of breast cancer patients which monocytes were submitted to multiplex analysis of cytokines

N.	cod. Pat	Age at blood collection	Morphologic classification	Molecular classification
1	1421188	65	IDC	Luminal B
2	1419756	42	IDC	Luminal B
3	1422271	50	ILC	Luminal B
4	1422853	74	ILC	Luminal A
5	1421991	65	IDC	triple-negative
6	1424413	36	IDC	Luminal A
7	1423857	64	ILC	Luminal A
8	1423627	42	ILC	Luminal A
9	1425030	51	IDC	Her2+
10	1424235	47	IDC	Luminal A
11	1411702	73	ILC	Luminal A
12	1425343	84	IDC	Her2+
13	1423772	88	IDC	Luminal A
14	1424414	52	IDC	Lum. A or B (indef.)
15	1501106	82	ILC	Luminal A
16	1425993	82	IDC	triple-negative
17	1402532	55	ILC	Lum. A or B (indef.)
18	1503117	73	mixed IDC/ILC	Lum. A or B (indef.)
19	1502383	48	IDC	Lum. A or B (indef.)
20	1411956	80	mixed IDC/ILC	Luminal A

IDC= Invasive Ductal Carcinoma; ILC= Invasive Lobular Carcinoma

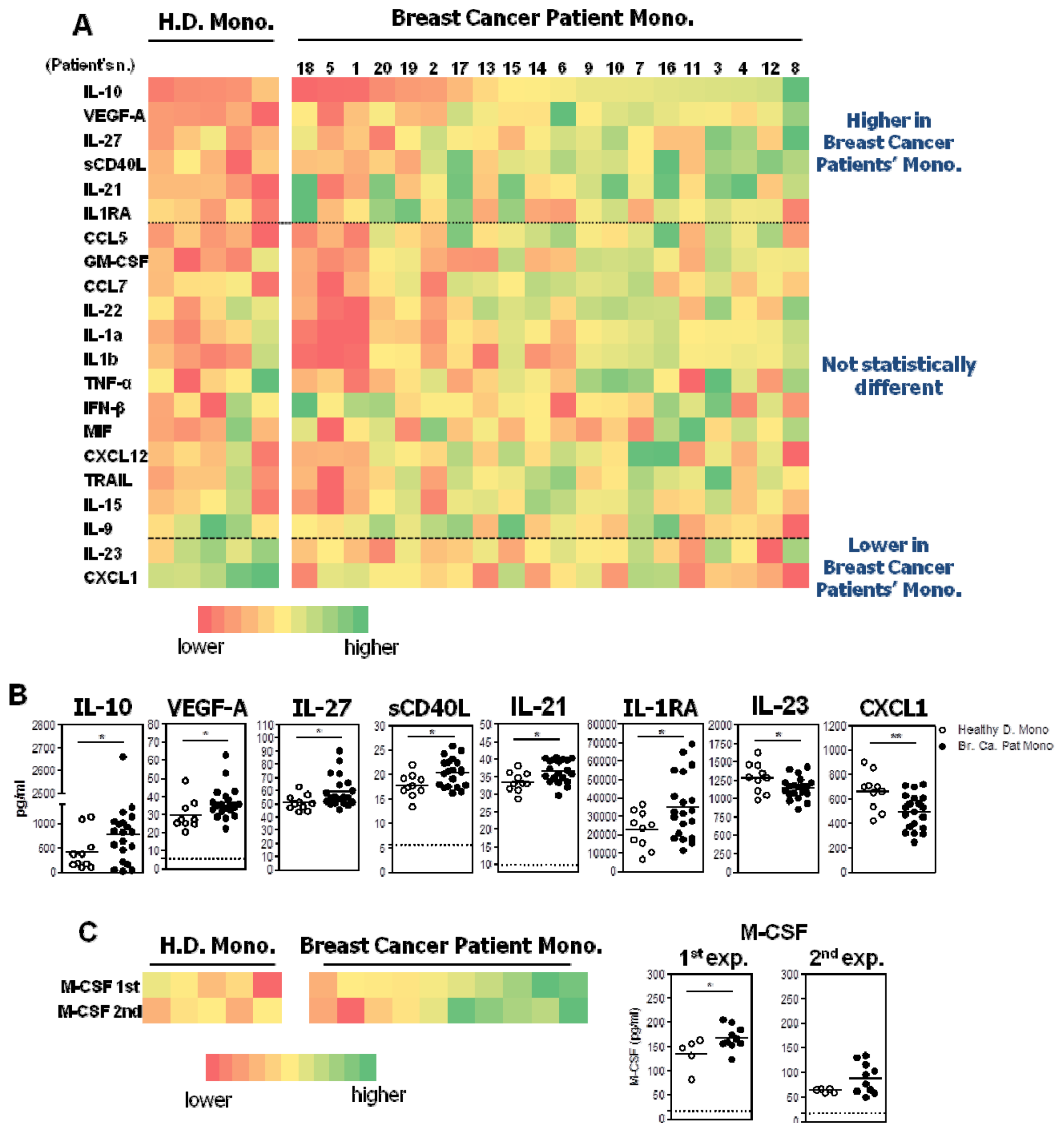


Figure 40. CD14+ blood monocytes from breast cancer patients produce elevated levels of suppressive cytokines. CD14+ monocytes were isolated from breast cancer patients' blood and incubated by 24 hours in presence of LPS. Later, supernatants were collected and submitted to multiplex for cytokine analysis comparing healthy versus patients' monocytes. In A and B, respectively, heat-map and graphics showing diverse cytokines produced by healthy versus patients' monocytes. In C, M-CSF production by monocytes represented in two rounds of experiments. For all graphics, *p<0.05 and **p<0.01.

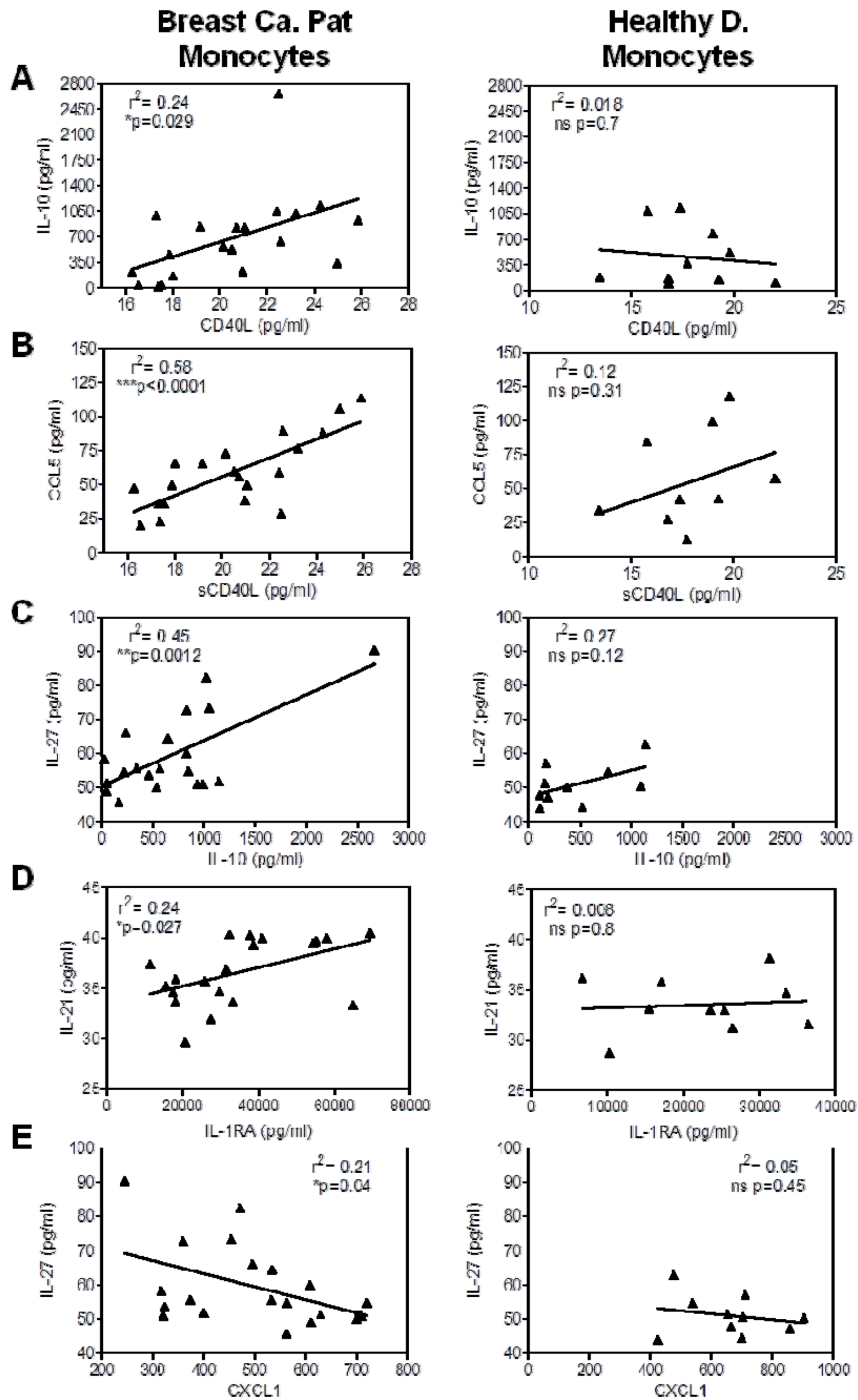


Figure 41. CD14$^+$ monocytes from breast cancer patients tended to display a differential profile of correlation among produced cytokines. CD14$^+$ monocytes were isolated from breast cancer patients' blood and activated by LPS for 24 hours. Cytokine levels were determined in cell culture supernatants by multiplex analysis comparing healthy versus patients' monocytes. A to E, graphics representing the correlation of several cytokines produced by patients and healthy donors' monocytes. For all graphics, ns=non significant, *p<0.05 and **p<0.01.

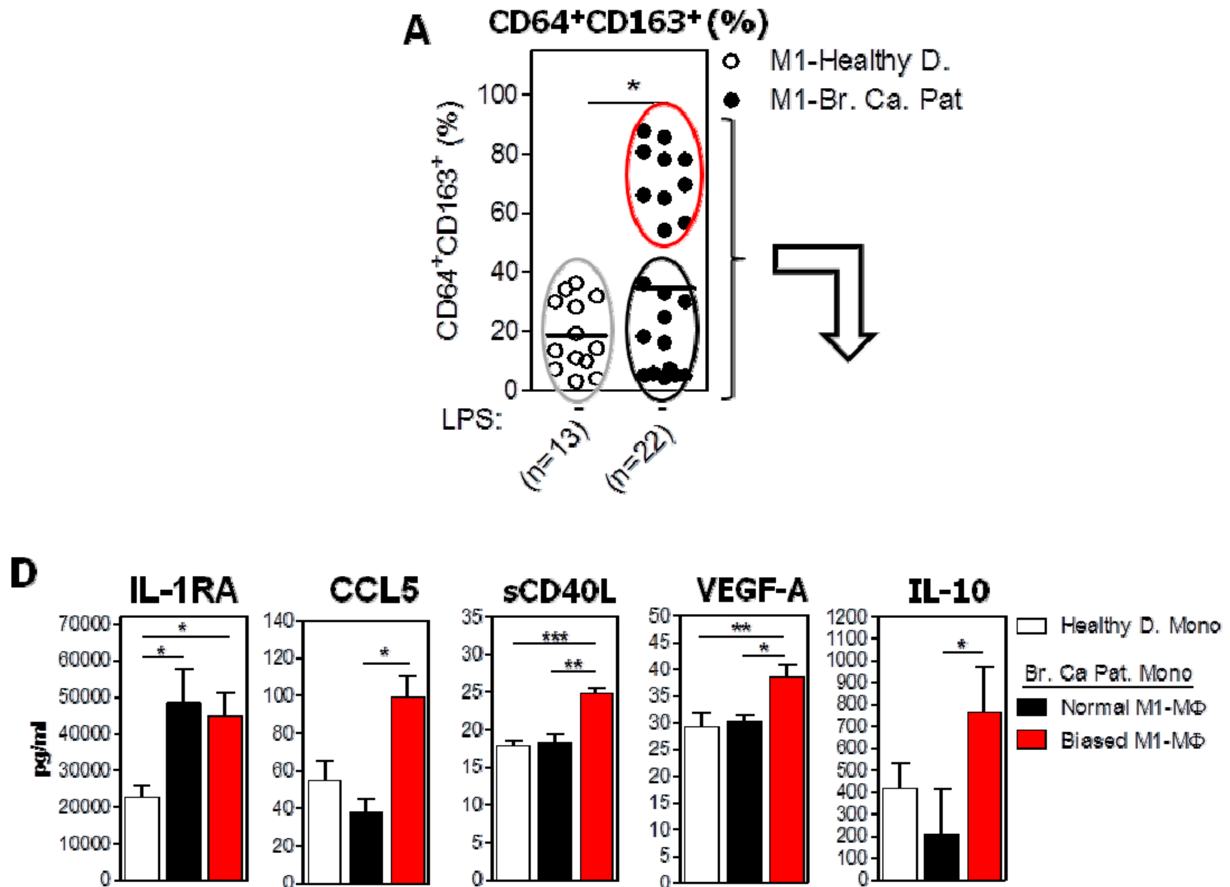


Figure 42. Monocytes from breast cancer patients that do not fully differentiate into M1-MΦ, produce elevated levels of CCL5, sCD40L, VEGF-A and IL-10. $CD14^+$ monocytes were isolated from healthy donors and breast cancer patients' blood. Part of cells was submitted to M1-MΦ differentiation *in vitro* (see methods) or was directly activated by LPS for 24 hours. Then, M1-MΦ were phenotypically evaluated (as Figure 38), while supernatants from LPS-activated monocytes from same donors were submitted to multiplex analysis of cytokines. In A, $CD64^+CD163^+$ frequency in M1-MΦ differentiated from healthy and patients' monocytes. In B, cytokines produced by healthy (white bar, n=10) and patients' monocytes with a normal M1-MΦ differentiation (black bar, n=5) versus those did not fully differentiated into M1-MΦ (red bar, n=3), (*p<0.05 and **p<0.01).

5 DISCUSSION

Our findings strongly point out possible explanations for the generation/accumulation of dysfunctional myeloid cells (MΦ and DCs) in tumor sites. Firstly, we identified candidate soluble factors derived from the tumor microenvironment *ex-vivo* that are able to bias monocytes towards immunosuppression. Secondly, we show that circulating blood monocytes from cancer patients are altered, displaying suppressive features instead of the expected ability to stimulate adaptive immunity. Thus, we hypothesize that this compromised differentiation process of monocytes, both locally, in the tumor microenvironment, and systemically, affecting the circulating monocytes, gives rise to biased myeloid APCs, which contribute to the failure of immune responses against cancer.

This hypothesis was elaborated from results obtained by two main questions investigated here: 1) How the tumor microenvironment contributes for TAMs' differentiation? 2) Is there a previous commitment of circulating monocytes that modifies TAMs' differentiation and, thus, cancer progression?

Investigating the leukocyte infiltration of breast tumors, we noted that $CD14^+CD64^+$ TAMs represented up to 30% of all $CD45^+$ cells in the infiltrate. This is in contrast to the composition of non-neoplastic breast tissue leukocyte infiltration, which shows only about 5% of CD14+ cells (RUFFELL et al., 2012). This increase in tumors suggests that, also in humans, these cells may have a role in tumor progression, contrasting to myeloid DC, $BDCA1^+CD14^{neg}$, which represented about 0.4% within $CD45^+$ leukocytes, but whose presence seems not to impact in cancer patient's survival, in contrast to what has been shown for plasmacytoid DC infiltration (TREILLEUX et al., 2004; LABIDI-GALY et al., 2011). Indeed, tumor-infiltrating myeloid DCs were described as immature and dysfunctional (BELL et al., 1999; GERVAIS, 2005; DIEU-NOSJEAN et al., 2008), and may contribute indirectly to tumor angiogenesis (FAINARU et al., 2008). Interestingly, we also identified $CD14^+BDCA1^+$ cells (0.8% among $CD45^+$ leukocytes), which have been called "inflammatory DCs", and were recently described in human ovarian cancer ascites showing a very similar pattern of gene signature to monocyte-derived DCs (SEGURA et al., 2013b).

The phenotypic definition of TAMs in human cancer is still very controversial. Over a long period, CD68 was used to define human TAMs, however, recent studies have used CD163, instead, to better characterize these cells (HEUSINKVELD; VAN DER BURG, 2011). This was mainly due to the fact that other immune cells and

fibroblasts may express CD68 (RUFFELL et al., 2012). In face of the heterogeneity of these cells, we decided to expand the analysis panel, using CD14, CD64 and CD163 to define these cells. The inclusion of CD64 was based on the description of recent papers, used to distinguish Macrophages from DCs (TAMOUTOUNOUR et al., 2012; reviewed by DE CALISTO et al., 2012). Our findings underscore the heterogeneity of TAMs, based on the expression of CD163. Interestingly, depending on the patient, $CD14^{+}CD64^{+}$ TAMs expressed CD163 at different levels, defined here as $CD163^{low}$ and $CD163^{high}$ subsets. Actually, CD163 has been recently associated to TAMs subsets that present anti-inflammatory features (REINARTZ et al., 2014), a definition originating from studies of M2-MΦ differentiated *in vitro*, under the influence of M-CSF (JAGUIN et al., 2013; LACEY et al., 2012; VOGEL et al., 2014). Normally, it is expected that monocytes, which are $CD163^{+}$, when migrating into sites of chronic infections or inflammation, up-regulate CD163 and become M2-like MΦ ($CD163^{high}$) to avoid further tissue damage and to promote clearance, helping to re-establish homeostasis. However, it is interesting to note that after the control of infection/inflammation, M2-MΦ progressively disappear from tissues, while, in tumor sites, $CD163^{high}$ TAMs accumulate contributing to the local anti-inflammatory environment.

The relevance of $CD163^{high}$ TAMs for the natural history of human breast cancer was strongly supported by our *in situ* IHC analysis that revealed a positive association between poor prognosis and high infiltration by $CD163^{+}$ TAMs. These findings are in accordance with other recent published studies using CD163 as a prognostic marker for breast (KRÜGER et al., 2013; MEDREK et al., 2012; SOUSA et al., 2015; TIAINEN et al., 2015), renal (DANNENMANN et al., 2013), ovarian (REINARTZ et al., 2014), and pancreatic cancers (INO et al., 2013). It is important to mention, that patients presenting the more aggressive molecular subtypes of breast cancer, Luminal B, $Her2^{+}$ and basal-like, presented an elevated frequency of $CD163^{+}$ TAMs. These findings are in accordance with Medrek and collaborators (2012) describing an increased frequency of $CD163^{+}$ TAMs and poor rate of survival in patients with triple-negative breast cancers, contrary to what was found in Luminal A breast cancers. Still, it is important to consider that newly infiltrating $CD163^{+}$ monocytes (not yet differentiated into TAMs) may be part of the total $CD14^{+}CD64^{+}$ cells we evaluated here, since both TAMs and monocytes share these markers. In fact, no study clearly described the participation of blood monocytes in the

development of human breast cancers, mainly due to the lack of specific markers to define this subpopulation and their similarities and differences when compared to TAMs. Indeed, when analyzing under microscopy the sample of one patient, we have identified two morphologically different CD163+ cells among sorted CD11b+HLA-DR+CD14+ cells, but only one among those that did not express CD163 (data not shown).

Although we found a variable composition of $CD163^{low}$ versus $CD163^{high}$ TAMs, the accumulation of total $CD14^{+}$ TAMs, independently of CD163 status of expression, was associated to low numbers of $CD3^{+}$ T cells infiltration in tumors, suggesting that TAMs may inhibit T lymphocyte migration into tumors. The selectivity of T cell infiltration inhibition was supported by the fact that TAMs had no impact on the frequency of total $CD45^{+}$ leukocytes found in the samples, where neither B cells nor myeloid DCs numbers were affected by the frequency of TAMs. It would be very interesting to define more precisely the link between TAM infiltration and the pattern of infiltrating $CD3^{+}$ T cells, since several studies have shown that higher Th1 and $CD8^{+}$ infiltration are strongly correlated to good patient's outcome, whereas Th2 and Treg infiltrate are normally correlated to worse prognosis (reviewed by FRIDMAN et al., 2012). Here, on the other hand, we found that regardless of the TAMs subset, the presence of these cells is correlated to low T cell infiltration, while only the presence of $CD163^{high}$ TAMs was associated with worse prognosis.

