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iMACS DEFICIENCY IN NOD MICE : IMPLICATION IN CYCLOPHOSPHAMIDE-INDUCED DIABETES ?

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INTRODUCTION

The role of dendritic cells (DCs) and macrophages in pancreatic β -cells destruction leading to type 1 diabetes remains unclear.

NOD (non obese diabetic) mice displays a spontaneous autoimmune diabetes and exhibits functional dendritic cells abnormalities. Here, we have investigated the role of a peculiar set of natural suppressor macrophages: iMacS, or inhibitory macrophages, which are CD11b⁺Gr-1⁺CD11c⁻ cells that were shown to differentiate in DCs *in vitro*. They were recently considered responsible of immunosuppressions observed during infections, tumour growth and cyclophosphamide (CyP) treatments. iMacS could trigger apoptotic death of T cells and may be involved in the control of T cell expansion, in a MHC-unrestricted manner and through a secretion of NO but also through unknown cellular contact signals.

The aim of our study was to characterize the phenotype of iMacS in NOD mouse and to investigate their possible role in autoimmune diabetes pathogenesis.

MATERIALS & METHODS

Isolation of lin⁻c-kit⁺ hematopoietic progenitor cells (HPCs): Lin⁻ (CD8, CD3, CD4, CD11a, CD45R, CD11b, Gr-1) c-kit⁺ cells were isolated from mononuclear non-adherent cells from pooled femur bone marrow of three-month-old NOD and Balb/c mice as previously described. These cells are then cultured during 6 days in DMEM supplemented with 10% FCS, 20 ng/ml GM-CSF, 20 ng/ml SCF and 25 ng/ml TNF α or IFN γ (Endogen, Sigma).

CyP treatment: Female NOD and Balb/c mice were injected i.p. three times at three-day intervals with 100 mg of CyP/kg or NaCl for control mice.

CD11b⁺ selection: pure CD11b⁺ cell populations were selected by incubation of 6-day-cultured lin⁻c-kit⁺ cells with MACS super-magnetic microbeads conjugated with anti-CD11b antibodies (Miltenyi Biotech).

Proliferation assays: Viable spleen cells from control mice were aliquoted at 2.10⁵ cells/well alone or with 2.10⁵ CD11b⁺ cells, irradiated or not, isolated from syngeneic CyP-treated mice, in the presence or absence of stimulus, for 3 days. Stimuli were ConA at 1 μ g/ml or rIL-2 at 25U/ml + anti-mouse CD3 ϵ MoAb at 2 μ g/ml. Proliferative activities were quantified by incubation with 1 μ Ci/well of 3H-thymidine.

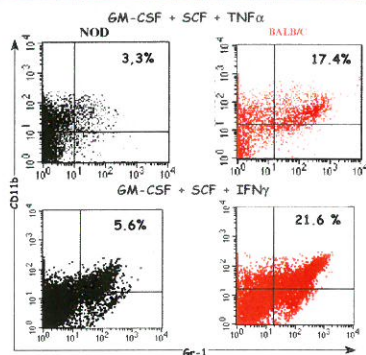
Nitrite production: NO secretion by iMacS was assessed by measuring nitrite accumulation in 72-hour-culture supernatants using Griess coloration (Promega). The detection threshold was 2 μ M.

Immunofluorescence labelling and flow cytometric analysis: 6-day-cultured lin⁻c-kit⁺ and splenocytes from CyP-treated or control mice were incubated 5 min. at 4°C with FcBlock (BD Biosciences) and labelled with various conjugated-antibodies or isotype controls. Flow cytometric analysis was performed on FACScan™ using the CELLQuestPro software (BD Biosciences). FITC-intracellular staining detecting iNOS or MOMA-2 were performed after surface staining using Cytofix/Cytoperm™ products (BD Biosciences).

Statistical analysis: The statistical significance of differences was evaluated using Mann Whitney's U-Test or Student's t-test. p<0.05 was considered as significant.

RESULTS

I-iMacS differentiation from HPCs in NOD mice



III-CyP treatment enhanced diabetes in NOD mice

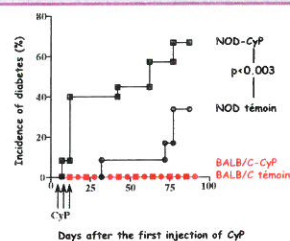


Figure 3: Cumulative incidence of diabetes in CyP-treated and control NOD and Balb/c mice. Mice were considered diabetic when glycemia was > 13.5 mM. The time-course of diabetes was analyzed using Kaplan-Meier curves that represented the percentage of diabetic mice up to 80 days after the first injection of CyP.

Simultaneously, we have studied iMacS induced by a classical immunosuppressive treatment with CyP. We have confirmed that such a treatment (100mg/kg, 3 times at 3 day intervals) increase diabetes in NOD mice.

IV-iMacS recruitment in spleen following CyP treatment

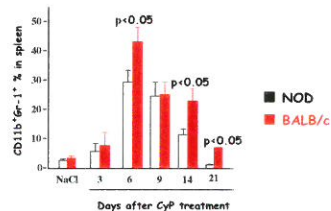


Figure 4: Kinetic of recruitment of CD11b⁺Gr-1⁺ cells in the spleen after CyP treatment. Splenocytes were analyzed at day 3, 6, 9, 14 and 21 after the last delivery of CyP by flow cytometry. At the same time, control mice were analyzed. The data are means of percentages \pm SEM of CD11b⁺Gr-1⁺ in total viable spleen cells.

