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EUROPEAN HEMATOLOGY ASSOCIATION
haematologica
Journal of the European Hematology Association

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Haematologica 2017 [Epub ahead of print]

Citation: Stephen-Victor E, Das M, Sharma M, Galeotti C, Fohrer-Ting H, Sendid B, Darnige L, Terris B, Badoual C, Bruneval P, Kaveri SV, and Bayry J. Demystification of enigma on antigen presenting cell features of human basophils: data from secondary lymphoid organs. Haematologica. 2017; 102:xxx doi:10.3324/haematol.2016.163451

Demystification of enigma on antigen presenting cell features of human basophils: data from secondary lymphoid organs

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Running head: APC features of human basophils from SLO

Word count: 1373

Number of Figures: 3

Number of Tables: 0

Supplemental files: 1

Acknowledgments

We thank Ms. Anupama Karnam and Dr. Jonathan Pol for their help; and Dr. V Languillat-Fouquet and Pr. H Martelli, Service Chirurgie Pédiatrique, Hôpital Bicêtre, France for providing spleen sections of patients with spherocytosis. Supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Université Pierre et Marie Curie and Université Paris Descartes. ES-V is a recipient of fellowship from Indo-French Center for Promotion of Advanced Research (CEFIPRA) and CG. is a recipient of fellowship from La Fondation pour la Recherche Médicale (FDM20150633674), France.

Antigen presenting cells (APCs) play a key role in the uptake of antigens, and processing and presentation of antigen-derived peptides in the context of MHC molecules to T cells. The co-stimulatory molecules CD80 and CD86 provide signals for T cell activation and proliferation. Dendritic cells (DCs), macrophages/monocytes and memory B cells are classically known for their role as APCs. In contrast to this dogma, recent reports from mice show that basophils can also function as APCs. These reports were revolutionary, as basophils were previously known as accessory cells that produce IL-4 required for the Th2 responses mediated by classical APCs.[1,2] In fact, it was shown that murine basophils express MHC class II, and co-stimulatory molecules CD80 and CD86 required for the T cell stimulation.[3-5] Additionally, these basophils were also reported to process and present antigens that are associated with classical Th2 responses such as allergens and proteases, and mediate Th2 differentiation *in vitro* and *in vivo*. Although *in vivo* data were later contradicted,[6,7] the *ex-vivo* and *in vitro* data clearly demonstrated functioning of murine basophils as APCs.

In contrast to mice, several data from humans have shown that basophils either from healthy donors or from allergic and lupus patients lack the features of APCs.[8-12] These basophils were negative for the expression of antigen presenting molecule HLA-DR, and co-stimulatory molecules CD80 and CD86, and failed to polarize Th2 as well as Th17 responses when co-cultured with $CD4^+$ T cells. However, all these reports were based on basophils from the circulation, while APC functions of murine basophils were demonstrated from the secondary lymphoid organs like spleen and lymph nodes. One of the hypotheses is that basophils upon migration to the secondary lymphoid organs might gain characteristics of APCs. Therefore, the

debate on whether human basophils display the features of APCs or not remains an unresolved issue unless and until their features are probed in secondary lymphoid tissues of humans.

In order to address unequivocally whether human basophils like their murine counterparts display features of APCs or not, we studied basophils from human secondary lymphoid tissues such as spleen, lung-draining lymph nodes and tonsils (*See Online Supplementary Methods*). In general, c-kit (CD117) and BDCA-4 (CD304) markers were used to distinguish basophils from mast cells and plasmacytoid DCs (pDCs), while CD203c distinguished basophils from other immune cells. Basophils were identified as $CD203c^{+}Fc\varepsilon RI^{+}BDCA\text{-}4^{-}$ $c\text{-}kit^{-}$ cells. As myeloid DC population was also reported to express FcεRI and that pDCs also express BDCA-2 (CD303); BDCA-1 (CD1c), BDCA-3 (CD141) and BDCA-2 markers were additionally used to distinguish splenic basophils from different DC subsets. The phenotype of basophils was analyzed in steady state and under various stimulatory conditions. Basophils were also sorted from the spleen and the morphology of cytospin preparations of sorted basophils was analyzed by May-Grünwald Giemsa staining. The APC features of sorted splenic basophils were investigated in the functional assays by probing their ability to stimulate $CD4^{+}$ T cell activation, proliferation and cytokine production (*See Online Supplementary Methods*).