We also noted that CD163 expression was associated with a “suppressive phenotype”, since $CD163^{high}$ TAMs presented higher PD-L1 expression at the basal level and after LPS activation and increased their production of IL-10, when compared to $CD163^{low}$ TAMs. Other works also showed TAMs can produce immunosuppressive factors such IL-10 and TGF-beta (BISWAS et al., 2006; TORROELLA-KOURI et al., 2009). Ogino and collaborators recently showed (2013) in an elegant study of colorectal carcinoma, that $CD14^{+}CD163^{high}$ TAMs were high producers of IL-10 *ex-vivo*. Other study in a mouse model demonstrated that IL-10 produced by TAMs from mammary carcinoma can block the ability of intratumoral DCs to produce IL-12, thus avoiding anti-tumor $CD8^{+}$ T cell responses even under chemotherapy (RUFFELL et al., 2014). Suppressive TAMs were also reported in pancreatic cancer (SANFORD et al., 2013) and in HPV-associated tumor model (LEPIQUE et al., 2009). Some studies reported other possible mechanisms for TAMs' suppressive abilities. Isolated TAMs were able to suppress T cell expansion *via* B7-

H4 up-regulation in ovarian cancer (KRYCZEK et al., 2006) and *via* PD-L1 in hepatocellular carcinoma (KUANG et al., 2009). In a mouse model of squamous cell carcinoma (BELAI et al., 2014), a critical role of infiltrating TAMs in suppressing anti-tumor responses via PD-1/PD-L1 axis was described. Interestingly, we also found up-regulated PD-L1 in CD163^{high} TAMs. These studies open a range of possibilities by which TAMs could suppress T lymphocytes, but, nevertheless, IL-10 seems to be centrally involved in the phenomenon, as we will discuss ahead, but did not investigate thoroughly yet.

Even though the bipolar model (M1 x M2) facilitates the comprehension of MΦ polarization, it may not describe precisely the phenomenon in tumors. Actually, as extensively discussed lately (reviewed by RUFFEL; COUSSENS, 2015), TAMs can be better defined by their functional status at different steps of disease progression, considering a larger phenotypic spectrum, including the release of soluble factors and functional assays and not only by their surface phenotype. Although tumor-derived factors have been extensively studied, most of the work in humans have used cancer cell lines as the source and very few studies have analyzed the properties of the whole human tumor microenvironment, as reported previously by our group (LABIDI-GALY et al., 2011; SISIRAK; FAGET et al., 2012; SISIRAK et al., 2013). The later studies, obtained tumor derived factors from tumor samples kept in culture without manipulation, and focused their attention on pDCs, while we disrupted the tumor samples in small volumes of medium and used this medium as a carrier for the factors present in the microenvironment and studied their action upon monocytes. It should be stressed that, in both cases, these factors are the product of different cells as a result from the interaction of tumor cells, stroma, immune infiltrate and soluble molecules in the *millieu*.

We found that factors derived from tumor microenvironment (SNDil), depending on the patient, could skew the spontaneous monocyte differentiation into one of two distinct macrophage-like subsets: I) CD163^{low}CD86^{low}PD-L1^{low}IL-10^{low} and II) CD163^{high}CD86^{low}PD-L1^{high}IL-10^{high} SNDil-MΦ. It is noteworthy that, while CD163^{high} SNDil-MΦ shared similarities with M2-MΦ, CD163^{low} SNDil-MΦ were closer to undifferentiated M0-MΦ. Despite this heterogeneity, it is important to mention that SNDils never generated Mo-DCs nor M1-MΦ, since all SNDil-MΦ expressed CD64 and maintained and/or up-regulated CD163, markers already used

by other groups to exclude Mo-DCs or M1-MΦ differentiation (BUECHLER et al., 2000; VOGEL et al., 2014).

CD163 expression analysis on SNDil-MΦ revealed a very similar patter to that observed on TAMs. This suggests that signals from the tumor microenvironment trigger pathways that induce TAMs to up-regulate CD163 expression, a phenomenon that occurs both *in vivo* (in TAMs) and *in vitro* (in SNDil-MΦ). IL-10 could explain this phenomenon, since when the cytokine and its receptor were blocked during M2-MΦ *in vitro* differentiation, no CD163 up-regulation was noted. However, no IL-10 was detected in the SNDil. This indicated that though IL-10 might be involved, it is probably not the initiator of the phenomenon, but, likely, induced by other factor(s) in the SNDil. IL-10 signaling, besides up-regulating CD163, also increased PD-L1 expression, but did not interfere with CD80 and CD86. Similar results were already shown by other studies were IL-10 was shown to be involved in: PD-L1 up-regulation in HIV-infected human macrophages (RODRIGUEZ-GARCIA et al., 2011); CD163 expression in macrophages differentiated *in vitro* (BUECHLER et al., 2000) and CD163 expression on DCs isolated from human skin (LINDENBERG et al., 2013). Despite the need for M-CSF + IL-4 during M2-MΦ differentiation process, it seems that their capacity to produce IL-10 is critical to shape their final phenotype. Similarly, SNDil-MΦ also showed a positive correlation between their levels of IL-10 production and CD163 expression. In contrast, M1-MΦ and Mo-DCs, that produce low levels of IL-10, but high TNF-α amount, did not maintain/up-regulate CD163 or PD-L1 expression *in vitro*. It is interesting that, also in other studies, the regulation of CD163 was associated with the presence of pro-inflammatory *versus* anti-inflammatory cytokines, where pro-inflammatory cytokines such as IFN-γ and TNF-α induce CD163 down-regulation, whereas, IL-6, IL-10 and TGF-β were able to induce CD163 up-regulation on monocytes *in vitro* (BUECHLER et al., 2000; VOGEL et al., 2014). Thus, when analyzing our data on SNDil-MΦ and TAMs, we could suggest that the “final” phenotype of the cells depends on different steps: initially, the signals that trigger their differentiation and later on, their own capacity to produce cytokines (e.g. IL-10 or TNF-α) that will act to maintain/change or block their final differentiation.

As to other markers, SNDil-MΦ acquired a phenotype that suggested a bias in their ability to stimulate lymphocytes, since we found down-regulation of CD86, in both $CD163^{low}$ and $CD163^{high}$ and an increased of PD-L1 expression and IL-10 production in $CD163^{high}$ SNDil-MΦ when compared to control M0-MΦ. IL-10 can be

considered the major anti-inflammatory cytokine and is produced by several immune and non-immune cells, including tumor cells (GASTL et al., 1993). IL-10 inhibits the expression of MHC-II and the up-regulation of CD80 and CD86 (BUELENS et al., 1995; KOPPELMAN et al., 1997), as observed here. Previous data obtained by our group also showed that tumor cells products can induce de production of IL-10 and PGE_2 by monocytes (MÉNÉTRIER-CAUX et al., 1999) and were able to inhibit optimal DC maturation (THOMACHOT et al., 2004). Coherently, we also noted that $CD163^{high}$ SNDil-MΦ have not only a weak ability to induce naïve $CD4^+$ T cell proliferation, but also a high efficiency to suppress activated-$CD4^+$ T lymphocytes, as do M2-MΦ. These findings were associated to the ability to inhibit the production of IFN-γ, IL-2, GM-CSF, and IL-13 by anti-CD3/anti-CD28-activated $CD4^+$ T lymphocytes. Other authors also described that IL-10 itself can inhibit both proliferation of $CD4^+$ T cells and the production of IL-2, IL-5, TNF-α and IFN-γ (GROUX et al., 1996). Also, IL-10 inhibits the release, by monocytes/macrophages, of pro-inflammatory mediators such as TNF-a, IL-1b, IL-6, IL-8, G-CSF and GMCSF (MACATONIA et al., 1993); restrains DC maturation and differentiation from monocyte precursors (COMMEREN et al., 2003); and hinders human monocytes in their ability to secrete IL-1a, IL-1b, IL-6, IL-8, TNF-a, and G-CSF (DE WAAL MALEFYT et al., 1991). Thus, it is not surprising that most of the inhibitory properties of IL-10 are due to their effect on APCs (DE WAAL MALEFYT et al., 1991). Curiously, $CD163^{low}$ SNDil-MΦ also presented suppressive capabilities, though less accentuated than those of $CD163^{high}$ SNDil-MΦ or M2-MΦ. These findings suggest a “ranking” of suppressive skills, which seems to be related to the amount of IL-10 secretion by the APCs, highlighting, thus, the importance of additional investigation to determine the mechanism by which SNDil drives monocytes to be high producers of IL-10.

$CD163^{high}$ SNDil-MΦ and $CD163^{high}$ TAMs, though originating in different contexts, share similarities in phenotype and function: expression of high levels of PD-L1 and production of elevated amounts of IL-10 (when compared to their $CD163^{low}$ SNDil-MΦ and $CD163^{low}$ TAMs counterparts, respectively). For $CD163^{high}$ SNDil-MΦ, the role of the IL-10/IL-10R pathway was confirmed, since when this pathway was blocked by both anti-IL-10 and anti-IL-10R, we found an improvement of T cell expansion and a restoration of Th1 cytokines production (IFN-γ, TNF-α, and GM-CSF). Also, in the same context, we found an augmentation of other

cytokines/chemokines, probably produced by the APCs (IL-6, IL12p70, and CCL22). IL-10 seemed also to modulate PD-L1 and PD-L2 expression by M2-MΦ and SNDil-MΦ, since the blocking of its pathway reduced the expression of both surface molecules. Furthermore, the suppressive effects of these molecules were synergized by IL-10, since their blocking alone was not efficient to recover T cell functions, which only occurred with the presence of anti-IL-10/IL-10R mAb.

It is important to mention that SNDil-MΦ may suppress T cells by other signals, not identified in our experiments. Even if IL-10 blockage seemed to “break” the suppressive loop, it was not a complete restoration of function, thus it remains unclear what cytokines or surface molecules contribute to the suppression we observed. Herein, we should also consider the kinetics of cytokine production *versus* consumption and the turnover of surface receptors, since blocking anti-IL-10 and anti-PD-L1 mAbs were added only once, at the beginning of the co-cultures – maybe, new proliferating T cells appeared and, after 4 days of co-culture, were not covered by the added mAbs, masquerading the role of these molecules in the phenomenon.

In the attempt to explore additional surface markers to define SNDil-MΦ and TAMs, CD200R emerged as a likely candidate. CD200R was expressed by M1-MΦ, M2-MΦ and Mo-DCs, but not by monocytes or M0-MΦ and thus, might be used to discriminate these stages of differentiation. CD200R is the receptor for the membrane glycoprotein CD200, also called OX-2. As reviewed by Rygiel and Meyaard (2012), CD200 is basally overexpressed on B cell chronic leukemia, hairy cell leukemia, ovarian and melanoma cancers, but can also be up-regulated during cancer development, as in metastatic squamous cell carcinoma. In our context, CD200R expression was low on both CD163^{low} and CD163^{high} SNDil-MΦ, suggesting that SNDil-MΦ were not completely differentiated as compared to APCs differentiated *in vitro.* Yet, since we did not evaluate CD200R expression on TAMs from breast or ovarian tumors and no other study specifically addressed this question in humans, it is impossible to take this speculation further at the moment.

Once SNDil-MΦ were phenotypically and functionally characterized, our study attempted to decipherer which signals derived from the each tumor microenvironment might skew monocytes into suppressive cells. Analyzing more than 40 different molecules in tumor supernatants, we found that the expression of CD163 on SNDil-MΦ was positively correlated to TGF-β1 and CCL22, while the production of IL-10 was positively associated to TGF-β3 and CCL22 presence in SNDils.

Interestingly, a study published by our group reported that the production of CCL22 by tumor cells is due to the cooperation of monocytes and NK cells in the microenvironment, a mechanism dependent on IL-1β and TNF-α signaling (FAGET et al., 2011). Accordingly, it's already known that CCL22 can be also produced by diverse MΦ and DC subsets (MANTOVANI et al., 2000) and promote the recruitment of Treg cells to tumor sites (GOBERT et al., 2009). In addition, all APCs differentiated *in vitro* in our study were able to produce CCL22 (data not shown) and an increased production of CCL22 was detected in MΦ-T cells co-cultures in the presence of anti-IL-10/IL-10R mAbs. In this context, CCL22 seems to be involved in several processes of immune regulation; however its role in the differentiation process of monocytes was not yet addressed in the literature. Nevertheless, it's seems more reasonable to assume that CCL22 in the tumor microenvironment is, actually, related to the number of TAMs and their crosstalk with tumor cells than to the differentiation pathway of monocytes in the microenvironment.

Elevated levels of TGF-β were found in SNDils and the presence of the three isoforms (TGF-β1, TGF-β2, and TGF-β3) were associated to one or more phenotypic characteristics of SNDil-MΦ. It is already established that TGF-β is involved in the regulation of T cell expansion and function, preventing the development of autoimmune diseases (KEHRL et al., 1986; GORELIK et al., 2000), and, herein, we showed that its presence in the tumor microenvironment was also associated to the differentiation of blood monocytes into suppressive $CD163^{high}IL\text{-}10^{high}$ SNDil-MΦ. Other studies have revealed that the addition of TGF-β in monocyte cultures promotes a more suppressive phenotype only when in association to M-CSF (MIA et al., 2014), while another article described a down-regulation of CD163 expression in human monocytes incubated with TGF-β alone (PIOLI et al., 2004). Interestingly, Shabo and colleagues (2008) described that *in situ* CD163 positivity was associated to TGF-beta expression in breast cancer tissues. Other studies also described TGF-β as able to promote the chemotaxis of monocytes (WAHL et al., 1987), to participate in the process of Langerhans cells development (BORKOWSKI et al., 1996) and in the inhibition of function of several immune cells, including MΦ, NK and B cells (Reviewed by LI et al., 2006). In addition, in murine cancer models, tumor cells that were able to produce high amounts of TGF-β grew faster, by evading the immune surveillance of the host (CHANG et al., 1993; FAKHRAI et al., 1996). More importantly, a recent study by our group also revealed TGF-beta as a critical factor

that inhibits IFN-α production by plasmocytoid DCs in human breast cancer (SISIRAK et al., 2013). Nevertheless, the tumor microenvironment contains several soluble factors and it is reasonable to assume that not only TGF-β but a mix of distinct signals is responsible for phenotype of SNDil-MΦ. Curiously however, though other authors have described the presence of high concentrations of several anti-inflammatory molecules in the tumor microenvironment, including TGF-β, IL-10, and prostaglandin E2 (PGE_2) (GABRILOVICH, 2004; IKUSHIMA; MIYAZONO, 2010), in our case, other mediators may be involved. Though we did not evaluate the presence of PGE_2 in SNDils, IL-10 and TNF-α were not detected in any of the samples analyzed.

On the other hand, elevated amounts of CCL19, CCL21, IL-8, M-CSF, and VEGF were detected in SNDils that induced the differentiation of monocytes into $CD163^{high}IL\text{-}10^{high}$ SNDil-MΦ. Although CCL19, CCL21, IL-8 are chemokines involved in leukocyte recruitment, the potential of these factors to induce monocyte differentiation is less expected. Contrastingly, GM-CSF and M-CSF, respectively, are classically known as the main regulators of M1-MΦ and M2-MΦ differentiation *in vitro* (LACEY et al., 2012). Interestingly, GM-CSF was not detected in the SNDils, while M-CSF was present in almost all analyzed samples. Studies reported previously by our group showed that IL-6 and M-CSF derived from tumor cell lines were able to skew monocytes into macrophage-like cells (MÉNÉTRIER-CAUX et al., 1998; MÉNÉTRIER-CAUX et al., 2001). Indeed, the role of M-CSF was reported in several mouse models of cancer and human tumors, where TAMs play a critical role in tumor growth. The use of mAbs targeting the M-CSF receptor (also called CSF-1R) alone or in combination with additional therapies (chemotherapy, radiotherapy, others), showed the reduction of $CD163^+$ TAMs in cancer patients with diverse cancer types (RIES et al., 2014); prevention of pancreatic ductal adenocarcinoma development (MITCHEM et al., 2013); interference with the carcinogenic process in cervical cancer (STRACHAN et al., 2013); improvement of the survival of mammary tumor-bearing mice (DENARDO et al., 2011), and the regression of glioblastoma multiforme (PYONTECK et al., 2013). Considering the classical function of M-CSF in the differentiation, survival, and functionality of TAMs (DENARDO et al., 2011; RUFFELL et al., 2015) and its use *in vitro* for M2-MΦ differentiation, we may assume its critical role in the generation of SNDil-MΦ. However, we still need to verify this hypothesis

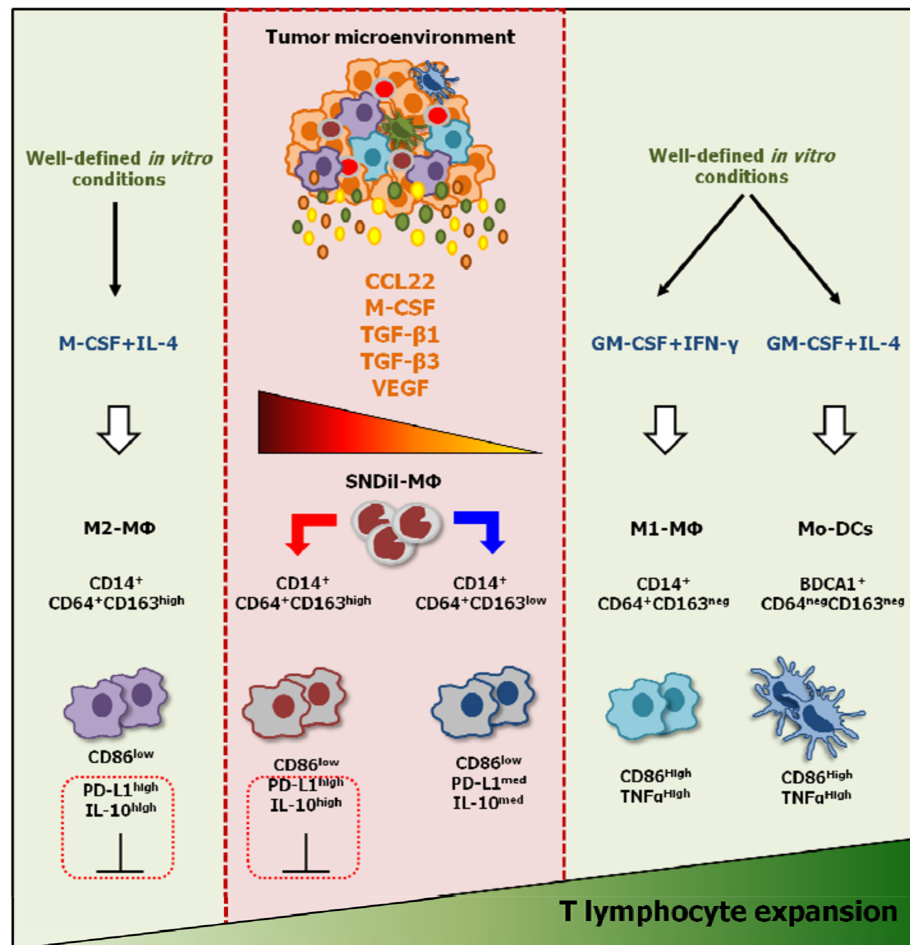
by blocking M-CSF in the supernatants of primary tumor microenvironment (or the receptor on monocytes) in our experimental setting.

