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

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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).

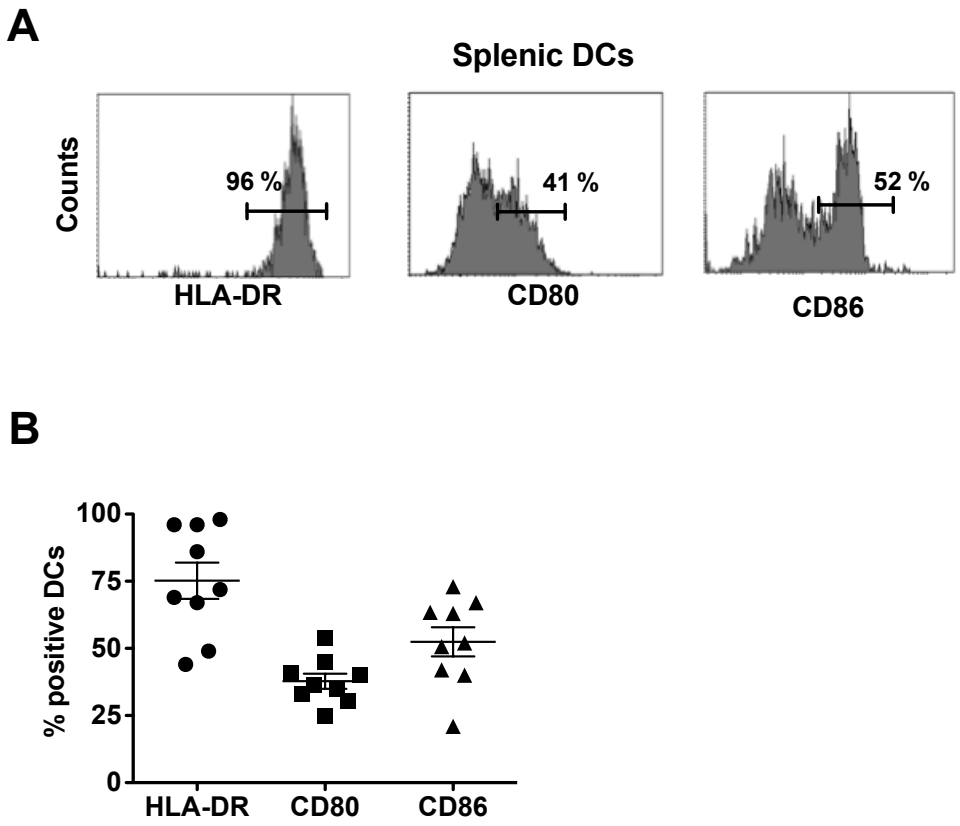
Antibodies

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.