Basophils are rare leukocytes and represent <1% blood leukocytes in the blood.[1] However, basophils were sparsely present in the human lung-draining lymph nodes (0.0013% (±0.0002 SD), n=3) and tonsils (0.004±0.0024%, n=4) (Figure 1 A-B). Therefore, we could not analyze APC features of basophils from these secondary lymphoid organs.

Basophils in the spleen (0.172±0.122%, n=11) (Figure 1 C-D) were nearly 130 times higher than lung-draining lymph nodes. We found that splenic basophils under steady state condition did not express HLA-DR, CD80 and CD86 (Figure 1 E-F). On the other hand, splenic DCs expressed high levels of HLA-DR and varying degrees of CD80 and CD86 (*Online Supplementary Figure S1*). These results thus indicate that under steady state, human basophils residing at secondary lymphoid tissues do not display phenotypic characteristics of APCs.

Basophils receive signals from various sources such as cytokines, toll-like receptor (TLR) agonists and IgE-bound antigens. Therefore, we explored if basophils express phenotypic markers of APCs upon activation by these different categories of stimulation. IL-3, in addition to supporting differentiation and survival of basophils, also primes their activation.[2] Previous reports have shown that murine basophils cultured in IL-3-conditioned medium express MHC class II and co-stimulatory molecules.[3-5] On the other hand, GM-CSF and IFN-γ were previously reported to induce HLA-DR and co-stimulatory molecules on human innate cells. Therefore, IL-3 and cytokine cocktail containing IL-3, GM-CSF and IFN-γ were used for the stimulation of basophils (*See Online Supplementary Methods*).

Human basophils express various TLRs including TLR4 and 2 and undergo activation upon TLR signaling.[13] Therefore, we stimulated basophils with lipopolysaccharide from *E. coli* (LPS, TLR4 agonist), FSL-1 (Pam2CGDPKHPKSF, TLR2/6 agonist) or CpG ODN (TLR9 agonist). Further, we have also used papain as a model allergen in our experiments and this cysteine protease allergen can directly activate basophils and promote murine basophil-mediated Th2 responses.[4] Since, IgE-bound antigens/allergens provide the most-potent activation signal for basophils,[13,14]

this stimulation condition was mimicked by crosslinking of surface FcεRI by using anti-IgE antibodies (*See Online Supplementary Methods*).

Splenocytes were stimulated for 4 to 24 hours and analyzed for the expression of HLA-DR and B7 molecules on the basophils. Notably, expression of HLA-DR, CD80 and CD86 on basophils did not change significantly despite stimulation with IL-3, LPS, papain, FSL-1. The expression of these markers on stimulated basophils was on par with steady state cells (Figure 2 A-D**)**. We confirm that basophils were indeed activated by these stimuli as analyzed by the expression levels of FcεRI and CD203c (Figure 2 E-F**)**. The expression of FcεRI was significantly increased on splenic basophils irrespective of stimulatory conditions. Although expression of CD203c was also enhanced upon stimulation, due to variations among the donors, significance was not reached in all stimulatory conditions. Similar results were also obtained when splenocytes were stimulated with anti-IgE antibodies or cytokine cocktail (Figure 2 G-L**)**. These results thus provide a pointer that human splenic basophils lack the phenotypic characteristics of APCs irrespective of activation signals they receive.

One of the hallmarks of APCs is their ability to stimulate $CD4^+$ T cell proliferation and cytokine production. Therefore, to prove indisputably that human basophils lack the functions of APCs, we sorted splenic basophils for co-culture with $CD4^+$ T cells (*See Online Supplementary Methods*). Basophils from splenocytes were first enriched using basophil isolation kit II (Miltenyi Biotec). Enriched cells were labelled and live basophils were sorted on FACSAria III flow cytometer (BD Biosciences) as cells positive for FcεRI and CD203c; and negative for BDCA-4 and c-kit. The purity of sorted basophils was 98-99 %. May-Grünwald Giemsa staining

of cytospin preparations confirmed morphology of basophils with basophilic granules in the cytoplasm. Nucleus was partly covered by the basophilic granules. Cells presented relatively narrow cytoplasm that was loosely permeated by intensely basophilic granules. The basic color of the cytoplasm was pale blue to pale pink (Figure 3 A). Isolated splenic basophils were functionally viable. Upon IL-3 stimulation, isolated cells presented with enhanced CD69 expression and released histamine in response to degranulation stimuli.