Another important molecule found in SNDils was the angiogenic factor VEGF. As reviewed by Toi and colleagues (2001), the presence of VEGF has been extensively reported in diverse tumors, including breast cancer. VEGF can be produced by tumor cells and TAMs and is normally associated to hypoxia and controlled by the hypoxia inducible factor 1α (HIF-1α) (ANN et al. 2014; PEREIRA et al. 2014). In breast cancer, Bos and collaborators (2001) reported that the increased expression of HIF-1α was associated to high levels of VEGF, and to more aggressive tumors. In addition, different non-neoplastic tissues can also produce VEGF and trigger the migratory capabilities of mouse and human monocytes (AVRAHAM-DAVIDI et al., 2013; BARLEON et al., 1996; CLAUS et al., 1990). More recently, Voron and colleagues (2015) reported that VEGF derived from tumor microenvironment was able to enhance the expression of inhibitory immune checkpoint molecules in $CD8^+$ T cells, accelerating the process of lymphocyte exhaustion. Although some authors already described the participation of VEGF in the inhibition of DC differentiation (GABRILOVICH et al., 1996), the majority of data in the literature was obtained by using supernatants from tumor cell lines. These observations are confirmed by our study, since we found this cytokine in the SNDils and its presence was associated with the more suppressive phenotype of SNDil-MΦ.

Of note, IL-4, IL-13 and IFN-γ, used for the *in vitro* differentiation of MΦ or Mo-DCs, were not detected in SNDils. Interestingly, the absence of IL-4 and IL-13 in SNDils, cytokines normally used in protocols for *in vitro* M2-MΦ differentiation, may indicate an important difference between the differentiation of SNDil-MΦ and M2-MΦ. Indeed, though SNDil-MΦ present similarities to M2-MΦ, their phenotypes differ partially, indicating that other cytokines/chemokines or their combination in the tumor microenvironment may play the role of IL-4/IL-13 during SNDil-MΦ differentiation.

Altogether, our findings showed that the tumor microenvironment can drive blood monocytes differentiation towards two distinct profiles of SNDil-MΦ, characterized by different levels of CD163 expression, IL-10 secretion, and suppressive capabilities. These cells seem to be in an intermediate stage of differentiation between *in vitro* polarized M1-MΦ and M2-MΦ, possibly controlled by different combinations and concentrations of CCL22, M-CSF, TGF-β1, TGF-β3, and VEGF (Scheme 3).

Scheme 3 – A proposed model to illustrate how the tumor microenvironment may induce suppressive SNDil-MΦ



Besides the characterization of tumor-infiltrating APCs and the effects of the tumor microenvironment in monocyte differentiation, we also propose here that tumor-derived factors may have a systemic role, affecting monocytes in the patients' bloodstream. Circulating monocytes are known as the most important progenitors of macrophages and some subsets of myeloid DCs. During decades, the development of protocols of differentiation *in vitro* allowed the use of APCs in immunotherapeutic approaches, as the current allogeneic DC based-vaccine used by our group (BARBUTO et al., 2004; NEVES et al., 2005). However, herein, we showed that breast cancer patients' monocytes present deviations that affect their differentiation

in vitro into Mo-DCs (GM-CSF + IL-4) and M1-MΦ (GM-CSF + IFN-γ), ending up with suppressive capabilities.

Data presented here and recently published by us (RAMOS et al., 2012a - Appendix A) showed that monocytes from breast cancer patients can be differentiated into Mo-DCs, since they presented CD14 down-regulation and showed similar levels of CD11c and HLA-DR as healthy donors' Mo-DCs. In the absence of any stimulus, Mo-iDCs from patients, however, already showed a semi-mature phenotype ($CD86^{+/high}PD\text{-}L1^{+/high}$), but failed to induce T cell proliferation while inducing high frequencies of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs. Tregs are recognized as central in the maintenance of tolerance to self (SAKAGUCHI et al., 2010), but may also be involved in the failure of the immune system to control infections (CAMPANELLI et al., 2006), tumor growth (RAMOS et al., 2012b) and to respond to therapeutic vaccination (PALUCKA; BANCHEREAU, 2012). Many evidences have demonstrated that Treg accumulation is associated to mechanisms of tumor escape for several types of cancer (CURIEL, 2007b; ZOU, 2005; ZOU, 2006). Indeed, an increased presence of Treg is not restricted to the tumor sites, but is also observed in the peripheral blood from patients with distinct malignancies. Some evidences suggested Treg cells as the main barrier for the success of immunotherapeutic interventions (DUNN; OLD; SCHREIBER, 2004; SAKAGUCHI, 2005; SHEVACH, 2002; ZOU, 2005). In mouse tumor models, the elimination of Tregs can improve antitumor immune responses and survival (RAMOS et al., 2012b; ZOU, 2006), while several human studies correlated Treg accumulation with a worse prognosis for diverse diseases, including pancreas (LIYANAGE et al., 2002), breast (GOBERT et al., 2009; LIYANAGE et al., 2002), lung (WOO et al., 2002) and ovarian cancer (CURIEL et al., 2004; KNUTSON et al., 2015). In this context, our findings open another explanation for the accumulation of Tregs in tumors and in the periphery – a deviation in the functional status of DCs in cancer patients.

Furthermore, TNF-α was not able at all to modify patients' Mo-iDCs bias, while LPS altered it only in part, by promoting the up-regulation of CD40 and CD86 and partially reducing their capacity to induce Treg *in vitro*. These findings offer a possible explanation support for the use of toll-like receptors agonists (TLR-L) in patients. Indeed, in last decades, numerous studies have proposed the use of diverse TLR-L in tumor models as monotherapy or as adjuvants to vaccines to restore intratumor DCs' functionalities (LE MERCIER et al., 2013).

We found more accentuated differences between healthy and patients' Mo-DCs in their immature status, which are in accordance with the immature status of tumor-infiltrating DCs in breast cancer (DIEU-NOSJEAN et al., 2008; TREILLEUX et al., 2004). For these reasons, we went further to investigate the possible mechanisms that contribute to Tregs induction by patient's Mo-iDCs *in vitro*. Importantly, the blockage of TGF-β1 and PD-L1 in independent settings significantly reduced the induction of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs by patients' Mo-iDCs. TGF-β is a multifunctional cytokine that directly regulates T cell growth and development (MASSAGUE, 2000), inhibits IL-2 production, and has potent anti-proliferative effects on $CD4^{+}$ T cells (GORELIK et al., 2002). TGF-β was, indeed, described as crucial to the generation of iTregs in the periphery (CHEN et al., 2003; GHIRINGHELLI et al., 2005). In accordance, the addition of neutralizing antibody against TGF-β1 in co-cultures of Mo-iDCs from patients also restored T cells proliferation. It is noteworthy, however, that anti-TGF-β1 mAb was not sufficient to completely prevent the induction of Tregs by patients' Mo-iDCs, suggesting the contribution of PD-L1 - or other molecule(s) - in the mechanism of Treg generation in our study.

Actually, high concentrations of anti-PD-L1 mAb led to a reduction in Treg induction by patient's Mo-iDCs. Additionally, it is interesting to note that anti-PD-L1 mAbs have no effect on healthy Mo-iDCs functions in co-cultures, Which could be inhibited, though, by the blockage of CD80 and CD86 that significantly decreased T cell activation and increased the frequency of induced $CD4^{+}CD25^{+}Foxp3^{+}$ T cells. As already mentioned, many studies have analyzed the PD-L1/PD-1 pathway in the function of immune effector and immune evasion by cancer cells (BLANK et al., 2007; DONG et al., 1999; KEIR et al., 2007). Though not investigated in our experiments, PD-1 is the lymphocyte receptor for PD-L1 and PD-L2, and when engaged by these ligands may result in inhibition of T-cell proliferation or exhaustion (BLANK et al., 2006; FREEMAN et al., 2000; SAKUISHI et al., 2010). Indeed, other groups also described the role of PD-L1 in the induction of $Foxp3_{+}$ regulatory T cells when expressed by murine (WANG et al., 2008) and human DCs (ARMANATH et al., 2010). More recently, clinical trials targeting the PD-1/PD-L1 axis with anti-PD1 monoclonal antibodies revealed their safety (TOPALIAN et al., 2012) and achieved promising results, with tumor regressions in patients with advanced cancers (BRAHMER et al., 2012; HERBST et al., 2014; LARKIN et al., 2015; LIPSON et al.,

2013), thus, highlighting immunotherapeutic approaches as successful to treat cancer patients.

Our observations, showing that monocytes from cancer patients' present a distinct program of differentiation in comparison to healthy donors' Mo-DCs are in agreement with those of other groups, which described that Mo-DCs generated from patients' monocytes were phenotypically altered, acquiring "macrophage-like" characteristics in direct correlation to tumor aggressiveness, as observed for melanoma (FAILLI et al., 2013), colorectal cancer (ORSINI et al., 2013) and cervical cancer (ROY et al., 2011). Furthermore, other groups also described defective subpopulations of DCs in the blood of patients with Chronic Lymphocytic Leukemia (ORSINI et al., 2003) and breast cancer (GABRILOVICH et al., 1997; SATTHAPORN et al., 2004), revealing that circulating myeloid APCs could be also affected by tumors. Such studies evaluated DCs at the final time-point of differentiation, but their findings corroborate our main hypothesis: that circulating monocytes are already biased in patients.

In accordance with patients' Mo-DCs deviation, we also showed here that blood monocytes from a fraction of breast cancer patients did not fully differentiate into M1-MΦ (under GM-CSF + IFN-γ), maintaining CD163 expression and producing higher IL-10 levels when compared to healthy donors. Although in a limited number of experiments, our data suggest that these phenotypic alterations of patients' M1-MΦ conferred also them, a suppressive function. As both Mo-DCs and M1-MΦ were differentiated in the presence of GM-CSF, which is the only obvious common factor between the two settings, and no differences were found on GM-CSF-R expression, it is possible to infer that the alteration in patients' monocytes is downstream in the GM-CSF pathway.

It is not new that circulating leukocytes from breast cancer patients have an altered profile of gene expression, but the exact pathways implicated on monocyte differentiation and its initiation still unclear. Sharma and colleagues (2005) identified more than 35 altered genes in peripheral blood cells from breast cancer patients at early stages of diseases, most of them associated to ribosomal and mitochondrial alterations. In another study, Aaroe and collaborators (2010) identified about 60 up-regulated genes in peripheral blood cells from breast cancer patients in comparison to healthy donors' blood cells, in which STAT3 was 2 fold increased. These aspects should be strongly considered in the development of immunotherapeutic approaches

using autologous monocytes/DCs against cancer mainly due to the alteration found in patients' blood, which generate defective APCs (comment from BARBUTO, 2013). These findings are in accordance to recent pre-clinical and clinical research that has provided evidence that cancer progression is driven not only by tumor genetic alterations and paracrine interactions within the microenvironment, but also by complex systemic processes, including the modulation of immune cells (review by MCALLISTER; WEINBERG 2014).

Concerning monocytes, systemic alterations could operate during hematopoiesis or when they circulate in bloodstream. We found that patients' monocytes produced high quantities of "anti-inflammatory related-molecules": IL-10, IL-27, sCD40L, IL-1RA, VEGF-A, and M-CSF. Importantly, these findings are in accordance with the biased differentiation of Mo-DC and M1-MΦ from breast cancer patients' monocytes. In fact, our data also showed that about 35% of the patients presented a non-altered profile of cytokine production, comparable to that of healthy donors. These findings are in accordance with a recent study published by Chittezhath and collaborators (2014), describing the molecular profile of circulating monocytes from renal carcinoma patients. In this study, $CD14^{+}$ monocytes from patients at stages 3 and 4 presented a mixed of pro-inflammatory and tumor-promoting phenotype, with increased expression of TNF-α, IL-1A, IL-1β, IL-8, CCL5, VEGF-A and IL-10, suggesting a mechanism dependent of the presence of IL-1β in patients' plasma. In addition, another very recent article from Bergenfelz and collaborators (2015), has identified an elevated frequency of Monocytic-Myeloid-Derived Suppressor Cells (Mo-MSDCs: $CD14^{+}HLA\text{-}DR^{low/-}CD86^{low/-}CD80^{low/-}CD163^{low/-}$) in the circulation of patients with metastatic breast cancer. The authors performed a microarray analysis of total $CD14^{+}$ circulating monocytes from metastatic breast cancer patients' revealing that the pro-inflammatory genes TNF, IL-1β, HLA-DR and CD86 were significantly down-regulated, while ARG1, HMGB1 and several matrix metalloproteinases were up-regulated in comparison to healthy donors' monocytes. Similar observations were also reported in murine models of tumor: Some groups demonstrated that circulating monocytes from mammary tumor-bearing mice showed a mix of pro/anti-inflammatory genetic programming, distinct of that from monocytes in tumor-free mice (CASO et al., 2010; TORROELLA-KOURI et al., 2013). In addition, Stone & Rosseti and colleagues (2014) also described that $CD19^{neg}MHC\text{-}II^{+}$ APCs from spleen of tumor-bearing mice (HPV16-associated tumor)

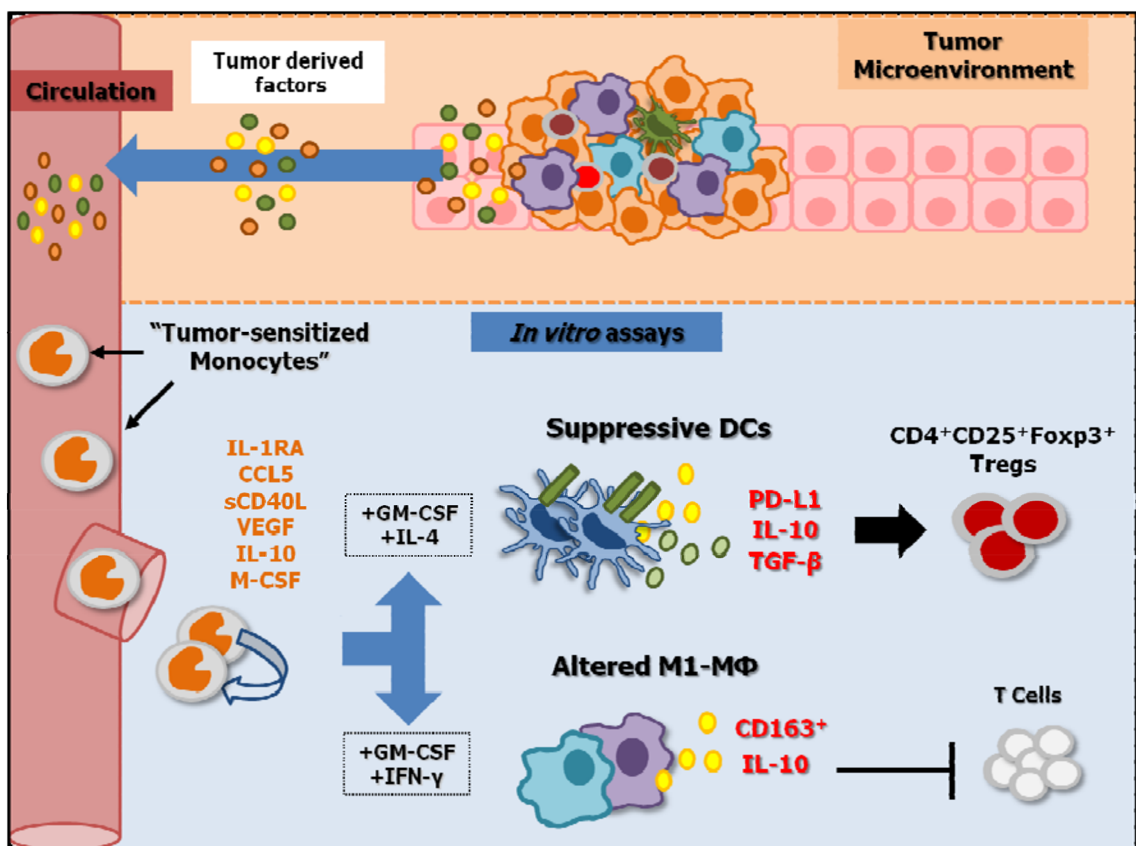
presented a suppressive profile of cytokine production, reinforcing the hypothesis of systemic effects of tumor growth.

More importantly, in a small number of donors, we observed that those patients' monocytes that did not fully differentiate into M1-MΦ, were already biased to produce elevated amounts of CCL5, sCD40L, IL-10 and VEGF-A. In this way, one possible strategy to investigate the role of these molecules in the phenomenon will be the blockage of the up-regulated cytokines during monocyte to MΦ or Mo-DCs differentiation in a large cohort of breast cancer patients and evaluate the phenotype of the resulting cells.

In addition, current projects in our group are also investigating possible alteration in intracellular pathways from patients' monocytes, aiming to characterize and define the downstream factors possible affected in diverse pathways, including NF-kB (FERREIRA, AC in preparation; MIGLIORI, IK in preparation), STAT/SOCS (TONIOLO, PA submitted) and MAPK (ZELANTE, B in preparation). The connection of these molecules to the IL-10 signaling pathway might offer mechanistic explanations for the phenomena, since IL-10 emerged as a major player in the modification of APCs in cancer patients, as it was produced by $CD163^{high}$ TAMs, $CD163^{high}$ SNDil-MΦ, biased M1-MΦ differentiated from patients' monocytes and by patients' monocytes under LPS activation. When IL-10 binds to its receptor, intracellular domain of IL-10R are phosphorylated by Jak1 and Tyk2 tyrosine kinases, triggering the activation of STAT1, STAT3 and STAT5, and favoring their translocation into the nucleus to induce target gene expression (FINBLOOM; WINESTOCK, 1995). STAT3 have been strongly associated to the impair of myeloid cells differentiation *via* tumor derived-factors, generating defective DCs (GABRILOVICH et al., 2012; KORTYLEWSKI et al., 2005). Recently, Farren and colleagues (2014) suggested that breast tumor derived-factors can induce a sustained STAT3 up-regulation on myeloid cells progenitors from humans and mice, avoiding the activation of ERK and NF-kB signaling and, thus, limiting their capacity to be differentiated into DCs or MΦ. STAT3 can also be activated by IL-6, but in a transient and rapidly manner, whereas IL-10R has a strong preference for STAT-3 and induces a sustained STAT3 phosphorylation (BRAUN et al., 2013), putting forward IL-10 as a central player. Finally, though IL-10 appears to have a decisive role, the original factors responsible for its production, probably from the tumor microenvironment, are still unclear.

Thus, we showed that breast cancer patients' monocytes did not present alteration in CD163 expression, but are already altered to produce preferentially anti-inflammatory cytokines, which in turn, may affect their differentiation into biased M1-MΦ ($CD163^{+/-}IL10^{+}$) and Mo-DCs ($CD86^{+/-}PD\text{-}L1^{+}TGF\beta^{+}$), in an autocrine/paracrine manner, generating APCs defective as activators of T cells or biased to induce $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs (Scheme 4).

Scheme 4 – A proposed model illustrating how tumor microenvironment derived factors may act systemically altering the differentiation of breast cancer patients' monocytes.



One possible explanation for monocyte deviation came from studies demonstrating that increased amounts of anti-inflammatory cytokines in circulation are associated to cancer progression. The detection of higher amounts of TGF-β (MA et al., 2013), M-CSF (KAMINSKA et al., 2006; ŁUKASZEWICZ-ZAJĄC et al., 2010) and IL-6 (PLANTE et al., 1994; SCAMBIA et al., 1995) in patients' serum from diverse types of cancer strongly suggest that tumor presence may affect another

cells in distant organs/tissues, a finding also present in breast cancer patients for IL-6 and VEGF (ADAMS et al., 2000; BENOY et al., 2002), CCL2 and CCL5 (DEHQANZADA et al., 2007), M-CSF (RICHARDSEN et al., 2015; TAMIMI et al., 2008) and TGF-β (DIVELLA et al., 2013; IVANOVIĆ et al., 2003). Accordingly, similar molecules were increased in SNDils that induced a suppressive phenotype in SNDil-MΦ, leading us to hypothesize that systemic alterations found in circulating monocytes from patients could be due to the combination between high local and plasmatic levels of factors like these. Thus, during tumor development, circulating monocytes are concomitantly induced to produce anti-inflammatory/angiogenic molecules and have their potential to differentiate into Mo-DCs and M1-MΦ deeply altered. As this phenomenon was not found in all patients, we may assume that diverse intracellular pathways could be modified by tumor products, in a "patient-dependent fashion". More interestingly, no correlation was noted between the altered cytokine production by patients' monocytes and the morphological or molecular characteristic of tumors in a small group of patients. On the other hand, the hypothesis of the systemic effect of tumors is strongly supported by studies published by us, where we described that the bias in monocytes from cancer patients' depends on the tumor burden, since a functional recovery of Mo-DCs was observed three months after tumor resection in a case of renal cell carcinoma (CLAVIJO-SALOMON et al., 2015), or after immunotherapeutic DC-vaccination in melanoma and renal cell carcinoma patients (NEVES et al., 2005).

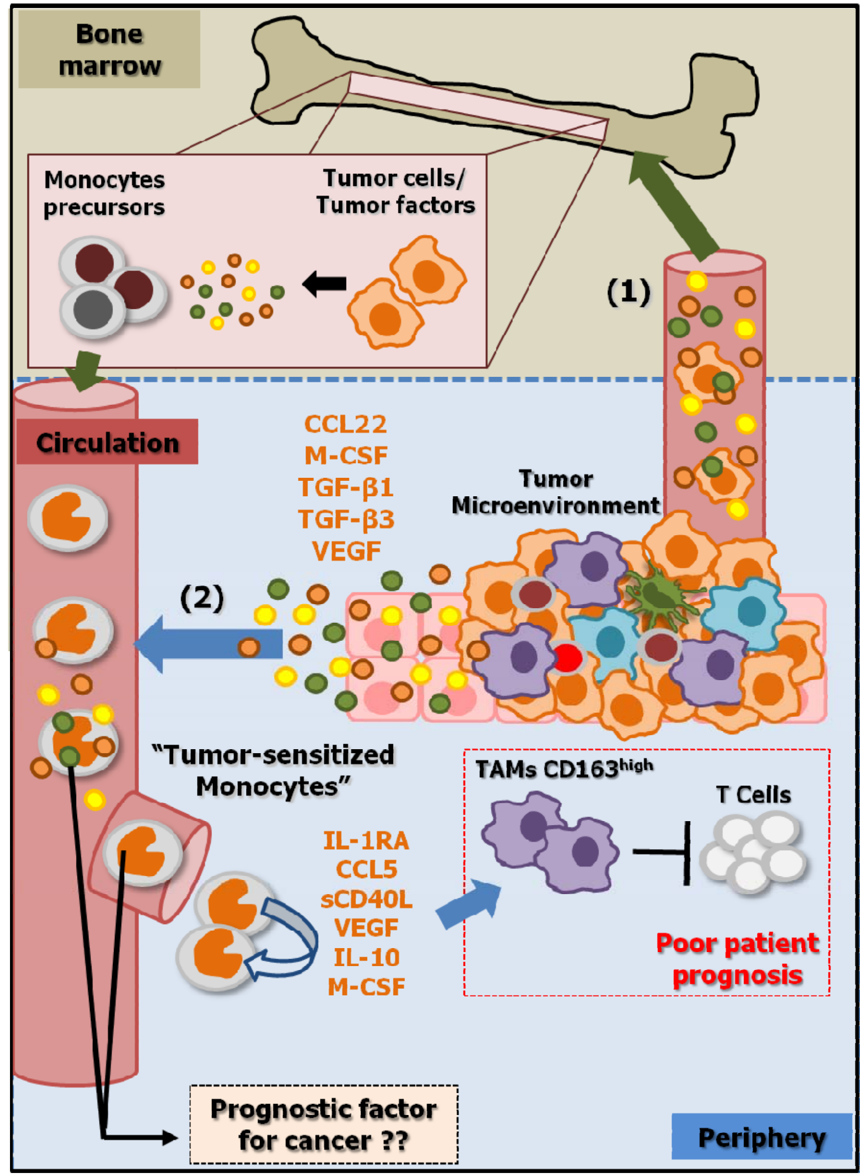
As monocytes have a short life-spam in bloodstream (1-2 days), the contact with soluble factors, possibly produced in the cancer microenvironment, need to be strong enough to imprint alterations in these cells. Hence, another possible explanation for the bias found in patients' monocytes is that tumor-derived factors are capable to sensitize these cells already in the bone marrow. It sounds more plausible if this premise is correlated to articles describing the presence of tumor cells in bone marrow of patients. Interestingly, as shown by Wang and colleagues very recently (2015), different human breast tumor cell lines can form micrometastasis in the bone marrow of mice in the very early process of carcinogenesis. Additionally, important studies in large cohorts of patients have reported that tumor cells can reside in the bone marrow of breast cancer patients, a phenomenon also correlated to a worse patients' prognosis (BRAUN et al., 2000; BRAUN et al., 2005; HARTKOPF et al., 2014; JANNI et al., 2011). These points are in accordance with a very recent and

surprisingly study published by Narod and colleagues (2015), describing that a group of breast cancer patients with ductal carcinoma *in situ* (neoplastic cells yet restricted to mammary ducts) may die from metastatic cancer even if never experiencing an invasive breast carcinoma, a phenomenon that was not prevented by local radiotherapy or mastectomy. Therefore, assuming this, factors from “bone-marrow resident tumor cells” could easily have access to monocyte progenitors and alter their physiology already during their bone marrow differentiation/maturation. As the present study did not deeply evaluate if tumor-derived factors are present in the bone marrow or in the blood circulation, we must consider both possibilities, presently, as educated speculations.

Overall, we observed that the various immune aspects of breast cancer patients are very heterogeneous, like cancer itself. We, furthermore, described interesting points that are in accordance but not directly associated to the molecular or morphological cancer classification currently used. Thus, we could envisage a future scenario where the parameters we analyzed could integrate an independent “myeloid immune classification” of tumors.

Finally, we proposed here a complementary hypothesis for the tumor escape mechanism (Scheme 5). In cancer patients, tumor products acting locally, at the microenvironment, but also systemically (either in the bloodstream Scheme 5 – (2) and/or in the bone marrow (Scheme 5 – (1)) cause monocytes functional and differentiation biases. Though not confirmed, we also identified possible molecules as mediators of the phenomena and, though, as targets for the manipulation to obtain functional immune responses against cancer. Furthermore, the determination of altered intracellular signaling pathways and their possible targets, through which tumors are able to modify the function of circulating monocytes, can provide new targets for immunotherapeutic approaches for cancer. More importantly, we believe that our data provide enough evidence to justify the investigation of blood monocytes as a prediction factor to be integrated into strategies of prevention and/or treatment of breast cancer.

Scheme 5 – Tumor products may access bone marrow and blood circulation and affect monocyte physiology from cancer patients.



6 CONCLUSION

Main findings obtained in our study:

- Breast and ovarian tumors shown high infiltration by Tumor-associated Macrophage (TAMs) with very heterogeneous CD163 expression;
- Higher presence of TAMs $CD163^{high}$ is correlated to lower infiltration of T $CD3^{+}$ lymphocytes in breast cancer tissues;
- $CD64^{+}CD163^{high}$ TAMs express high PD-L1 levels and up-regulate its expression and IL-10 production under LPS stimulation *ex-vivo;*
- Higher CD163 expression *in situ* is correlated with poor breast cancer patients' outcome within 12,5 years of retrospective analysis;
- Primary tumor microenvironment derived factors can induce SNDil-MΦ $CD163^{high}PD\text{-}L1^{high}CD86^{low}IL\text{-}10^{high}$ phenotype on conditioned monocytes;
- SNDil-MΦ $CD163^{high}$ suppress $CD4^{+}$ T cell expansion via partially role of IL-10;
- The increased presence of IL-8, CCL19, CCL21, VEGF, M-CSF, TGF-β3 TGF-β1 and CCL22 molecules in tumor microenvironment is associated to SNDil-MΦ $CD163^{high}IL\text{-}10^{high}$ phenotype;
- Breast cancer patients' monocytes originate biased dendritic cells that induce higher frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells with TGF-β1 and PD-L1 participation;
- Breast cancer patients' monocytes fail to fully differentiate into M1-MΦ, maintaining partial CD163 expression and producing high amounts of IL-10 cytokine;
- Circulating blood monocytes from breast cancer patients display a different profile of cytokine production in comparison to healthy donors, by secreting higher amounts of IL-10, VEGF-A, IL-27, sCD40L, IL-21, IL-1RA and M-CSF under 24 hours of LPS activation.

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APPENDIX- A

JLB

Article

Monocyte-derived dendritic cells from breast cancer patients are biased to induce $CD4^+CD25^+Foxp3^+$ regulatory T cells

Rodrigo Nalio Ramos, Lilian Sally Chin,* Ana Paula S. A. dos Santos,*,† Patrícia Cruz Bergami-Santos,* Fábio Laginha,‡ and José Alexandre M. Barbuto*,1*

*Department of Immunology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil; †Department of Physiology, Federal University of Maranhao, MA, Brazil; and ‡Department of Radiology, Perola Byngton Hospital, Sao Paulo, SP, Brazil

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ABSTRACT

DCs orchestrate immune responses contributing to the pattern of response developed. In cancer, DCs may play a dysfunctional role in the induction of $CD4^+CD25^+$ $Foxp3^+$ Tregs, contributing to immune evasion. We show here that Mo-DCs from breast cancer patients show an altered phenotype and induce preferentially Tregs, a phenomenon that occurred regardless of DC maturation stimulus (sCD40L, cytokine cocktail, TNF-α, and LPS). The Mo-DCs of patients induced low proliferation of allogeneic $CD3^+CD25^{neg}Foxp3^{neg}$ cells, which after becoming $CD25^+$, suppressed mitogen-stimulated T cells. Contrastingly, Mo-DCs from healthy donors induced a stronger proliferative response, a low frequency of $CD4^+CD25^+Foxp3^+$ with no suppressive activity. Furthermore, healthy Mo-DCs induced higher levels of IFN-γ, whereas the Mo-DCs of patients induced higher levels of bioactive TGF-β1 and IL-10 in cocultures with allogeneic T cells. Interestingly, TGF-β1 blocking with mAb in cocultures was not enough to completely revert the Mo-DCs of patients' bias toward Treg induction. Altogether, these findings should be considered in immunotherapeutic approaches for cancer based on Mo-DCs. *J. Leukoc. Biol.* 92: 673–682; 2012.

Introduction

DCs are unique, professional APCs adapted to initiate, coordinate, and regulate adaptive immune responses by inducing naive T cells' differentiation [1, 2] into Th1 [3], Th2 [4], Th17 [5], and other $CD4^+$ cell subtypes [6]. Recent data suggest that DCs are also crucial for the induction and maintenance of T cell tolerance and have important physiological roles in the prevention of autoimmunity [7]. Furthermore, DCs can contribute to the subversion of the immune responses in cancer [8, 9], in ways that could resemble their role in maintaining self-tolerance.

It did not take long after the discovery of DCs for their potential in cancer immunotherapy to be recognized [10, 11]. However, as DCs have the potential to induce not only immunity but also immune tolerance, depending on their functional status [12], many attempts to use DCs in cancer immunotherapy may have failed to reach all of the expected success as a result of a possible functional deficit of the cells used. Further, many studies have demonstrated that DCs are essential for Treg induction in vitro [13, 14], apparently depending on various distinct mechanisms [15] but also frequently, on external sources of cytokines [16].

Optimization of DC differentiation protocols has focused largely on the capacity of human DCs to improve protective immunity against cancer [17]; however, their ability to induce regulatory cells concurrently has received less attention. Tregs contribute to the maintenance of immunologic self-tolerance [18] and play a relevant role in immune responses to infectious agents [19, 20]. Harnessing their function could help in the management of transplantation reactions, whereas their action can represent an obstacle for successful immunotherapy in patients with cancer [21, 22]. Although $CD8^+$ Tregs have been described [23], most studies focused on $CD4^+$ Tregs. These have been described on the basis of their origin and generation [24] and mechanism of action [25]. Inducible Tregs that are comprised of IL-10-producing Tr1 cells [26], TGF-β-producing Th3 cells [27], and inducible $Foxp3^+$ T cells [28], described in different situations, including cancer [29–31], seem to depend largely on the context of antigen presentation, a phenomenon where DCs play a most relevant role.

DCs are affected by the presence of tumors [32, 33]. Various observations indicate a deficient functional activity of these cells with defective stimulation of immune responses as a re-

Abbreviations: Foxp3=forkhead box p3, FSC=forward-scatter, MFI=median fluorescence intensity, Mo-DC=monocyte-derived DC, Mo-iDC=monocyte-derived immature DC, Mo-mDC=monocyte-derived mature DC, PD-L1=programmed cell death ligand 1, sCD40L=soluble CD40 ligand, SSC=side-scatter, Tr1=T regulatory 1, Treg=regulatory T cell

1. Correspondence: Laboratory of Tumor Immunology, Dept. of Immunology, Institute of Biomedical Sciences, University of São Paulo, Avenida Professor Lineu Prestes, 1730, São Paulo, 05508-900, Brazil. E-mail: jbarbuto@icb.usp.br

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sult of a decreased frequency of functionally competent mDCs (semimature or iDC induction) within tumors. These DCs are characterized, mainly, by a poor up-regulation of MHC class II and costimulatory molecules and absent or low-production proinflammatory cytokines, such as IL-12, thus favoring tumor evasion from the immune response. Another functional consequence of tumor–DC interactions could be the favoring of the already-described expansion of $CD4^+CD25^+Foxp3^+$ Tregs in patients with solid tumors. Indeed, some studies have shown the active participation of DCs in the induction of Foxp3 expression by $CD4^+$ T cells in humans [34] and mice [35]. However, DCs from healthy donors only seem to induce Tregs in the presence of TGF-β [36] or overstimulation with anti-CD3, anti-CD28, and IL-2.

Although DCs from cancer patients are recognized as functionally deficient, it is not so clear whether Mo-DCs from these patients show similar deficiencies. Here, we generated DCs (Mo-DCs) from breast cancer patients and show that these are strongly biased to the induction of functional Tregs, thus possibly contributing to the maintenance of a pro-tumor immune status in the patients and possibly, posing an obstacle for DC-based therapeutic approaches for cancer.

MATERIALS AND METHODS

Eligibility criteria

Patients, from the Mastology Center at the Perola Byington Hospital (Sao Paulo, Brazil), with a recent histopathological diagnosis of invasive breast carcinoma and who had not received chemotherapy, radiotherapy, or immunotherapy before study enrollment, were included. Those with uncontrolled metastatic lesions in the brain, with hypercalcemia, with other previous or concomitant neoplasia, who were pregnant or lactating, with autoimmune diseases, or were HIV-seropositive were excluded from the study. As controls, we chose healthy, female volunteers over 20 years old. All patients and healthy volunteers gave written, informed consent. The protocol was approved by the Institutional Ethics Committee (2009/902 CEP).

DC generation

PBMCs were separated from blood collected in heparin (50 U/ml) by centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Mononuclear cells were resuspended and seeded in culture six-well plates in RPMI-1640 culture medium (Gibco, Grand Island, NY, USA), supplemented with 10% FCS (Gibco) plus antibiotic-antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin; Gibco). After that, culture plates were incubated at 37°C overnight. In case of monocyte use, after overnight incubation, nonadherent cells were removed, and the adhered monocytes were harvested, washed, and used for the experiments. In case of Mo-iDC use, nonadherent cells were removed, the medium was replaced, and GM-CSF (50 ng/ml; R&D Systems, Minneapolis, MN, USA) and IL-4 (50 ng/ml; R&D Systems) were added. Mo-iDCs were harvested after 7 days, without any additional manipulation, phenotypically characterized, and used in cocultures. Mo-mDCs were harvested at Day 7, after receiving maturation stimuli at Day 5 with sCD40L (1 mg/mL; Invitrogen, Carlsbad, CA, USA); a cytokine cocktail containing IL-1β (10 ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), and TNF-α (10 ng/ml; R&D Systems); only TNF-α (50 ng/ml; R&D Systems); or LPS (500 ng/ml; *Escherichia coli* 0111:B4; Sigma-Aldrich, St. Louis, MO, USA).

For some experiments, the starting cell population was that of $CD14^+$ cells, isolated from PBMCs by magnetic beads (CD14 isolation kit; Miltenyi Biotec, Germany).

Purification of T cells and isolation of $CD25^{neg}$ cells

$CD3^+$ T cells were purified from nonadherent PBMCs by magnetic bead selection using the CD3 isolation kit (Miltenyi Biotec), reaching over 97% purity. $CD25^{neg}$ cells were separated from previously $CD3^+$-isolated or $CD4^+$ (Miltenyi Biotec) cells by depletion of $CD25^+$ cells using the CD25 Microbeads II kit (Miltenyi Biotec) with 98% of purity. Among these $CD3^+CD25^{neg}$ and $CD4^+CD25^{neg}$ cells, >78% were $CD45RA^+$. All phenotypic analyses were done by flow cytometry.

T cell proliferation by CFSE dilution

Isolated $CD3^+$ T cells were labeled with 5 μM CFSE and cultivated with patients or healthy Mo-iDCs. After 5 days, lymphocytes were stained with anti-CD4 and anti-CD8 antibodies, and CFSE dilution was determined by flow cytometry analysis. PHA was used as a polyclonal-positive stimulus. At least 20,000 events were acquired/antibody analyzed.

Mo-DCs and T cell cocultures

Monocytes or Mo-iDCs and Mo-mDCs, after differentiation in vitro (for 7 days), were harvested from plates and cultured with $CD3^+CD25^{neg}$ or $CD4^+CD25^{neg}$ T cells at a 1:10 ratio in RPMI 1640, supplemented with 10% FCS plus antibiotic-antimycotic solution. No other stimuli were added to the cocultures. After 6 days of culture, $CD3^+CD25^{neg}$ or $CD4^+CD25^{neg}$ T cells were phenotypically evaluated as to their phenotype (CD4, CD25, Foxp3, CD127, and CTLA4 expression) or used in functional suppression assays.

Flow cytometry

Monocytes and DCs. Cell preparations (2.5×10^5 cells/condition) were labeled with each of the various specific fluorescent antibodies (CD1a, CD11c, CD14, CD40, CD80, CD86, PD-L1, CD83, CD123, HLA-DR) or isotype controls (BD Biosciences, San Jose, CA, USA) and analyzed in the FACSCalibur cytometer (BD Biosciences) using the FlowJo software, Ver. 7.2.4 (Tree Star, Ashland, OR, USA).

Lymphocytes. Foxp3 expression in T cells was assessed using the anti-human Foxp3 staining kit (Clone 236A/E7; eBioscience, San Diego, CA, USA). Mouse IgG1κ (BD Biosciences) was used as isotype control in all intracellular staining analyses. Samples were also stained with CD25, CD3, CD4, CTLA4, CD127, and CD45RA (BD Biosciences). At least 20,000 events were acquired/antibody analyzed.

Cytokine assay

Coculture supernatants were harvested after 5 days, and IFN-γ (BD PharMingen, San Diego, CA, USA), IL-10 (BD PharMingen), and bioactive TGF-β1 (eBioscience) were quantified by sandwich ELISA, according to the manufacturers ' instructions. OD was determined in a VersaMax microplate ELISA reader and converted to pg/ml using a standard curve and SoftMax Pro software.

Suppression assay

To assess the ability of T cells, generated after cocultures with Mo-iDCs from patients and controls, to suppress other T cell responses, $CD25^+$ cells were sorted magnetically from cocultures using the CD25 Microbeads II kit (Miltenyi Biotec) and added to allogeneic, healthy, CFSE-labeled PBMCs (1×10^5/well) at a 1:10 ratio in 96-well U-bottom plates in the presence of PHA (1 μg/ml) at 37°C and 5% CO_2. On Day 3, the cells were harvested, and the T cell proliferative response was determined by flow cytometry, evaluating the dilution of CFSE.

TGF-β blocking in cocultures

To block TGF-β activity in cocultures of Mo-DCs and T lymphocytes, 80 ng/ml neutralizing anti-TGF-β (R&D Systems) or control IgG mAb (BD Biosciences) were added on Day 0 of cultures.