We co-cultured sorted splenic basophils with allogeneic $CD4^+$ T cells at a ratio of 1:10 under various stimulatory conditions such as IL-3, LPS, papain or FSL-1. $CD4^+$ T cells were isolated from peripheral blood mononuclear cells of healthy donors by using CD4 MicroBeads (Miltenyi Biotec). Consistent with the phenotypic characteristics, stimulated basophils failed to induce $CD4^+$ T cell proliferation (Figure 3 B), T cell activation marker CD69 (Figure 3 C) and secretion of IL-2 (Figure 3 D). In addition, basophils did not induce Th2 (IL-13), Th17 (IL-17A) or as expected Th1 (IFN-γ) cytokines (Figure 3 E-G). CpG or cytokine cocktail-stimulated basophils also presented similar results. Splenic DCs used as control, induced $CD4^+$ T cell proliferation, expression of CD69, and secretion of IL-2, IFN-γ and IL-13 (Figure 3 B-F).

Several models of allergy or parasitic infection have demonstrated induction of Th2 response *in vivo* in the spleen[7] suggesting that spleen has an important role in Th2 responses. Furthermore, previous studies in helminth-infected or papain-immunized mice have used splenic basophils to demonstrate their APC features[3-5] and capacity to induce Th2 responses.[3,5] These lines of evidence thus validate the use of human splenic basophils for the analysis of APC characteristics.

Altogether, our results indicate that in human, the role of basophils in Th2 responses is restricted to provision of IL-4 in the microenvironment[15] but not as APCs.

Author Contributions

ES-V, MD, MS, CG, HF-T performed experiments; ES-V, MD, MS, LD, PB, SVK, JB analyzed experimental data; BS, BT, CB and PB provided essential tools and materials for the research; JB wrote the paper; all authors revised and approved the manuscript critically for important intellectual content and approved the final version.

Conflicts of interest

None

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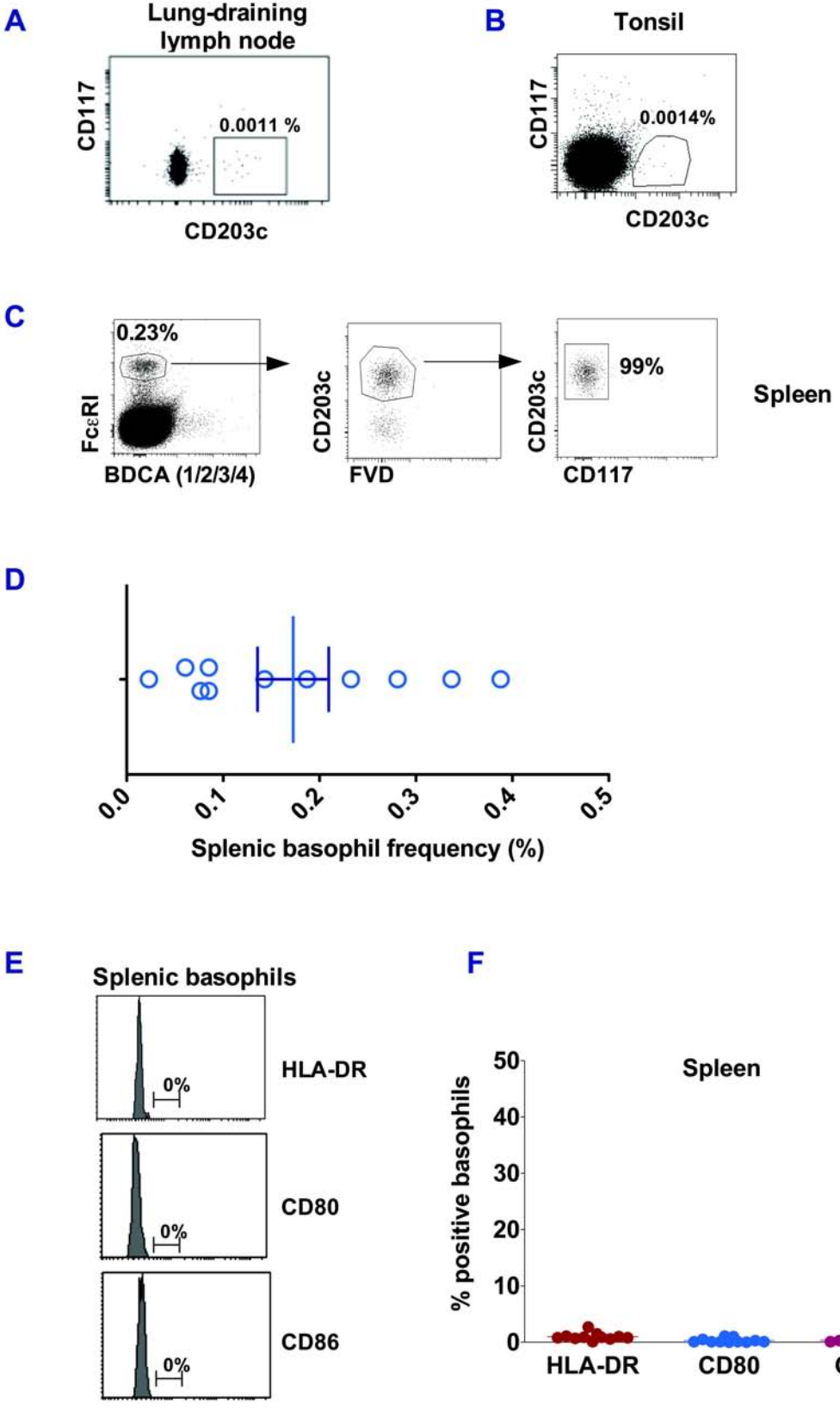
Figure Legends

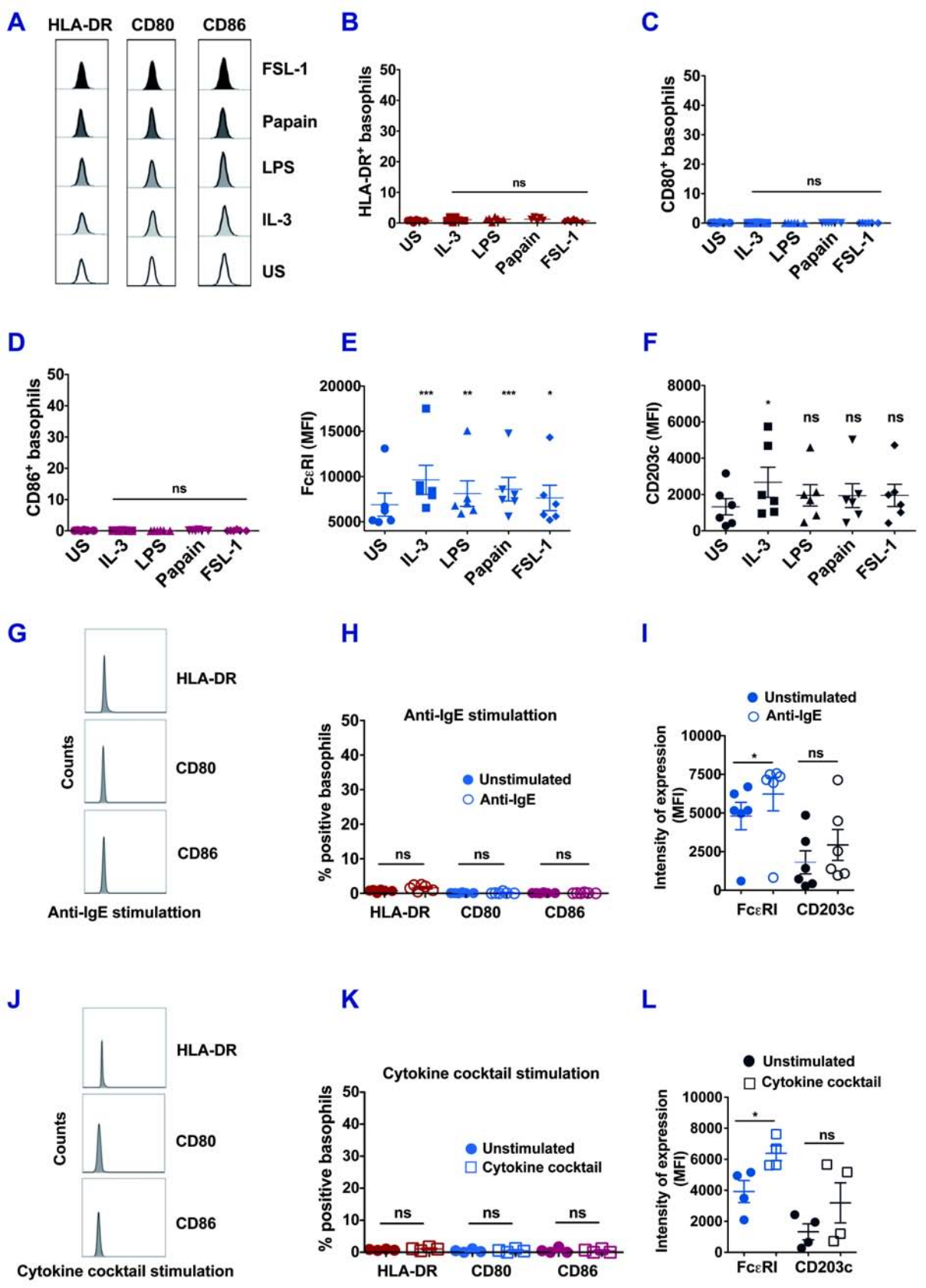
Figure 1. Human basophils from secondary lymphoid organs lack the phenotypic features of APCs. (A, B) The frequency of basophils in lung-draining lymph nodes and tonsils. Representative data of four subjects. (C) $CD203c^{+}Fc\varepsilon RI^{+}BDCA\text{-}(1/2/3/4)^{-}CD117^{-}$ splenic basophils negative for fixable viable dye (FVD). (D) The frequency of splenic basophils from 11 subjects. (E, F) Representative histograms and mean±SEM (n=11 subjects) of HLA-DR, CD80 and CD86 expressions on steady-state splenic basophils.