Statistical analysis

Results were checked for normality by the Kolmogorov-Smirnov test, and comparisons between results obtained from healthy donors and breast cancer patients were performed by a two-tailed unpaired *t*-test (*$P<0.05$; **$P<0.01$; ***$P<0.0001$). Effects of neutralizing anti-TGF-β were compared using the paired *t*-test (*$P<0.05$; **$P<0.01$). Phenotype of monocytes, Mo-iDCs, and Mo-mDCs was compared using the one-way ANOVA test with the Tukey post-test.

RESULTS

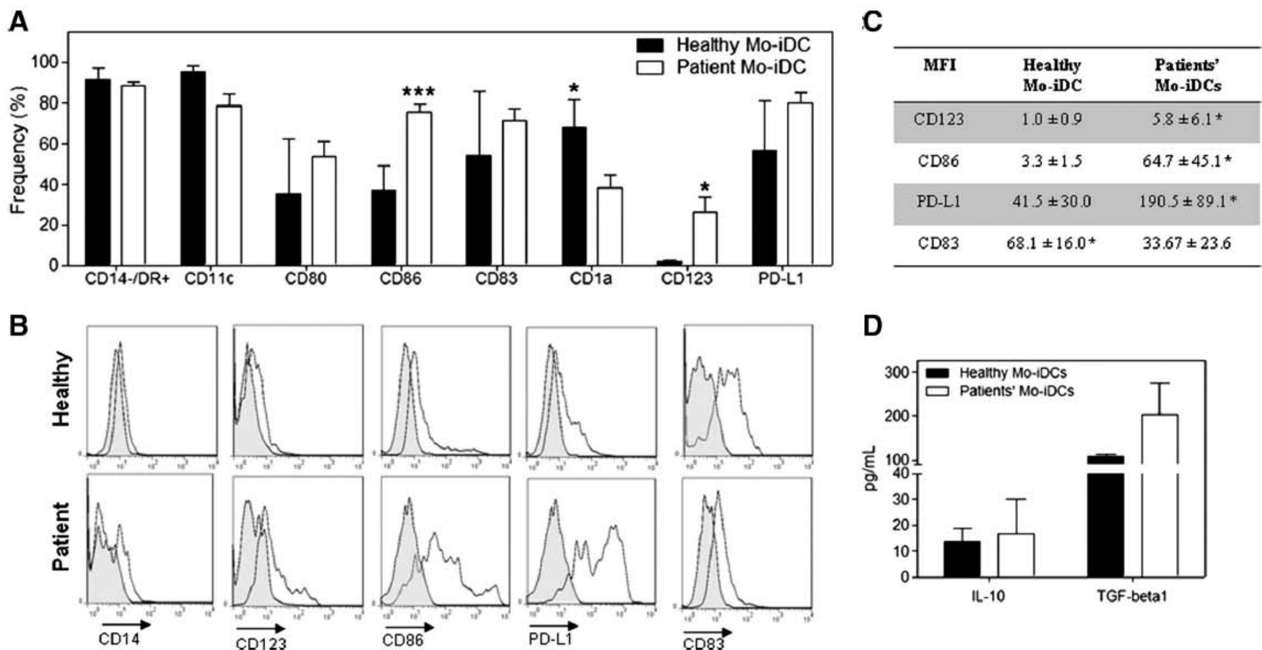
Mo-iDC cells from patients are phenotypically different from those derived from healthy donors

Mo-iDCs were generated in vitro from breast cancer patients ($n=9$) and from healthy donors ($n=5$) by culture for 7 days in the presence of IL-4 and GM-CSF. Already at the FSC and SSC profiles of the cells, we noticed that the Mo-iDCs of patients represented a nonhomogeneous population compared with the relatively homogeneous cells of healthy donors. On the other hand, the analysis of CD14 and HLA-DR expression indicated similar frequency of cells with a Mo-iDC phenotype (CD14-HLA-DR$^+$) in both groups (healthy donors: 87.3%±0.3 vs. patients: 87.1%±3.0; **Fig. 1A**). Of the molecules evaluated in DCs, CD80, HLA-DR, and CD11c were not significantly different between healthy donors and patients, but the expression of CD86 and CD123 clearly distinguished these groups, both in frequency of positive cells (CD86=healthy donors: 37.2%±4.0 vs. patients: 74.6%±6.5; $P<0.0001$; CD123=healthy donors: 1.9±0.47 vs. patients: 28.4±7.16; $P<0.05$) and MFI (CD86=healthy donors: 4.1±2 vs. patients: 64.4±44; $P<0.05$; CD123=healthy donors: 1±0.8 vs. patients: 5.8±6; $P<0.05$), whereas for PD-L1, just the MFI distinguished the groups (healthy donors: 41.5±30 vs. patients: 190.5±89.1; $P<0.05$; Fig. 1A–C). On the other hand, the expression of CD1a was significantly lower in frequency (healthy donors: 68±13 vs. patients: 38.1±18; $P<0.05$) and the CD83 activation marker, lower in MFI (healthy donors: 68±16 vs. patients: 33.2±24; $P<0.05$) in Mo-iDCs from patients (Fig. 1A–C). Nevertheless, the maturation status of both cell populations was similar, with their immature phenotype indicated by low values of MFI for all costimulatory molecules tested.

In the ELISA assay of the culture supernatants (at Day 5), for the production of IL-10 and bioactive TGF-β1, although Mo-iDC patients seemed to produce higher levels of the latter (healthy donors: 107.4±4.73 pg/ml vs. patients: 203.3±71.7 pg/ml; $P<0.05$; Fig. 1D), the difference was not significant.

Mo-iDCs from patients induce lower lymphocyte activation and proliferation but higher proportions of CD4$^+$CD25$^+$Foxp3$^+$ cells

To investigate further the possible functional deviation of the Mo-iDCs of patients, our next step was the analysis of their ability to activate and stimulate lymphocyte proliferation in



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MFI	Healthy Mo-iDC	Patients' Mo-iDCs
CD123	1.0 ±0.9	5.8 ±6.1*
CD86	3.3 ±1.5	64.7 ±45.1*
PD-L1	41.5 ±30.0	190.5 ± 89.1*
CD83	68.1 ± 16.0*	33.67 ± 23.6

Figure 1. Mo-iDCs from cancer patients are more heterogeneous, express high levels of CD86, CD123, and PD-L1, and produce IL-10 and TGF-β1. Monocytes from healthy donors and from cancer patients were cultured in the presence of IL-4 and GM-CSF for 7 days and characterized. Flow cytometry analysis of Mo-iDCs (CD14^{low}/HLA-DR$^+$ cells) from healthy donors and breast cancer patients showing: (A) frequency of cells positive for characteristic markers, (B) representative histograms, and (C) MFI values for the markers; (D) ELISA assay of Mo-iDC supernatants for the presence of IL-10 and bioactive TGF-β1 (healthy donors, $n=5$; patients, $n=9$; *$P<0.05$; ***$P<0.0001$).

vitro. Allogeneic $CD3^+$ lymphocytes were labeled with CFSE and cocultivated with Mo-iDCs for 5 days (at a 10:1 lymphocyte:DC ratio). After that, cells were recovered and labeled with anti-CD4 and anti-CD8 antibodies and their proliferation analyzed by CFSE dilution. Analysis of nonstimulated and PHA-stimulated lymphocytes indicated ~16% and ~77% of $CD4^+$ proliferation and ~12% and ~76% of $CD8^+$ proliferation, respectively. Mo-iDCs from healthy donors induced higher frequency (healthy donors: 57%±12 vs. patients: 24.1%±6.6; $P<0.01$) and absolute number (healthy donors: $7.9\times10^4\pm5.3\times10^3$ vs. patients: $6.3\times10^4\pm3.3\times10^3$; $P<0.01$) of proliferating $CD4^+$ lymphocytes (**Fig. 2A–C**). Similar differences were observed in $CD8^+$ lymphocyte stimulation, also in frequency (healthy donors: 60%±13.8 vs. patients: 22.8±6.5%; $P<0.05$) and absolute number (healthy donors: $8.07\times10^4\pm7.5\times10^3$ vs. patients: $6.14\times10^4\pm3.2\times10^3$; $P<0.05$), comparing Mo-iDCs from healthy donors and breast cancer patients (Fig. 2A–C).

When Mo-iDCs were used to stimulate allogeneic $CD3^+CD25^{neg}$ lymphocytes, cells from patients induced the expression of the CD25 activation marker in significantly less lymphocytes than Mo-iDCs from healthy donors (healthy donors: 35.7%±7.9 vs. patients: 11.8%±5.9; $P<0.0001$). This was also reflected in the total cell count in these conditions (patients: $8.04\times10^5\pm10.8\times10^5$ vs. healthy donors: $34.8\times10^5\pm11.3\times10^5$; $P<0.01$; Fig. 2D–F).

Furthermore, Mo-iDCs from cancer patients induced a significantly higher occurrence of $CD4^+Foxp3^+$ among the $CD25^+$ fraction of allogeneic cocultured T cells compared with those induced by the healthy Mo-iDC donors: healthy, 2.5%±0.7 ($1.0\times10^5\pm6.6\times10^4$), vs. patient, 56.8%±4.1 ($4.6\times10^5\pm3.1\times10^5$; **Fig. 3A** and **B**). To exclude possible non-DCs contaminating the cultures, we differentiated Mo-iDCs from $CD14^+$-preisolated cells. In coculture with allogeneic T lymphocytes, these induced the same Treg frequency as the nonisolated population (Fig. 3C). Accordingly, cocultures with patient Mo-iDCs showed increased concentrations of IL-10 (healthy donors: 14.0±2.6 pg/ml vs. patients: 61.8±28.1 pg/ml; $P<0.05$) and bioactive TGF-β1 (healthy donors: 57.6±3.3 pg/ml vs. patients: 298.1±129 pg/ml; $P<0.05$), whereas those with healthy Mo-iDC donors presented higher levels of IFN-γ (healthy donors: 471.8±620 pg/ml vs. patients: 14.8±15.6 pg/ml; $P<0.05$; Fig. 3D).

Tregs induced by Mo-iDCs from cancer patients showed a reduced proliferative activity in the cocultures and are able to suppress T lymphocyte responses to PHA

T lymphocytes were separated, labeled with CFSE, and cocultured with Mo-iDCs. After 6 days, proliferation was evaluated by CFSE dilution within $Foxp3^+$ and $Foxp3^{neg}$ cells. As expected, proliferation of $Foxp3^+$ cells was significantly lower than that of $Foxp3^{neg}$ cells in healthy Mo-DC-stimulated cultures of donors and patients (**Fig. 4A**). Higher expression of the CTLA4 molecule, a characteristic Treg marker, was also observed in gated $CD4^+CD25^+Foxp3^+$ cells in healthy Mo-DC cocultures of donors and patients compared with the $Foxp3^{neg}$ population (Fig. 4A, bottom graphs).

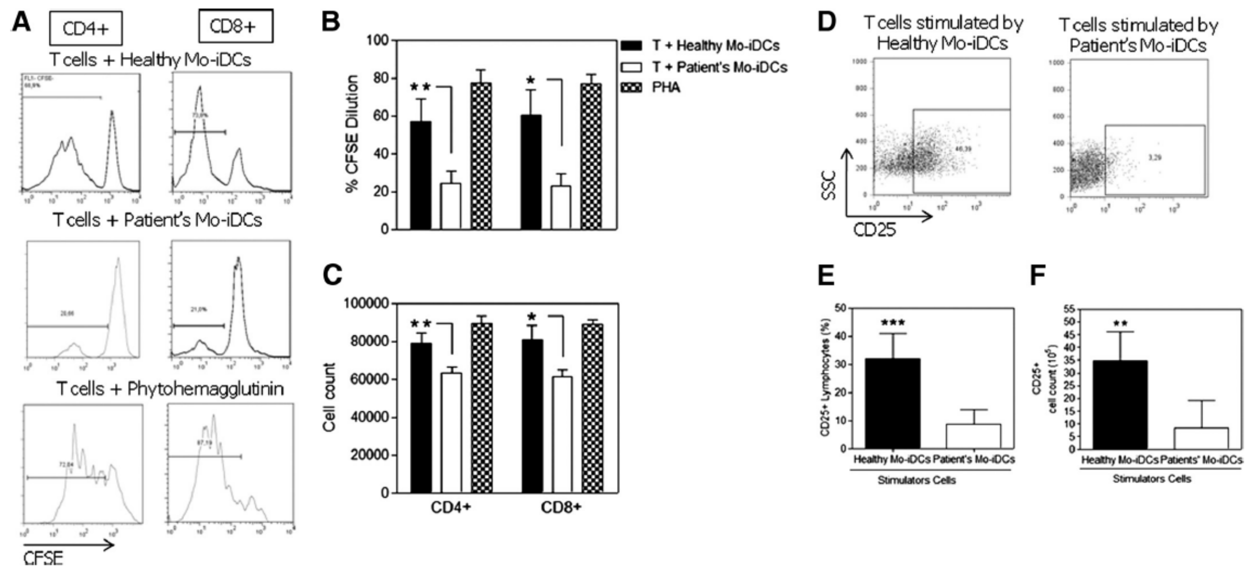

Figure 2. The Mo-iDCs of patients induce low proliferation and CD25 expression by allogeneic T cells. Mo-iDCs from controls and patients were cocultured with allogeneic CFSE-labeled $CD3^+$ or $CD3^+CD25^{neg}$ cells for 5 days. At the end of culture, CFSE dilution and CD25 expression by $CD3^+$ cells were evaluated by flow cytometry. (A) Representative experiment of CFSE dilution in $CD4^+$ and $CD8^+$ T cells. FL1, Fluorescence 1. (B) Pooled data from five independent experiments showing frequency of cells with CFSE dilution and (C) absolute number of dividing cells (n=5; $*P<0.05$; $**P<0.01$). (D) Representative experiment showing CD25 expression by $CD3^+$ cells after coculture of $CD3^+CD25^{neg}$ lymphocytes with Mo-iDCs from healthy donors or from breast cancer patients. (E) Average frequency and (F) absolute number of $CD25^+$ cells in six independent experiments (n=6; $**P<0.01$; $***P<0.0001$).

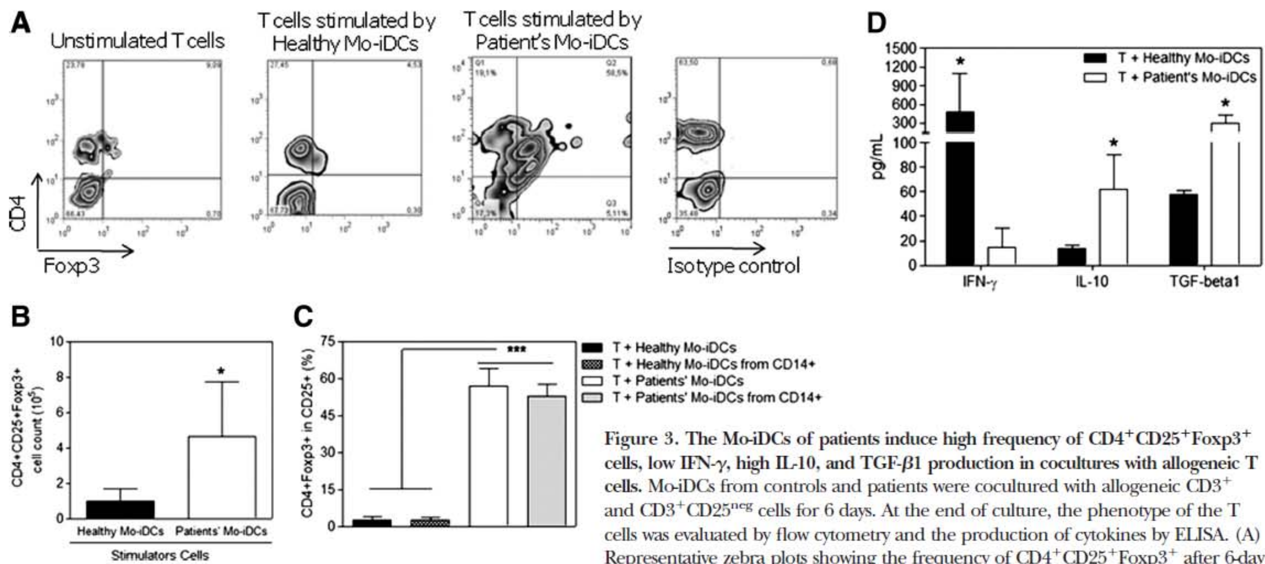

Figure 3. The Mo-iDCs of patients induce high frequency of $CD4^+CD25^+Foxp3^+$ cells, low IFN-γ, high IL-10, and TGF-β1 production in cocultures with allogeneic T cells. Mo-iDCs from controls and patients were cocultured with allogeneic $CD3^+$ and $CD3^+CD25^{neg}$ cells for 6 days. At the end of culture, the phenotype of the T cells was evaluated by flow cytometry and the production of cytokines by ELISA. (A) Representative zebra plots showing the frequency of $CD4^+CD25^+Foxp3^+$ after 6-day cultures among unstimulated T lymphocytes or T lymphocytes cocultured with Mo-iDCs from healthy donors or from breast cancer patients. (B) Absolute number and (C) average frequency of $CD4^+CD25^+Foxp3^+$ cells in 6-day cocultures of allogeneic T cells with iDCs differentiated from adherent monocytes (n=6) or from $CD14^+$-preisolated monocytes obtained from healthy donors or from breast cancer patients (n=3; *$P<0.05$; ***$P<0.0001$). (D) ELISA assays of coculture supernatants for the presence of: IL-10, bioactive TGF-β1, and IFN-γ (n=5; *$P<0.05$).

To test the suppressive function of lymphocytes stimulated by different Mo-iDCs, $CD25^+$ cells were isolated by magnetic microbeads (97% of purity) from the healthy Mo-iDC–T cell cocultures of patients and added (at a 1:10 ratio) to allogeneic PBMCs, previously labeled with CFSE and stimulated with PHA. After 3 days in culture, cells were analyzed by cytometry for CFSE dilution. $CD25^+$ cells from the Mo-iDC–T cocultures of patients inhibited mitogen-induced T cell proliferation, whereas cells from the healthy coculture of donors were stimulatory in the same settings (Fig. 4B and C).

TGF-β neutralization with mAb partially inhibits Treg induction by Mo-iDCs from breast cancer patients

TGF-β is recognized as a relevant cytokine for the induction of Tregs and is produced in high levels by Mo-DCs from breast cancer patients. Therefore, we tested whether a neutralizing antibody against this cytokine was able to inhibit Treg generation by Mo-DCs from patients cocultured with allogeneic $CD4^+$-isolated lymphocytes. Indeed, the Mo-iDC–T cell cocultures of patients treated with the neutralizing antibody presented significantly higher cell proliferation (**Fig. 5A**), without reducing the induced $CD25^+$ T cell proportion (Fig. 5B). Beyond that, the presence of anti-TGF-β antibodies also led to significantly lower frequency (Fig. 5C) and absolute number (Fig. 5D) of $CD4^+CD25^+Foxp3^+CD127^{low}$ cells in cocultures, however, not in a similar frequency to levels induced by healthy Mo-iDCs.

DCs differentiated from the monocytes of breast cancer patients present a bias for the induction of Tregs even after maturation stimulus

Maturation of DCs is associated with a decrease in their ability to induce regulation. Therefore, monocytes and Mo-iDCs and sCD40L, cocktail, TNF-α, and LPS-treated Mo-iDCs from breast cancer patients were used as stimulators of allogeneic T cells to analyze the generation of $CD4^+CD25^+Foxp3^+$ cells. The phenotypic analysis demonstrated the dynamic increase of molecules (MFI) during monocyte-to-Mo-DC differentiation (**Fig. 6A**). In functional assays, healthy Mo-mDC$_{TNF\text{-}\alpha}$induced a higher frequency of $CD25^+$ cells (Fig. 6B) and lower frequency (Fig. 6C) and absolute number (Fig. 6D) of $CD4^+CD25^+Foxp3^+$ cells, as compared with all groups of the activated Mo-mDCs of patients. Moreover, analysis between the APCs of patients showed a reduced ability of monocytes to induce $CD4^+CD25^+Foxp3^+$ in comparison with Mo-iDCs and all Mo-mDC groups in frequency and absolute number (Fig. 6C and D). Regarding the activation status between Mo-DCs from patients, Mo-mDCs$_{LPS}$ were the only ones capable of significantly increasing the frequency of $CD25^+$ cells (Fig. 6B) and reducing $CD4^+CD25^+Foxp3^+$ frequency (Fig. 6C) in relation to the ability of Mo-iDCs; however, the absolute number of $CD4^+CD25^+Foxp3^+$ induced was not altered (Fig. 6D). Furthermore, the absolute number of induced Tregs was not different, comparing all activation stimulus used in the Mo-iDCs of patients (Fig. 6D), failing to revert the bias present in those cells.