Figure 2. Expression of HLA-DR, B7 co-stimulatory molecules and activation-associated markers on human splenic basophils under various stimulatory conditions. (A-L) Spleenocytes were stimulated with IL-3, LPS, papain, FSL-1 (A-F), or anti-IgE (G-I), or cytokine cocktail (IL-3, GM-CSF and IFN-γ) (J-L) for 4 to 24 hours. Representative histograms showing the expression of HLA-DR, CD80 and CD86 on splenic basophils (A, G, J); mean± SEM values of % of splenic basophils positive for HLA-DR, CD80 and CD86 (B, C, D, H, K); and mean± SEM values of intensity of expression (MFI) of FcεRI and CD203c (E, F, I, L) on stimulated splenic basophils are shown. Data are from four to six subjects. US, unstimulated cells; * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns, not significant; by One-way analysis of variance (B-F) or two-tailed Mann-Whitney test (H, I, K, L).

Figure 3. Human splenic basophils lack the functional features of APCs. (A) May-Grünwald Giemsa staining of cytospin preparations of sorted splenic basophils. (B) Ability of splenic basophils to stimulate $CD4^{+}$ T cell proliferation as analysed by CFSE staining. Representative of

three experiments. (C) CD69 expression on $CD4^+$ T cells co-cultured with sorted splenic basophils. (D-G) Induction of IL-2, and Th2 (IL-13), Th1 (IFN-γ) and Th17 (IL-17A) cytokines in sorted splenic basophil-$CD4^+$ T cell co-cultures (n=3). US, unstimulated cells; *** $P<0.001$; ns, not significant; by One-way analysis of variance.


A
Lung-draining lymph node
CD117
0.0011 %
CD203c
B
Tonsil
CD117
0.0014%
CD203c
C
0.23%
FcεRI
BDCA (1/2/3/4)
CD203c
FVD
CD203c
99%
CD117
Spleen
D
0.0
0.1
0.2
0.3
0.4
0.5
Splenic basophil frequency (%)
E
Splenic basophils
0%
HLA-DR
0%
CD80
0%
CD86
F
Spleen
% positive basophils
50
40
30
20
10
0
HLA-DR
CD80
CD86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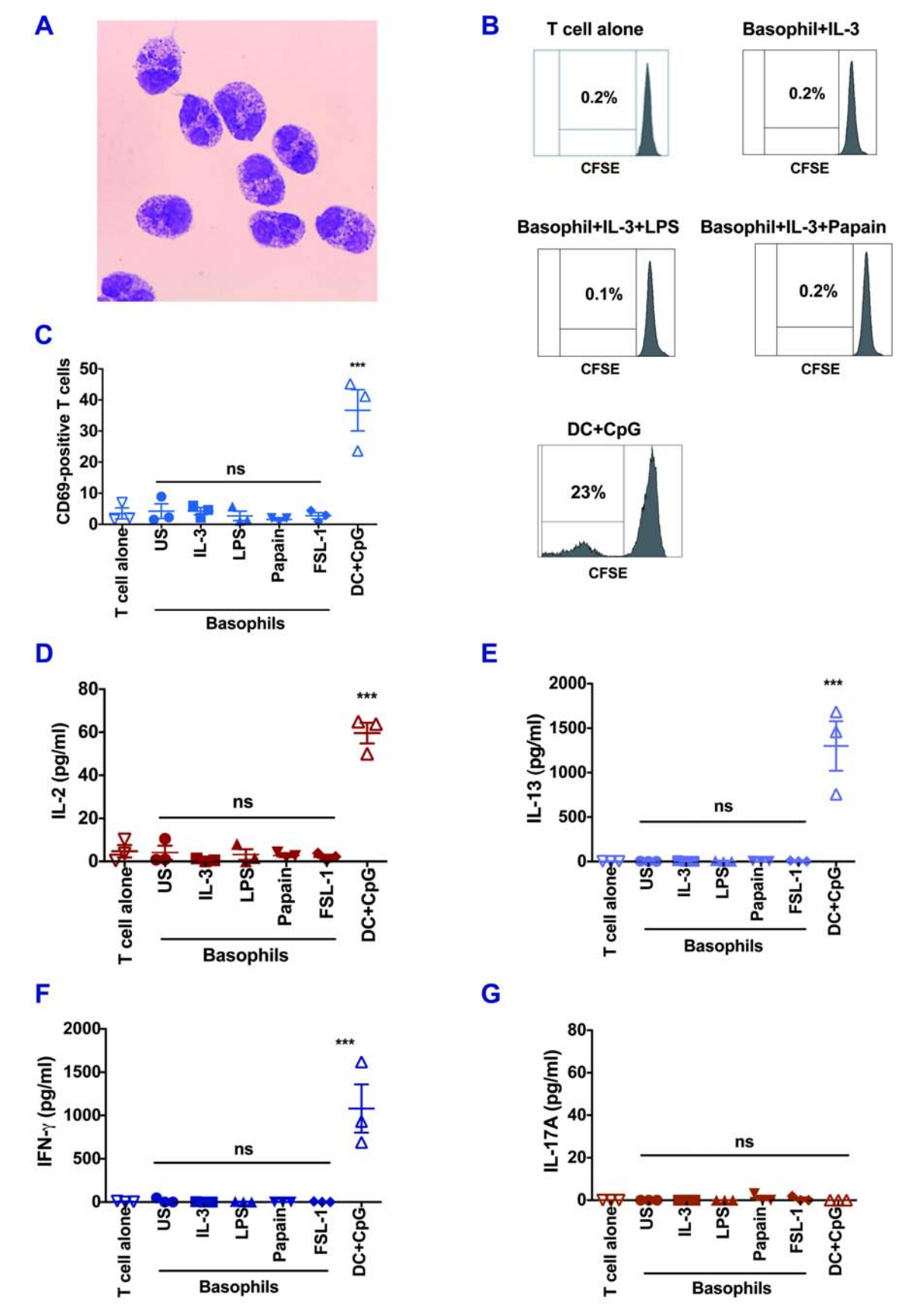
HLA-DR
CD80
CD86
FSL-1
Papain
LPS
IL-3
US
B
HLA-DR+ basophils
ns
US
IL-3
LPS
Papain
FSL-1
C
CD80+ basophils
ns
D
CD86+ basophils
ns
E
FcεRI (MFI)
F
CD203c (MFI)
G
HLA-DR
CD80
CD86
Counts
Anti-IgE stimulattion
H
Anti-IgE stimulattion
% positive basophils
Unstimulated
Anti-IgE
HLA-DR
CD80
CD86
I
Intensity of expression (MFI)
FcεRI
CD203c
J
Cytokine cocktail stimulation
K
Cytokine cocktail stimulation
Cytokine cocktail
L


A
B
T cell alone
Basophil+IL-3
0.2%
CFSE
Basophil+IL-3+LPS
Basophil+IL-3+Papain
0.1%
DC+CpG
23%
C
CD69-positive T cells
***
ns
T cell alone
US
IL-3
LPS
Papain
FSL-1
DC+CpG
Basophils
D
IL-2 (pg/ml)
E
IL-13 (pg/ml)
F
IFN-γ (pg/ml)
G
IL-17A (pg/ml)

SUPPLEMENTARY METHODS

Human spleen, lung-draining lymph nodes and tonsils

Samples of human spleen and lung-draining lymph nodes from healthy individuals or untreated subjects presented for pathological investigations were obtained from Service d'Anatomie Pathologique, Hôpital Européen Georges Pompidou, Paris, France and Service de Pathologie, Hôpital Cochin, Paris. For our work, only healthy tissues (noninvaded) were used. In addition, spleen sections were also obtained from two patients with spherocytosis (from Service de Chirurgie Viscérale Pédiatrique, Hôpital Bicêtre, Paris), an auto-hemolytic anemic condition characterized by the production of spherocytes where instead of bi-concave disk shaped erythrocytes, subjects are presented with sphere-shaped erythrocytes. Tonsils were from tonsillectomy subjects (Service d'Anatomie Pathologique, Hôpital Européen Georges Pompidou, Paris) presented with a history of hypertrophic and hyperplastic tonsils. Subjects' consent was obtained and since the study did not require additional sampling, an approval from an ethics committee was not required under French law according to the article L.1121-1 of the public health code. The article states that: The research organized and performed on human beings in the development of biological knowledge and medical research are permitted under the conditions laid down in this book and are hereinafter referred to by the term "biomedical research". The article further states that it does not imply under conditions: For research in which all actions are performed and products used in the usual way, without any additional or unusual diagnostic procedure or surveillance.