DISCUSSION

We show here that Mo-DCs from cancer patients have a bias toward the induction of Tregs, a characteristic that is bound to impair the effectiveness of any therapeutic trial based on the cells of these patients. Indeed, Tregs are recognized as central in the maintenance of tolerance to self [37] but may also be involved in the failure of the immune system to eliminate or control infections [19, 20] and tumors [21] and to respond to

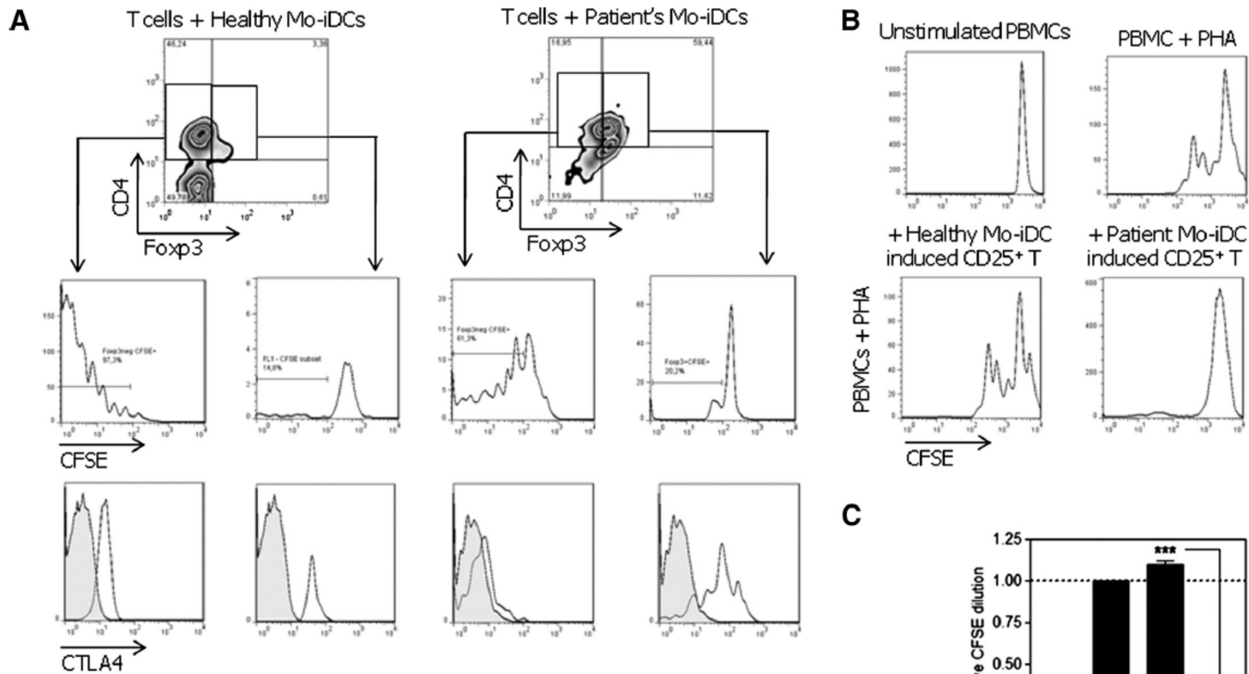

Figure 4. The Mo-iDC-induced $CD4^+CD25^+Foxp3^+$ cells of patients proliferate poorly and have suppressive function upon mitogen-stimulated T lymphocytes. (A) Mo-iDCs from healthy donors or from cancer patients were cocultured for 6 days with CFSE-labeled (lab.) T lymphocytes. CFSE dilution and CTLA4 expression were evaluated among $CD4^+CD25^+Foxp3^+$ and $Foxp3^{neg}$ cells (one representative experiment of three). Mo-iDCs-induced (ind.) $CD25^+$ cells were isolated and added to allogeneic, PHA-stimulated, CFSE-labeled cells (one sorted $CD25^+$ cell: 10 CFSE-labeled PBMC) and cultured for 3 days. (B) Representative experiment and (C) pooled data from four experiments showing CFSE dilution of PHA-stimulated PBMCs under the influence of $CD4^+CD25^+Foxp3^+$ obtained from cocultures with Mo-iDCs from healthy donors or from breast cancer patients (n=4; ***P<0.0001).

therapeutic vaccination [22]. Thus, the optimization of the DC phenotype, when these are used for therapeutic vaccination in cancer, is a crucial point for the achievement of better clinical responses.

Here, we induced the differentiation of blood monocytes obtained from breast cancer patients and from healthy donors into DCs, cultivating blood precursors in the presence of GM-CSF and IL-4, an established protocol for the generation of Mo-DCs [38]. Interestingly, under phase-contrast microscopy (data not shown) and by flow cytometry, Mo-iDCs, obtained from patients, presented a distinct morphology, being quite heterogeneous in FSC and SSC. On other hand, the frequency of cells within the FSC and SSC gate characteristic of DCs was similar between patients and healthy donors, as was the frequency of $CD14^{neg}HLA\text{-}DR^+$ cells within these gates, suggesting, thus, that the overall differentiation process was not affected significantly in the cells of cancer patients.

Although the frequency of Mo-iDCs was similar, cells derived from patients showed a higher frequency of CD86 costimulatory molecule expression and the classical plasmacytoid marker, CD123. Furthermore, whereas cells derived from healthy donors presented a single population of $CD86^+$ cells, with intermediate fluorescence intensity, those derived from patients seemed to be divided in two subpopulations: one with high and the other with low levels of CD86 expression. Indeed, the modulation of costimulatory molecules in APCs, particularly in DCs, seems to be essential to initiate and coordinate the nature of T cell responses [1]. CD86 and CD80 bind to stimulatory (CD28) and inhibitory (CTLA4) receptors on T cells with different affinities [39]. In human DCs, the induction and up-regulation of CD86 were shown to influence T cell activation significantly [40], whereas studies in knockout mice have indicated that the ability of DCs to generate/expand Treg subsets can be related to the balance of CD80 and CD86 [41, 42]. Our data indicate that the Mo-iDCs of patients present an altered CD86 pattern of expression and are poor T cell activators but very good Treg expansors/activators, suggesting that the modulation of costimulatory molecule expression could, indeed, be a pathway for the deviation of DC function in cancer patients. Actually, we have already observed an altered expression of CD86 in Mo-DCs from advanced cancer patients that was, apparently, corrected by an immunotherapeutic approach [9]. Simultaneously, we also need to consider the higher MFI expression of PD-L1 in Mo-iDCs from cancer

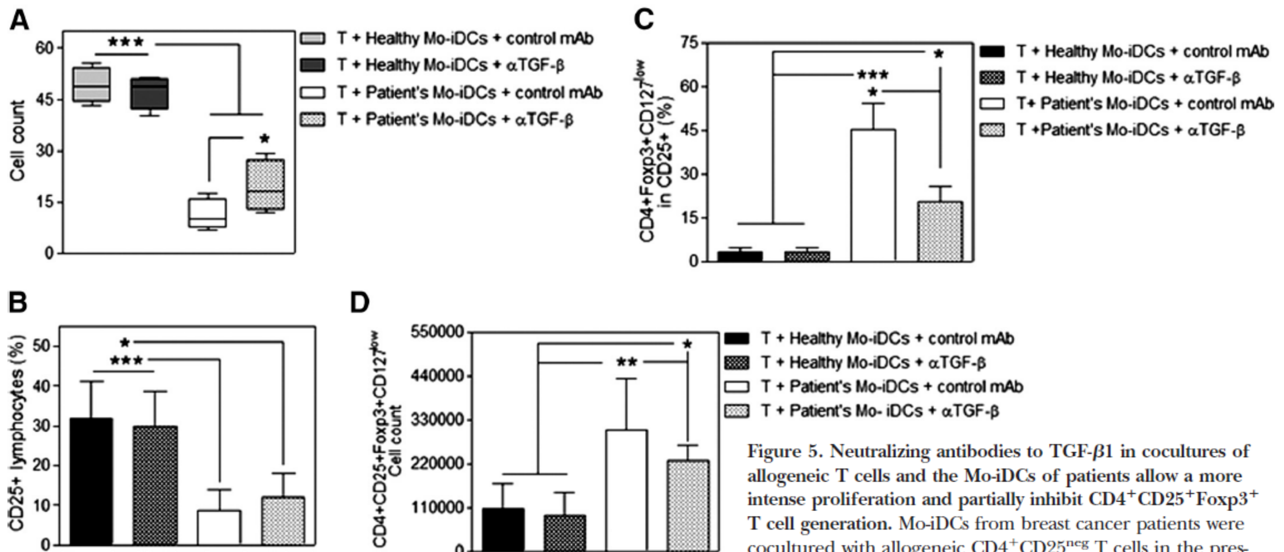

Figure 5. Neutralizing antibodies to TGF-β1 in cocultures of allogeneic T cells and the Mo-iDCs of patients allow a more intense proliferation and partially inhibit $CD4^+CD25^+Foxp3^+$ T cell generation. Mo-iDCs from breast cancer patients were cocultured with allogeneic $CD4^+CD25^{neg}$ T cells in the presence of 80 ng/ml neutralizing anti-TGF-β1 antibodies or control mAb. After 6 days, cells were harvested, counted, and labeled for flow cytometry. (A) Box and whisker plot showing absolute cell numbers recovered in cultures. (B) CD25 expression by stimulated T lymphocytes. (C) $CD4^+CD25^+Foxp3^+CD127^{low}$ cell frequency and (D) absolute numbers among recovered cells (healthy donors, $n=4$; patients, $n=6$; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$).

patients as possibly involved in the tolerogenic function of these cells, as in murine models, the expression of this molecule by DCs has been related to $Foxp3^+$ Treg via the PD-1R [43]. Interestingly, in humans, recent studies describe PD-L1 overexpression by peritumoral monocytes [44] and by tumor cells [45]. Furthermore, the high expression of CD123 molecules deserves investigation, as it has been reported that CD123 expression is related to a high expression of IDO, which confers a tolerogenic function to human myeloid DCs [46] and seems to be involved in the recruitment of $Foxp3^+$ cells to ovary tumor sites [47]. Therefore, although the ability of DCs to induce Treg activation depends on the balance of these various costimulatory molecules [48], the exact role of the balance between these molecules, mainly in human DCs and particularly, in cancer patients, is still unclear.

Additionally to the membrane phenotype differences, IL-10 and bioactive TGF-β1 cytokine production by the Mo-iDCs of patients were also characteristic, as elevated levels of both of these suppressor cytokines were found in their culture supernatants when compared with healthy Mo-iDCs. These cytokines are also involved in the commitment of näive T cells to suppressive properties [49], with IL-10 involved in the generation of Tr1 cells and TGF-β in the generation of Th3 $Foxp3^+$ cells. TGF-β is a multifunctional cytokine that regulates T cell growth and development [50], inhibits IL-2 production, and has potent, antiproliferative effects on $CD4^+$ T cells [51], principally by inducing Tregs [28, 36, 52], thus providing a possible explanation for the preferential generation/expansion of Tregs by Mo-iDCs from cancer patients. Indeed, the addition of a neutralizing antibody against this cytokine in cocultures of the Mo-DCs of patients and allogeneic T cells allowed a significantly higher proliferation of T cells, which also presented a significantly lower frequency of $CD4^+CD25^+Foxp3^+$ cells. It is noteworthy, though, that the addition of the neutralizing anti-TGF-β1 antibody was not enough to completely revert the induction of Tregs by the Mo-DCs of patients, thus suggesting the contribution of more than one mechanism for the generation of Tregs by the Mo-DCs of patients.

It is noteworthy that the $CD25^+$ cells, generated by coculture with the Mo-iDCs of patients, were able to suppress a mitogen-induced T cell proliferation, in contrast with $CD25^+$ cells stimulated by healthy donor Mo-iDCs that had no suppressive activity. Considering that the CD25 marker selected activated cells and Tregs, we thus confirm the suppressive activity of $CD25^+Foxp3^+$ lymphocytes induced by the Mo-iDCs of patients in vitro. Indeed, $Foxp3^+$ T cells are phenotypically and functionally heterogeneous and may contain $Foxp3^{low}$ non-Tregs (30–50% of total $CD4^+Foxp3^+$ T cells) that can produce proinflammatory cytokines [53], a phenomenon that could explain the lack of suppressive activity by these cells when induced by the healthy DCs of donors.

Although interesting, these data could still be considered less significant, as the bias was noted when Mo-iDCs were studied, a population whose ability to induce Tregs has long been described [13, 14]. Furthermore, as Mo-mDCs are used in therapeutic protocols for cancer, one could argue that the phenotype of immature cells is of lesser relevance. Notwithstanding the fact that the bias was noted when immature cells of healthy controls and patients were compared, thus indicating a phenomenon beyond the simple maturation status of the cell, we decided to induce the Mo-DCs of the maturation of patients (using sCD40L, a cytokine cocktail, TNF-α, and LPS) and then, evaluate their ability to induce Tregs, also comparing it with that of monocytes. In these experiments, we noted

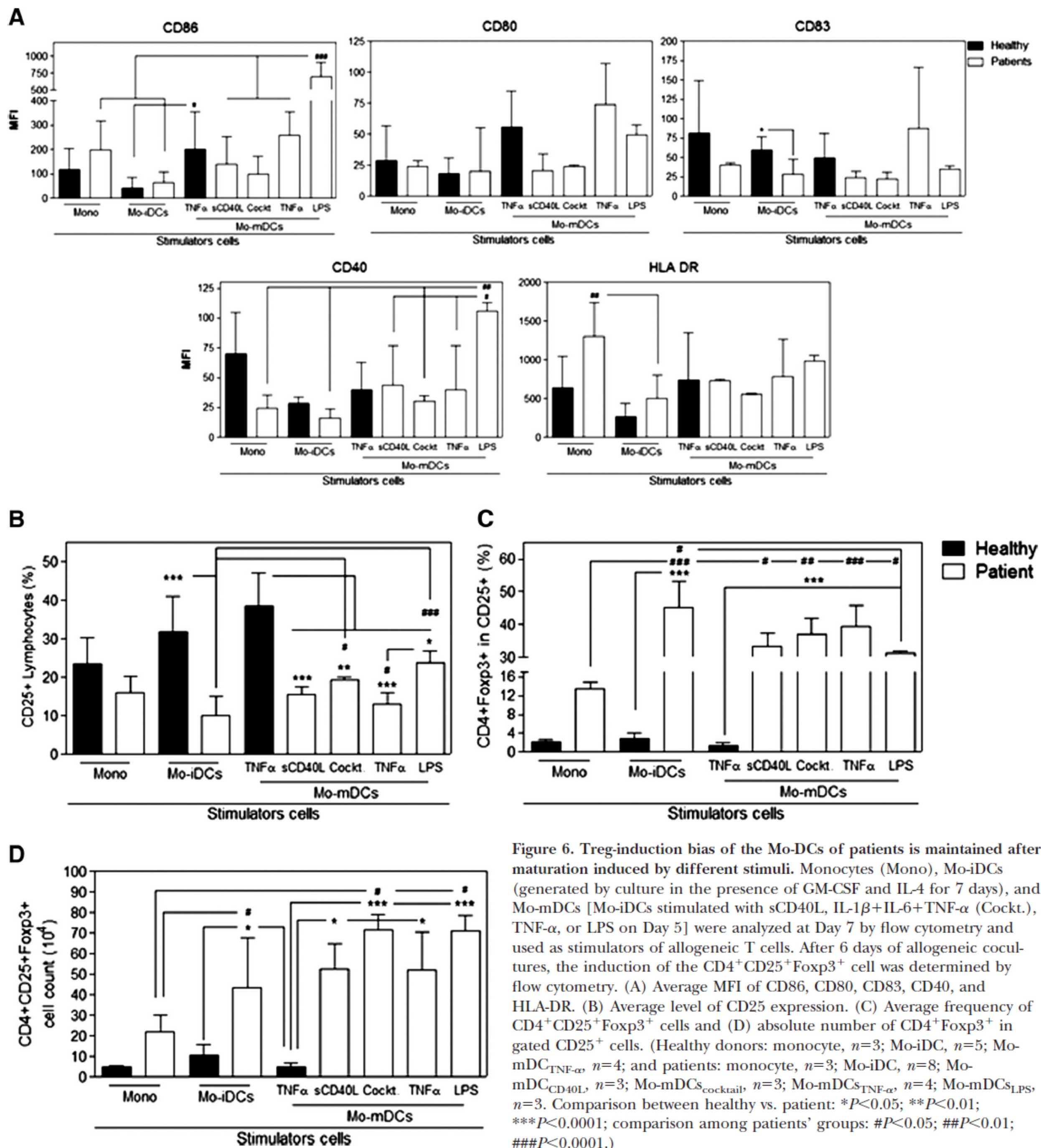

Figure 6. Treg-induction bias of the Mo-DCs of patients is maintained after maturation induced by different stimuli. Monocytes (Mono), Mo-iDCs (generated by culture in the presence of GM-CSF and IL-4 for 7 days), and Mo-mDCs [Mo-iDCs stimulated with sCD40L, IL-1β+IL-6+TNF-α (Cockt.), TNF-α, or LPS on Day 5] were analyzed at Day 7 by flow cytometry and used as stimulators of allogeneic T cells. After 6 days of allogeneic cocultures, the induction of the $CD4^+CD25^+Foxp3^+$ cell was determined by flow cytometry. (A) Average MFI of CD86, CD80, CD83, CD40, and HLA-DR. (B) Average level of CD25 expression. (C) Average frequency of $CD4^+CD25^+Foxp3^+$ cells and (D) absolute number of $CD4^+Foxp3^+$ in gated $CD25^+$ cells. (Healthy donors: monocyte, n=3; Mo-iDC, n=5; Mo-mDC$_{TNF\text{-}\alpha}$, n=4; and patients: monocyte, n=3; Mo-iDC, n=8; Mo-mDC$_{CD40L}$, n=3; Mo-mDCs$_{cocktail}$, n=3; Mo-mDCs$_{TNF\text{-}\alpha}$, n=4; Mo-mDCs$_{LPS}$, n=3. Comparison between healthy vs. patient: *P<0.05; **P<0.01; ***P<0.0001; comparison among patients' groups: #P<0.05; ##P<0.01; ###P<0.0001.)

that Mo-mDCs from patients conserved the bias toward the induction of a similar number of Tregs, indicating the existence of a profound alteration of the monocytes of patients, which cannot be corrected easily by standard culture protocols. Besides that, it is also relevant that monocytes from healthy donors and from patients initially present a similar capacity to induce $CD4^+CD25^+Foxp3^+$ T lymphocytes. This capacity, however, decreases in the cells of healthy donors, as they differentiate toward DCs (as one would expect), but increases in cells of patients.

Taken together, our data show important differences in phenotype and function of DCs derived from cancer patient monocytes that appear to be dependent of their surface phenotype and pattern of cytokine induction/production. These features could have a definitive role in the tumor-escape mechanism. If the apparent bias of Mo-DCs from cancer patients cannot be corrected by targeted in vitro manipulations, the effectiveness of cancer vaccination strategies based on these cells would probably be compromised significantly. On the other hand, the determination of the mechanisms, through which tumors are able to modify the circulating ability of monocytes to differentiate in vitro into DCs, could point to new, tumor-escape strategies, whose identification could provide new targets to be considered in immunotherapeutic approaches to cancer.

AUTHORSHIP

R.N.R. performed all experiments, analyzed results, prepared the figures, and wrote the manuscript. L.S.C. helped perform some experiments and analyze the results. A.P.S.A.S. and F.L. helped to obtain patients ' samples and analyzed and discussed the results. P.C.B-S. helped discuss the results. J.A.M.B. designed the research, discussed the results, and wrote the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

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PRESS RELEASE

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CONTACT:
Cody Mooneyhan
cmooneyhan@faseb.org
301-634-7104

CANCER "TURNS OFF" IMPORTANT IMMUNE CELLS, COMPLICATING EXPERIMENTAL VACCINE THERAPIES

New research published in the Journal of Leukocyte Biology suggests monocyte-derived dendritic cells may not be as effective in inducing desired immune responses as previously expected, complicating experimental cancer vaccine therapies while offering hope for those with autoimmune disorders

Bethesda, MD—A research report published in the September 2012 issue of the *Journal of Leukocyte Biology* (https://www.jleukbio.org) offers a possible explanation why some cancer vaccines are not as effective as hoped, while at the same time identifies a new therapeutic strategy for treating autoimmune problems. In the report, scientists suggest that cancer, even in the very early stages, produces a negative immune response from dendritic cells, which prevent lymphocytes from working against the disease. Although problematic for cancer treatment, these flawed dendritic cells could be valuable therapeutic tools for preventing the immune system from attacking what it should not, as is the case with auto immune disorders and organ transplants.

"Immunotherapy of cancer has been an elusive research target that, though promising, never seems to 'get there,'" said José Alexandre M. Barbuto, Ph.D., from the Laboratory of Tumor Immunology, Department of Immunology, Institute of Biomedical Sciences at the University of São Paulo, in São Paulo, Brazil. "This study helps us to better understand the mechanisms by which tumors avoid immune recognition and rejection and may, therefore, teach us how to actually engage effectively the immune system in the fight against tumors, thus achieving much better clinical responses and, consequently, quality of life, in our therapeutic approaches."

To make this discovery, researchers obtained a small sample of blood from breast cancer patients and from healthy volunteers. The blood cells were then separated and induced to become dendritic cells. Researchers then used these laboratory-generated dendritic cells to induce responses from other immune system cells, namely lymphocytes. While dendritic cells from the healthy donors induced vigorous lymphocytic responses, dendritic cells from cancer patients induced mainly the activation of a specific type of lymphocyte, a regulatory lymphocyte that works as a "brake" for other types of lymphocytes.

"Understanding why the immune system does not recognize and eliminate cancer is critical to developing effective immunotherapies to fight the disease," said John Wherry, Ph.D., Deputy Editor of the Journal of Leukocyte Biology. "Immunologists have been trying to unravel the answer the this question for decades and have realized that the problem is both on the immune system side and because cancer cells appear to actively 'fly under the radar' avoiding immune system detection. This article offers insights into the underlying mechanisms regulating a key immune cell type, the dendritic cell, involved in initiating anti-tumor responses."

The *Journal of Leukocyte Biology* (http://www.jleukbio.org) publishes peer-reviewed manuscripts on original investigatins focusing on the cellular and molecular biology of leukocytes and on the origins, the developmental biology, biochemistry and functions of granulocytes, lymphocytes, mononuclear phagocytes and other cells involved in host defense and inflammation. The *Journal of Leukocyte Biology* is published by the Society for Leukocyte Biology.

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APPENDIX - B

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Review Article

What Are the Molecules Involved in Regulatory T-Cells Induction by Dendritic Cells in Cancer?

Rodrigo Nalio Ramos, Cristiano Jacob de Moraes, Bruna Zelante, and José Alexandre M. Barbuto

Department of Immunology, Institute of Biomedical Sciences, University of Sao Paulo, Avenida Professor Lineu Prestes, 1730 Sao Paulo, SP, Brazil

Correspondence should be addressed to José Alexandre M. Barbuto; jbarbuto@icb.usp.br

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Dendritic cells (DCs) are essential for the maintenance of homeostasis in the organism, and they do that by modulating lymphocyte priming, expansion, and response patterns according to signals they receive from the environment. The induction of suppressive lymphocytes by DCs is essential to hinder the development of autoimmune diseases but can be reverted against homeostasis when in the context of neoplasia. In this setting, the induction of suppressive or regulatory T cells contributes to the establishment of a state of tolerance towards the tumor, allowing it to grow unchecked by an otherwise functional immune system. Besides affecting its local environment, tumor also has been described as potent sources of anti-inflammatory/suppressive factors, which may act systemically, generating defects in the differentiation and maturation of immune cells, far beyond the immediate vicinity of the tumor mass. Cytokines, as IL-10 and TGF-beta, as well as cell surface molecules like PD-L1 and ICOS seem to be significantly involved in the redirection of DCs towards tolerance induction, and recent data suggest that tumor cells may, indeed, modulate distinct DCs subpopulations through the involvement of these molecules. It is to be expected that the identification of such molecules should provide molecular targets for more effective immunotherapeutic approaches to cancer.

1. Background

Regulatory T cells (Tregs) are crucial to the maintenance of tolerance to autoantigens [1]. The failure of Treg function or their depletion has been implicated in the development of many autoimmune diseases in humans and in mouse models [2]. However, Treg-mediated suppressive activity can also contribute to the immune escape of pathogens or tumors [3, 4]. Nowadays, regulatory T cells (Tregs) are considered one of the major obstacles to the success of immunotherapeutic approaches to cancer [5–8]. Several studies have described the direct association between Treg increase and tumor development, implicating this phenomenon as one of the most important escape mechanisms in different tumor types [7, 9, 10]. Many evidences have demonstrated that Treg accumulation is not restricted to the tumor site but is observed in the peripheral blood as well, from patients with distinct malignant tumors, including pancreas and breast [11], lung [12], and ovarian cancer [4, 12]. Indeed, elimination of Tregs in mouse tumor models can improve antitumor immune responses and survival [9, 13].

Dendritic cells (DCs) are believed to act as sensors of the homeostatic equilibrium of their environment, where they capture antigens to present to T lymphocytes. Thus, depending on the status of the tissue, they might induce immunity or tolerance to the antigens they present. Indeed, many *in vitro* studies have demonstrated that DCs are essential for regulatory T-cells induction [14, 15], apparently depending on various distinct mechanisms [16], but also, frequently, on external sources of cytokines, among which TGF-beta seems to play a predominant role [17]. Not surprisingly, therefore, during tumor development the balancing role of DCs in the T helper versus Treg stimulation seems to be deeply modified [8, 18].

However, despite all the accumulated data, the precise role of DCs in the imbalance between T helper and Tregs

in cancer is still unclear. Do the observed biases of DC function in tumor bearers reflect a previous disturbance in their immune homeostasis or are these deviations of DC function the cause of the other immunological abnormalities? How significant is the contribution of these DC deficits to the escape of tumors from the body's control? Though the answer to these questions is not available yet, the increasing knowledge and characterization of DC behavior in the presence of tumors allows us to predict that it will be, and, furthermore, that, once reached, it will provide us with powerful tools for the clinical management of cancer. With these goals in view, we discuss, here, the impact of tumor presence in the membrane phenotype and function of DCs and their bias to induce/expand regulatory T cells.

2. The Tumor Microenvironment: A Tolerogenic Milieu

Several studies have described the potential impact of tumor-derived products in the suppression of immunity. Signals derived from tumors not only act directly upon immune effector cells but also induce the conversion and/or the recruitment of cells with suppressive functions to their microenvironment [19]. In consequence, tumors are typically characterized by the presence of higher concentrations of anti-inflammatory molecules, such as TGF-beta, IL-10, and prostaglandin E2 [20–23], increased amounts of angiogenic factors, as the vascular endothelial growth factor (VEGF) [24], and augmented CCL22 chemokine gradient [25] in addition to the local expression of immune-inhibitory molecules, including CTLA4 and PD-1/PD-L1 [26, 27]. Altogether, these constitute, nowadays, the most highly sought targets to achieve the breakdown of tumor-associated microenvironment-induced tolerance. Still, in order to obtain an immune recovery in face of tumors, we still need to identify the source of the tolerogenic signals. Though tumors cells may produce such mediators, also tumor-infiltrating leukocytes may be their source, and, indeed, the study of such populations has revealed that regulatory Foxp3^{+} T cells (Tregs) [28], anti-inflammatory M2-macrophages [29], plasmacytoid dendritic cells (pDCs) [30], and immature myeloid DCs [31] accumulate in human neoplastic tissues and patients' blood [4] and have been associated with poor prognosis for the patients specific cancer types.

As mentioned, the presence of tolerance-inducing conditions seems not to be restricted to the tumor microenvironment. Several studies have demonstrated the increase of anti-inflammatory cytokines and the higher frequency of suppressive cells in the bloodstream and lymph nodes from cancer patients. The detection of higher amounts of cytokines like TGF-beta [32], M-CSF [33], and IL-6 [34, 35] in patients' serum could suggest that the tumor presence affects cells in distant organs, thus resulting in systemic alterations which could allow tumors not only to grow locally unchecked but also to metastasize without an effective immune barrier. In agreement with that are: the higher frequency of myeloid-derived suppressor cells (MDSCs) (a group of immature but potent suppressor cells capable of down-regulating anti-tumor immunity) found in cancer patients' circulation [36]; the decreased frequency of circulating and tumor-infiltrating myeloid DCs [37, 38]; and the CD4 lymphopenia observed in cancer patients [39–41]; all three important alterations of immune homeostasis in cancer patients that, consequently, hamper the effectiveness of their treatment.

3. DCs: Targets to the Tumor Tolerogenic Milieu

Dendritic cells (DCs) are the best adapted professional antigen-presenting cells (APCs) able to initiate, coordinate, and regulate the adaptive immune responses by inducing naive T-cells differentiation into diverse T helper lymphocyte subtypes [42–46]. Generally, at homeostasis condition, tissue-resting DCs are in immature status (lower MHC class II and costimulatory molecules expression) and strategically located to sense and acquire antigenic products from the environment. Using nonspecific receptors, immature DCs can recognize pathogens or danger-associated molecular patterns (as known, PAMPs and DAMPs, resp.) and migrate to lymphoid organs, at the same time as they increase their expression of MHC, CD80, CD86, and CD40 surface molecules and become ready to activate naïve T lymphocytes [44]. DCs are also crucial for the induction/maintenance of T-cell tolerance to antigens acquired in "healthy" tissues, thus performing an essential role in the prevention of autoimmunity [47].

It is also evident that the term DC is applied to several distinct subpopulations, classified, still incompletely, in relation to their tissue localization, migratory ability, surface markers' expression, and the profile of soluble factors they release. Though still uncertain, it is becoming increasingly clear that any classification of DCs will be insufficient to accommodate all the plasticity of these cells. Therefore, a better approach to the problem would be to describe, as well as possible, the DCs found in a certain condition, and from that, to correlate their phenotype in that specific situation with the known functions of these cells. This has been done in relation to DCs within tumors and has shown that tumors modify significantly the phenotype of DCs within their microenvironment [8, 22]. Various observations point to a mainly functional deficit of these cells in immune stimulation, due to a decreased frequency of mature, functionally competent DCs within tumors [31] and in peripheral blood [48]. Actually, we have already shown an altered expression of CD86 in Mo-DCs from advanced cancer patients, which was, apparently, corrected by an immunotherapeutic approach [49]. Importantly, the presence of pDCs in tumor sites has been also related to poor prognosis in cancer patients [30], and their functional investigation revealed a considerable low to absent IFN-alpha production in breast and ovarian cancer [50, 51]. The tumor-associated stroma and cancer cells *per se* can generate signals that drive DC to a tolerogenic pathway, characterized, mainly, by a poor upregulation of MHC class II and costimulatory molecules and absent or low production of proinflammatory cytokines [52], thus favoring tumor

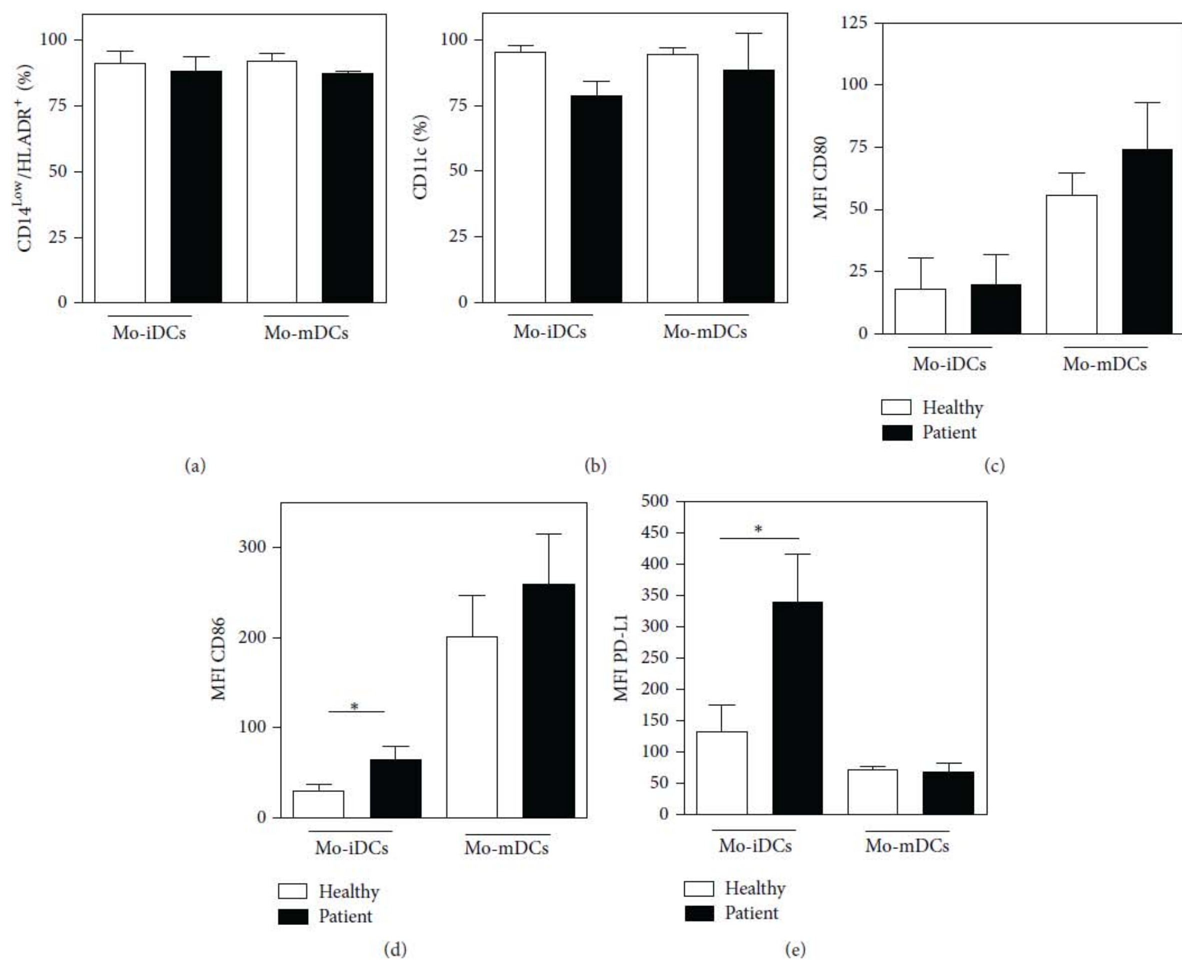

Figure 1: Patients' immature Mo-DCs express higher levels of PD-L1. Blood monocytes from control, and breast cancer patient subjects were cultured in the presence of IL-4 and GM-CSF for seven days and, subsequently, characterized. Flow cytometry analysis showing grouped frequency of CD14^{Low}/HLADR^{+} (a) and CD11c^{+} (b) cells and Mean Intensity Fluorescence (MFI) values of CD14^{Low}/HLADR^{+} gated cells to CD80 (c), CD86 (d), and PD-L1 (e) molecules in Mo-iDCs from healthy donors and breast cancer patients ($^{*}P < 0.05$, two-tailed unpaired t-test; healthy $n = 5$; patients $n = 9$). (Mature Mo-DCs were activated by TNF-alfa for 48 hours.)

evasion from the immune system. Interestingly, in tumor-bearing mouse, the presence of DCs is also crucial for cancer vascularization, and when DCs are depleted, the elimination of malignant cells can be enhanced [53, 54]. Additionally, another elegant study showed that human myeloid DCs expressing OX40L stimulate Th2 immunity *in vitro*, under the influence of thymic stromal lymphopoietin (TSLP) derived from breast tumor cells [55]. Such findings may explain the bias towards a Th2 inflammatory tumor microenvironment found in breast cancer.

Since it became possible to achieve DC differentiation from human blood monocytes (Mo-DCs) [56], the immunostimulatory potential of these cells could be harnessed for cancer immunotherapy [57–60]. On the other hand, *in vitro* findings, describe that tumor cells present during human monocyte differentiation cause alteration in their molecular expression and unsuccessful DC differentiation, even under exogenous cytokine addition [61–63]. In addition, we have shown that breast cancer patients' monocyte-derived DCs are phenotypic altered and biased to induce Tregs [64], even though differentiated without the presence of tumor cells in the culture.

Immature Mo-DCs from patients express higher levels of CD86 and PD-L1 membrane molecules after 7 days in the presence of IL-4 and GM-CSF (Figure 1). Though the expression of CD86 could be interpreted as an enhanced costimulatory ability, the same cannot be implied for PD-L1. PD-L1, also known as B7-H1, has been described as an inhibitory molecule in T lymphocyte activation [65, 66] and also related to T effector to Treg conversion [67] and the induction of T cell anergy by Mo-DCs [68]. Furthermore, its expression has been described as enhanced in monocytes from peritumoral stroma in hepatocellular carcinoma [26] and in lung cancer infiltrating DCs [69].

4. Regulatory T-Cells Induction by Tumor-Affected DCs

The induction and expansion of Tregs by DCs are generally related to their role in the maintenance of tolerance to self [16]. Several studies have been developed, trying to identify the signals that drive DCs into that function and, thus, eventually allow the use of such educated DCs to control unwanted immune responses, like those against transplanted tissues or in autoimmune diseases [70]. Actually, the acquisition of the ability to promote Tregs is an integral part of the physiologic function of DCs, as can be noted, for instance, in the presence of apoptotic cells [71, 72]. In this search, anti-inflammatory cytokines as IL-10 [73, 74], TGF-beta [75], and vitamin D3 addition [76, 77] have been shown to affect mouse and human DCs, causing them to stimulate regulatory or suppressive T lymphocytes [78]. Intriguingly, even inflammatory cytokines, as TNF-alpha, have been associated with tolerogenic DC induction in autoimmune disorders like the murine Experimental Autoimmune Encephalomyelitis (EAE) [79]. Paradoxically, the same functional status of DCs, which is the still unreached aim of research in autoimmunity and transplantation studies, is the natural status of DCs in cancer, which is, again, beyond our powers of effective modulation. Tumor cells are associated with lower activation of immune cells and hinder APC activation [32, 80, 81] and, also can attract regulatory T cells to their microenvironment [4, 11, 12], all phenomena which would be more than welcome in the aforementioned autoimmune and transplant recipients. Regarding APCs, *in vitro* studies showed Treg induction by human Mo-DCs stimulated by pancreatic or lung tumor cells [61, 62], the ability of human intratumoral pDCs, to expand Tregs *ex vivo* in breast cancer [50] and to induce suppressive activity by T cells in prostate cancer [82]. These findings show that tumor cells are able to promote Tregs induction by DCs in patients, and also to affect DCs from healthy donors, causing them to stimulate Tregs. Finally, our group has demonstrated that this effect of tumors upon DCs does not depend on the continuous presence of neoplastic cells, since Mo-DCs from breast cancer patients even when differentiated *in vitro* and, therefore, away from the direct tumor influence, are poor T-cell stimulators and biased to induce $CD4^+CD25^+Foxp3^+$ regulatory T cells when cocultured with naïve $CD4^+CD45RA^+$ lymphocytes (Figure 2). It should be noted that this bias was present, regardless of the maturation stimulus used to activate the patients' Mo-DCs [64]. Taken together, these data indicate that during tumor development a systemic tolerogenic status of DCs is favored, enhancing their ability to expand/recruit Tregs and whose specific mechanisms are still largely undetermined.