Isolation of splenocytes

Spleen pieces were mechanically disaggregated by gentleMACS dissociator (Miltenyi Biotec, program m_spleen_04) and filtered through 70-µm nylon membrane filter (BD Biosciences) to obtain single-cell suspension of splenocytes. Cells were then subjected to Ficoll-Paque PREMIUM density gradient centrifugation and all the cellular fractions including granulocytes and mononuclear cells were collected.

Isolation of cells from lung-draining lymph nodes and tonsils

Single-cell suspensions of lung-draining lymph nodes and tonsils were obtained by mechanical disaggregation. Small lumps were homogenized with the help of syringe plunger and passing the cells through a 70-µm nylon membrane filter to obtain single-cell suspensions. Isolated cells were then subjected to flow-cytometry to analyze the phenotype of basophils. Samples were acquired using LSR II (BD Biosciences) flow cytometer and data were analyzed by BD FACS DIVA software (BD Biosciences) and Flowjo.

Isolation of splenic basophils and dendritic cells

Basophils from splenocytes (250×10^6) were first enriched using basophil isolation kit II (Miltenyi Biotec). Enriched cells were labelled and live basophils were sorted on FACSAria III flow cytometer (BD Biosciences) as cells positive for FcεRI and CD203c; and negative for BDCA-4 and c-kit. The purity of sorted basophils was 98-99 %.

Splenic DCs were isolated using dendritic cells isolation kit II (Miltenyi Biotec).

Morphological and functional analysis of sorted splenic basophils

The cytospin preparations sorted basophils were stained with May-Grünwald Giemsa to determine classical features of basophils and to demonstrate morphology of the cells with basophilic granules in the cytoplasm.

Stimulation of splenocytes

Splenocytes (10^6 cells/ml) were stimulated with IL-3 (100 ng/million cells), LPS (100 ng/million cells; *E.coli* 055:B5, Sigma-Aldrich), papain (100 µg/million cells; Merck Millipore), FSL-1 (0.5 µg/million cells; InvivoGen), CpG (2.5 µM/million cells; InvivoGen) and cytokine cocktail (100 ng/million cells each of IL-3, GM-CSF and IFN-γ) for four to 24 hours. Cells were also cultured with IL-3 (100 ng/million cells) for four to 24 hours and during last 30 minutes, cells were treated with anti-IgE antibodies (100 ng/million cells). Phenotype of splenic basophils was analyzed by flow cytometry.

Isolation of $CD4^+$ T cells from the blood

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags of healthy blood donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (Institut National de la Santé et de la Recherche-EFS ethical committee convention 15/EFS/012). $CD4^+$ T cells were isolated from PBMCs by positive selection using CD4 MicroBeads (Miltenyi Biotec). The cell purity was more than 97%.

CFSE-labelling of $CD4^+$ T cells

$CD4^+$ T cells were washed and resuspended in PBS followed by incubation with CFSE (5 μM, BD Biosciences) at 37 °C for 10 minutes. Cells were thoroughly washed and resuspended in X-VIVO medium at concentration of 10^6 cells/ml.

Basophil-$CD4^+$ T cell and DC-$CD4^+$ T cells co-culture

Sorted splenic basophils (0.1×10^5/well/200 μl) were co-cultured with CFSE-labelled allogenic $CD4^+$ T cells at a ratio of 1:10 in 96-well U-bottom plates in serum-free X-VIVO medium and stimulated for up to 7 days with different conditions: IL-3 (1 ng/0.1×10^5 basophils), IL-3 in combination with LPS (1 ng/0.1×10^5 basophils), papain (1 μg/0.1×10^5 basophils), CpG (25 nM/0.1×10^5 basophils) or FSL-1 (5 ng/0.1×10^5 basophils), and cytokines cocktail (1 ng/0.1×10^5 basophils each of IL-3, GM-CSF and IFN-γ). Splenic DCs (0.1×10^5/well/200 μl) were co-cultured with CFSE-labelled allogenic $CD4^+$ T cells at a ratio of 1:10 and stimulated with CpG (25 nM//0.1×10^5 cells) up to 7 days. After 7 days, cell-free supernatants were collected and the extent of proliferation and activation status of $CD4^+$ T cells was assessed by flow cytometry.

Quantification of cytokines

IL-2, IFN-γ, IL-13 and IL-17A in the cell-free supernatants of basophil-$CD4^+$ T cell and DC-$CD4^+$ T cell co-cultures were quantified by ELISA (Ready-SET-Go, eBioscience).

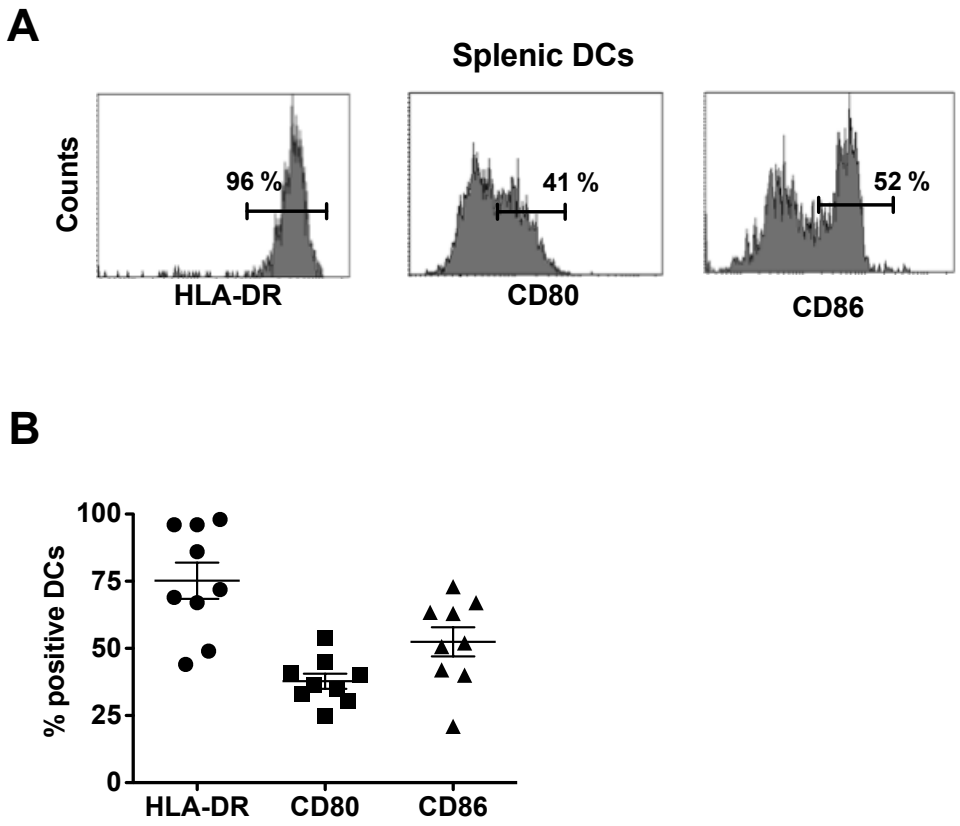
Antibodies

The following antibodies were used for flow cytometry. HLA-DR-FITC (Clone: TU36), CD86-FITC (Clone: FUN-1), CD80-PE (Clone: L307.4), CD117/c-kit-PerCP-Cy5.5 (Clone: YB5.B8),

CD3-PE (Clone: UCTH1), CD63-PE (Clone H5C6) and CD69-APC/Cy7 (Clone: FN50) monoclonal antibodies were from BD Biosciences. BDCA-1 (CD1c)-APC (Clone: AD5-8E7), BDCA-2 (CD303)-APC (Clone: AC144), BDCA-3 (CD141)-APC (Clone: AD5-14H2) and BDCA-4 (CD304)-APC (Clone AD5-17F6) monoclonal antibodies were obtained from Miltenyi Biotec. FcεRIα-BV510 (Clone: AER37 (CRA-1)) and CD203c-BV421 (Clone: NP4D6) were from BioLegand and Fixable viable dye-eFluor 780 was from eBioscience.

Statistical analysis

Statistical analysis was performed by Prism 5 GraphPad Software. Data are presented as mean ± SEM. One-way analysis of variance was used to determine the statistical significance of the data with more than three groups. $P<0.05$ was considered significant. Differences between two groups were determined by two-tailed Mann-Whitney test.



Supplementary Figure S1. Phenotype of human splenic DCs. (A, B) Representative histograms and mean (± standard error of the mean [SEM]) for nine subjects showing the expression levels of HLA-DR, CD80 and CD86 on steady-state splenic DCs.