5. Potential Mechanisms of Tumor-Affected DCs in the Induction of Tregs

Cytokines, as TGF-beta and IL-10 in addition to IL-2, are currently used to expand effectively murine and human Tregs *in vitro*. Interestingly, the same cytokines can also induce DCs to stimulate Tregs *in vitro* [17, 74, 75]. This may suggest that the major signals responsible for the generation and expansion of regulatory T cells *in vitro* and in vivo are already known. However, few data are available in regards to the mechanism of tumor-conditioned DCs in Tregs induction. Recent findings have demonstrated that infiltrating pDCs from ovarian [83] and breast tumor [84] can express high levels of ICOS-L, a phenomenon that could explain their ability to stimulate $Foxp3^+$ Tregs *in vitro*. Our own data also have shown that the Tregs induction by Mo-DCs from cancer patients could be partially reversed by blocking of TGF-beta *in vitro*, and not by LPS, proinflammatory cocktail, or sCD40L activation [64]. TGF-beta is a multifunctional cytokine that regulates T-cell growth and development [85], inhibits IL-2 production, and has potent antiproliferative effects on $CD4^+$ T cells [86], principally by inducing regulatory T cells [87]. However, since blocking of this cytokine was not enough to abolish the Treg-induction bias of the patients' Mo-DCs, it is likely that the TGF-beta signal may act together with other factors. Among the candidates for this cosignaling it is interesting to note that patients' Mo-DCs expressed higher levels of surface CD86 and PD-L1 (Figure 1), both molecules that have been also implicated in the balance of Tregs stimulation [88–91]. Thus, the TGF-beta signal may actuate together with surface molecules signals to "complement" the patients' Mo-DCs signalization in the induction/expansion of Tregs, as we showed here that DC-T cell contact is essential in that phenomenon (Figure 3).

6. Concluding Remarks

Tregs are recognized as central in the maintenance of tolerance to self [1] but may be also involved in the failure of the immune system to eliminate or control infections [3], tumors [13] and to respond to therapeutic vaccination [92]. Nowadays, it is also broadly accepted that DCs may play a crucial role in tolerance by the induction of Tregs at peripheral tissues and organs [16]. On the other hand, it is also known that tumor cells can alter profoundly the ability of DCs to instruct the immune system to generate adaptive antitumor responses [22], thus deviating the response to tolerance. The physiological DC ability to induce Treg activation depends on various cytokines and costimulatory molecules, but the exact balance between these, particularly, in DCs from cancer patients, is still unclear. CD86 and CD80 bind to both stimulatory (CD28) and inhibitory (CTLA-4) receptors on T cells, with different affinities [93]. In human DCs, the induction and upregulation of CD86 was shown to influence significantly T-cell activation [94], while studies in knockout mice have indicated that DCs ability to generate/expand Treg subsets can be related to the balance of CD80 and CD86 [89, 95].

Confirming the significant role of CTLA-4 signaling in the immunosuppression of cancer patients, the blockage of this molecule in clinical settings by monoclonal antibodies has been able to improve significantly the survival of metastatic melanoma patients [96, 97]. Additionally, PD-L1, ICOS-L, and TGF-beta seem to emerge as good candidates for the *in vitro* manipulation of DC phenotype/function for immunotherapeutic approaches. More recently, clinical trials targeting the PD-1/PD-L1 axis with anti-PD1 monoclonal

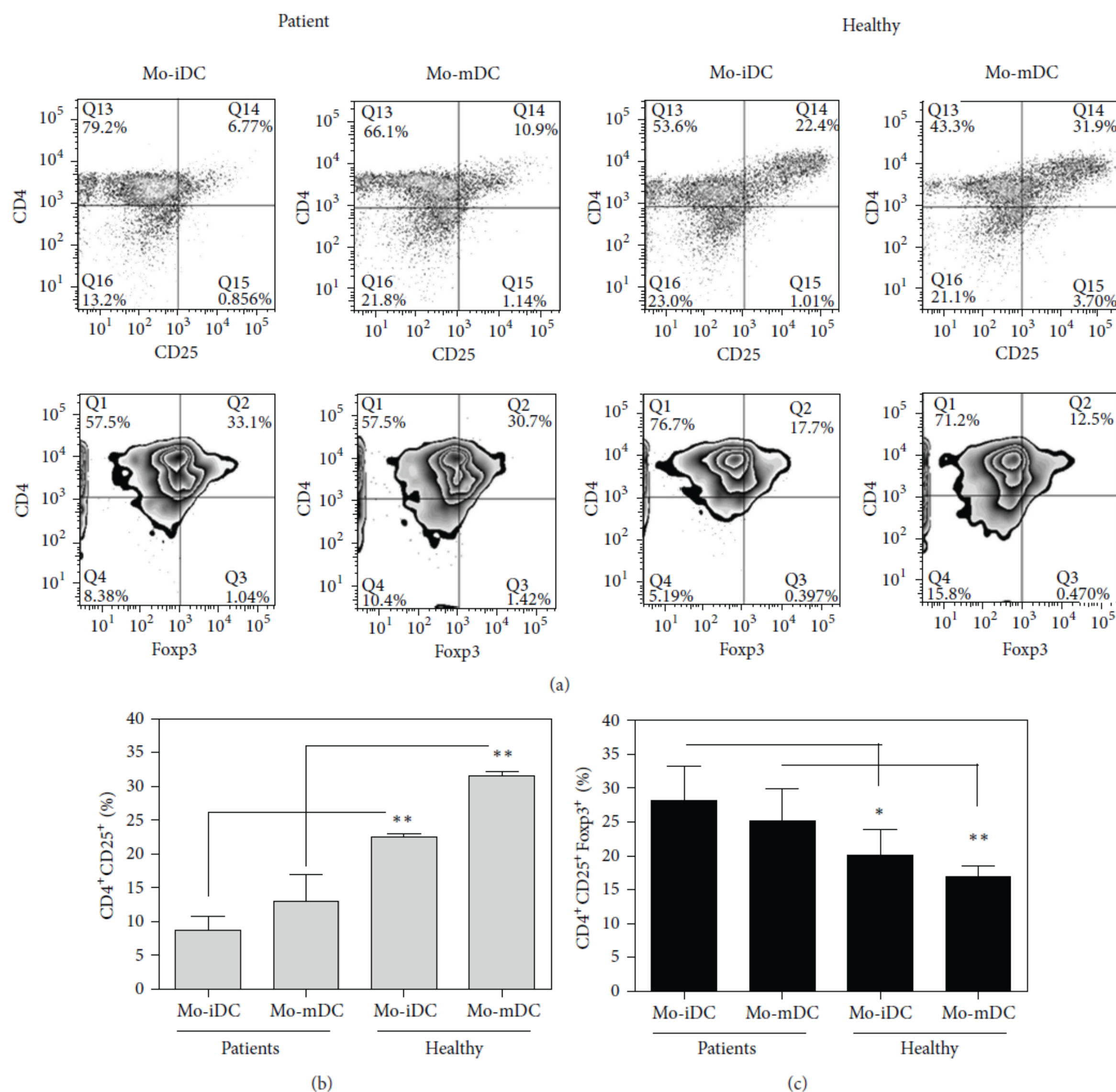

FIGURE 2: Patients' Mo-DCs fail to activate $CD4^+$ lymphocytes and induce higher Foxp3 expression even after maturation. Mo-DCs from controls and breast cancer patients were cocultured with allogeneic $CD4^+CD45RA^+$ cells for five days. At the end of culture the phenotype of lymphocytes was evaluated by flow cytometry. (a) Representative experiments of CD25 and Foxp3 expression in $CD4^+$ lymphocytes stimulated by immature DCs (Mo-iDCs) or mature DCs (Mo-mDCs) from healthy donors or breast cancer patients. Average frequency of $CD25^+$ cells (b) and $CD4^+CD25^+Foxp3^+$ cells (c) after $CD4^+CD45RA^+$ lymphocytes' coculture with Mo-DCs ($^*P < 0.05$; $^{**}P < 0.01$, two-tailed unpaired t-test; $n = 4$). (Mature Mo-DCs were activated by TNF-alfa for 48 hours.)

antibodies revealed their safety [98] and achieved promising results, with tumor regressions in patients with advanced cancer [99, 100], thus indicating another possible pathway to be explored in the clinic.

Nevertheless, these data are still sparse and much needs to be determined before an effective manipulation of DC phenotype and function is achieved. In order to accomplish this, however, studies addressing the intracellular signaling pathways in tumor-affected DCs are urgently needed and may shed light on the precise mechanisms of their response to tumors as well as provide molecular targets for their effective manipulation.

Abbreviations

APCs: Antigen-presenting cells
DCs: Dendritic cells
GM-CSF: Granulocyte macrophage colony stimulating factor

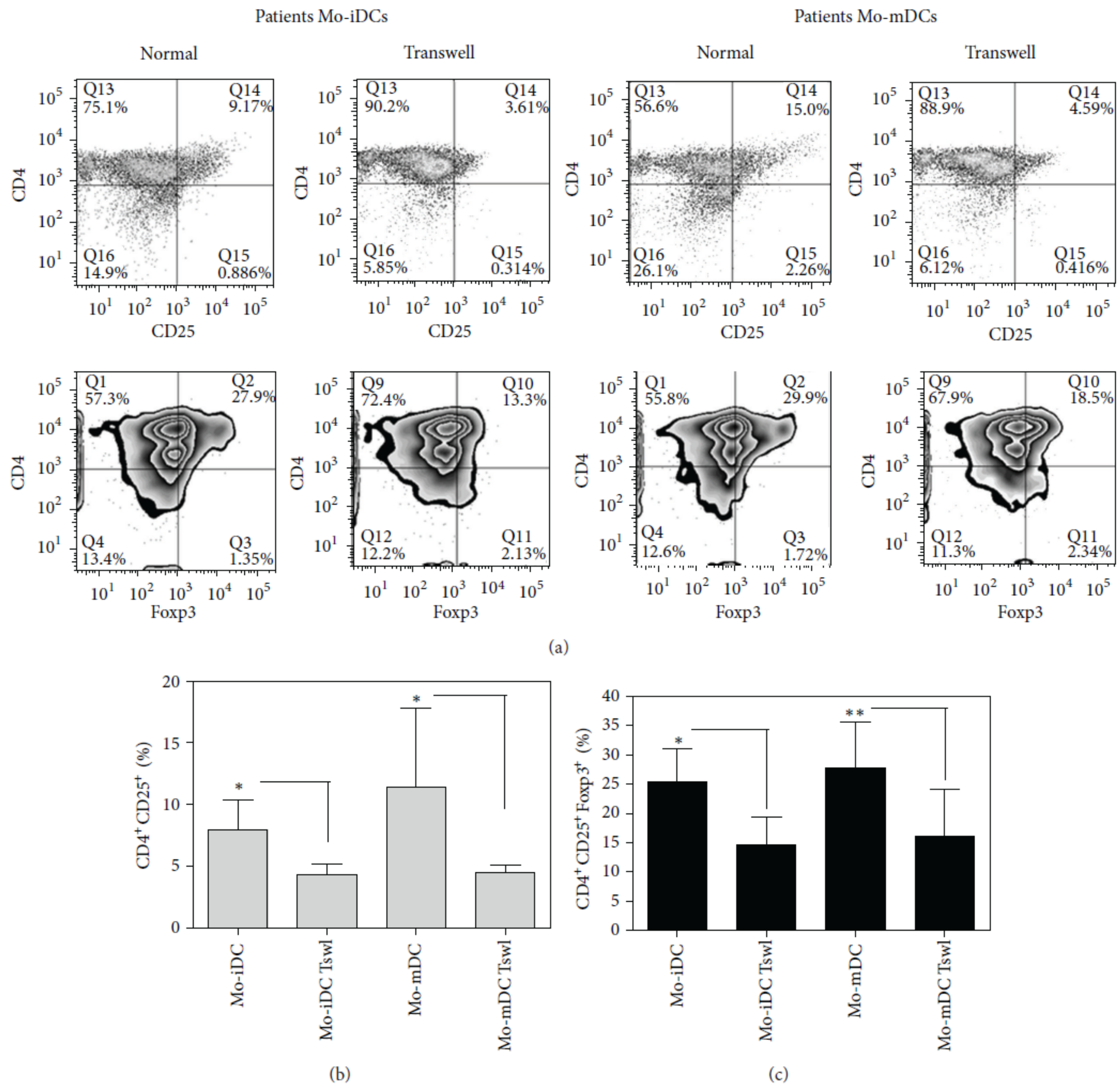

FIGURE 3: Patients' Mo-DCs induce expansion of regulatory T lymphocytes with the cooperation of contact molecules. Mo-DCs from breast cancer patients were cocultured with allogeneic CD4$^+$CD45RA$^+$ cells for five days in a transwell system or not. At the end of culture the phenotype of lymphocytes was evaluated by flow cytometry. (a) Representative experiments of CD25 and Foxp3 expression in CD4$^+$ lymphocytes stimulated by immature DCs (Mo-iDCs) or mature DCs (Mo-mDCs) from breast cancer patients in normal or transwell condition. Average frequency of CD25$^+$ cells (b) and CD4$^+$CD25$^+$Foxp3$^+$ cells (c) after CD4$^+$CD45RA$^+$ lymphocytes' coculture with patients' Mo-DCs ($^*P < 0.05$; $^{**}P < 0.01$, paired t-test; $n = 4$). (Tswl: transwell system; mature Mo-DCs were activated by TNF-alfa for 48 hours.)

IFN-gamma:	Interferon-gamma
MFI:	Median fluorescence intensity
MHC:	Major histocompatibility complex
Mo-DCs:	Monocyte-derived dendritic cells
Mo-iDCs:	Monocyte-derived immature dendritic cells
Mo-mDCs:	Monocyte-derived mature dendritic cells
PBMCs:	Peripheral blood mononuclear cells
pDCs:	Plasmacytoid dendritic cells
TGF-beta:	Transforming growth factor-beta
TNF-alpha:	Tumor necrosis factor-alpha
Tregs:	Regulatory T cells
TSLP:	Thymic stromal lymphopoietin
Tswl:	Transwell coculture system.

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APPENDIX - C

Main Abstracts in Congress and Seminars (up-dated: October, 2015)

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C. Breast Tumor microenvironment through local and systemic ways skew blood monocytes into suppressive CD163highIL-10high macrophages that impact in patients' survival. In: Innate Immunity 2015 - XL CONGRESS OF THE BRAZILIAN SOCIETY OF IMMUNOLOGY, Guarujá/SP – Brazil.
  * Oral presentation as finalist in the Theresa Kipnis prize among the 5 best projects in the congress - Honorable Mention.

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C. Tumor microenvironment acts direct and systemically skewing blood monocytes into suppressive CD163highIL-10high cells that may impact in breast cancer patients' survival. In: CRI-CIMT-EATI-AACR - The Inaugural International Cancer Immunotherapy Conference: Translating Science Into Survival, NYC/USA, 2015.

- Porchia, B.F. M. M.; Rosa, D.S.; Moreno, A.C.R.; Boscardin, S.B.; **Ramos, R.N**.; Diniz, M.O.; Aps, L.M.; Barbuto J.A.M.; Ferreira, L.C.S. "Impacto de uma formulação vacinai terapêutica na ativação de células dendríticas e indução de resposta imune efetora mediada por linfócitos T CD8 específicos: uma alternative inovadora para o controle de tumores induzidos pelo papiloma virus humano" In: II Bienal Internacional de Oncologia – AC Camargo Cancer Center – São Paulo/SP – Brazil.
  * Prize Dr. Fernando Gentil – AC Camargo Cancer Center

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C. Tumor microenvironment acts direct and systemically skewing blood monocytes into suppressive CD163HighIL-10High cells that may impact in cancer patients' survival. In: The symposium of "The Molecular, Integrative and Cellular Biology Doctoral School of Lyon", Lyon/France, 2014.
  *Oral Presentation

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C. Tumor microenvironment acts direct and systemically skewing blood monocytes into suppressive CD163HighIL-10High cells that may impact in cancer patients' survival. In: XXXIX Congress of the Brazilian Society of Immunology, Búzios/RJ - Brazil
  * Honorable Mention in the poster section among PhD studies.

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C. Tumor microenvironment induces CD163+PDL1+ M2-like macrophages and alters monocyte precursor's differentiation in primary breast and ovarian cancer patients. In: Keystone symposium on Molecular and Cellular Biology: "Inflammatory Diseases: Recent Advances in Basic and Translational Research and Therapeutic Treatments", Vancouver/Canada, 2014.

- Salomón, MAC ; **Ramos, R. N**. ; Romagnoli, G G ; Barbosa, B Z ; Barbuto, JAM . Human monocyte-derived dendritic cells require direct contact with natural killer cells to induce a Th1 profile of response. In: 15th International Congress of Immunology ICI, Milan/Italy, 2013.

- Salomón, M.A.C.; **Ramos, R. N.**; Romagnoli, G. G.; Barbuto, J.A.M. The Crosstalk Between Natural Killer and Monocytes During Dendritic Cells Differentiation. In: 16th International Congress of Mucosal Immunology (ICMI), Vancouver/Canada, 2013.

- Silva, F. S. ; **Ramos, R. N.** ; Bassi, E. J. ; Marques, M. M. ; Camara, N. O. S. ; Barbuto, JAM ; Maranduba, C. M. C. Stem Cells from Human Exfoliated Deciduous Teeth (SHED) decrease the ability of Monocyte-Derived Dendritic Cells (Mo-DCs) to induce T cell proliferation. In: XXXVII Congress of the Brazilian Society of Immunology, Campos de Jordão, SP-Brazil, 2012.

- Moraes, C. J.; **Ramos, R. N.** ; Bergami-Santos, P. C. ; Barbuto, JAM . Effects of thymosin-alpha1 and of the Stat3 inhibitor, JSI-124 on Mo-DCs phenotype and function. In: XXXVII Congress of the Brazilian Society of Immunology, Campos de Jordão, SP-Brazil, 2012.

- **Ramos, R. N**.; Barbosa, B. Z.; Moraes, C. J.; Laginha, F.; Barbuto, J. A. M. Role of PD-L1 in the imbalance of Treg/T helper induction by Mo-DCs from breast cancer patients. In: Annual Meeting of the Club Francophone des Cellules Dendritiques “Diversity and plasticity of DCs”, Paris/France, 2012.

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- Bergami-Santos, P. C.; **Ramos, R. N**.; Migliori, K. I.; Barbosa, B Z; Romagnoli, G G; Barbuto, JAM. Phenotypic and functional study of Heterokaryons used in therapeutic vaccines against advanced cancer. In: 4º Simpósio de Imunobiologia de Tumores, Botucatu, SP/Brazil, 2011.

- **Ramos, R. N.**; Chin, L. S.; Santos, A. P. S. A.; Bergami-santos, P. C.; Laginha, F.; Barbuto, JAM. Dendritic Cells differentiated from breast cancer patients monocytes (Mo-DCs) present a functional bias towards the induction of Regulatory T cells via TGF-beta participation. In: 25th Annual Meeting of the European Macrophage and Dendritic Cell Society, Brussels/Belgium, 2011.

- **Ramos, R. N.**; Moraes, C. J.; Santos, A. P. S. A.; Bergami-Santos, P. C.; Laginha, F.; Barbuto, JAM. Is The phenotypic delay in the differentiation of breast cancer patients monocytes into DCs (Mo-DCs) the source of functional bias by the induction of regulatory T cells? In: Harnessing Immunity to Prevent & Treat Disease, Cold Spring Harbor Laboratory – NYC/USA, 2011.

Scientific articles

- **Ramos, R.N**.; Rodriguez, R.; Léon, S.; Treilleux, I.; Ries, C.; Lavergne, E.; Chabaud, S.; Caux,C.; Barbuto, J.A.M.; Bendriss-Vermare, N.; Ménétrier-Caux, C.

Tumor microenvironment acts direct and systemically skewing blood monocytes into suppressive CD163highIL-10high cells that may impact in breast cancer patients' survival.
In preparation

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