Six days after the third injection of CyP, iMacS mobilization in spleen was maximal for both NOD and Balb/c mice but less important in NOD than in Balb/c mice. Moreover, iMacS mobilization decreased more rapidly in NOD mouse. The number of iMacS expressing a mature phenotype was also significantly lower in spleen of NOD mice (data not shown).

V-Inhibition of T-cell proliferations by CD11b⁺ cells of CyP-treated mice

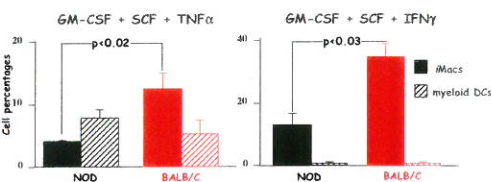


Figure 1: Purified lin⁻c-kit⁺ HPCs from NOD and Balb/c mice were cultured with GM-CSF, SCF and TNF α or IFN γ during 6 days. iMacS and myeloid DCs proportion were then analysed by flow cytometry. Data are means of percentages \pm SEM.

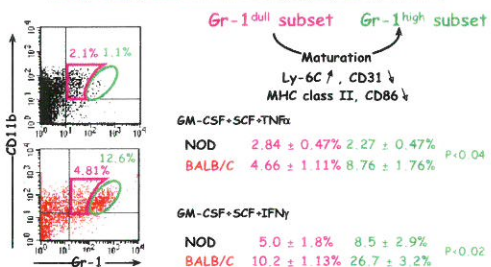
We have previously* shown that iMacS secreting NO and able to inhibit T-cell proliferations, could be derived from lin⁻c-kit⁺ HPCs, isolated from bone marrow, by culturing them with GM-CSF, SCF and TNF α or IFN γ for six days. Compared to Balb/c mice, NOD-HPCs cultured with TNF α were unable to differentiate into iMacS, whereas no difference seemed to be observed between myeloid CD11b⁺Gr-1⁺CD11c⁻ DCs obtained with the same protocol. With IFN γ , NOD-HPC could be derived in iMacS but significantly lesser than in Balb/c.



Figure 5: A/ Inhibition of syngeneic splenocyte proliferative activity by splenic CD11b⁺ isolated from CyP-treated mice 6 days after the last CyP injection. The % of inhibition was calculated as followed: 100 - (SI (spleno + CD11b⁺) / SI (spleno) * 100). SI: stimulation indices. B/ Intracellular expression of iNOS by iMacS. Splenic CD11b⁺ recovered 6 days after the last injection of CyP, were cultured in basal condition during 48h. Histogram-plots showed intracellular iNOS expression by gated CD11b⁺Gr-1⁺ cells (gray curves). Black curves represent the background staining with isotype controls. Similar results were obtained in at least two other independent experiments.

iMacS are known to inhibit ConA or CD3+IL-2 activated T-cell proliferations. iMacS from NOD mouse seemed to inhibit activated T-cell expansion less stronger than those from Balb/c (A). We have then investigated the NO secretion by iMacS. NOD iMacS secreted similar quantities of NO in response to activated T-cells (data not shown). Regarding their expression of inducible NO synthase, no difference was observed between NOD and Balb/c iMacS (B).

II-iMacS maturation from HPCs in NOD mice



We have also previously* shown that, in Balb/c mice, the majority of iMacS express high level of Gr-1 (Gr-1^{high} subset) and the minority express low level of Gr-1 (Gr-1^{dull} subset). Gr-1^{high} cells are more mature than the Gr-1^{dull} population as more of them exhibit a Ly-6C⁺ mature phenotype and lesser express CD31, MHC class II and costimulatory molecules which characterize immature myeloid cell precursors. In both culture situations, NOD-HPCs could not differentiate efficiently into iMacS exhibiting a mature phenotype.

VI-Inhibition of T-cell proliferations by irradiated CD11b⁺ cells of CyP-treated mice

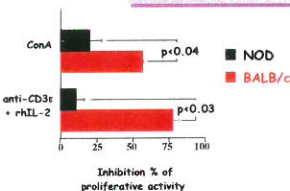


Figure 6: Inhibition of syngeneic splenocyte proliferative activity by splenic irradiated CD11b⁺ cells isolated from CyP-treated mice 6 days after the last CyP injection. The % of inhibition was calculated as followed: 100 - (SI (spleno + CD11b⁺) / SI (spleno) * 100). SI: stimulation indices.

To investigate cellular contact signals, we irradiated iMacS before co-culture. When irradiated, these cells do not secrete NO and could not express iNOS. In this context, contrary to Balb/c iMacS, irradiated iMacS from NOD mouse could not inhibit efficiently activated T-cell expansion.

CONCLUSION & PERSPECTIVES

In conclusion, iMacS from NOD mice exhibit a strong maturation and functional deficiency when derived from HPCs or mobilized after CyP treatment. iMacS from NOD mice could not control T-cell expansions if NO secretion was blocked, suggesting that their functional defects implicated anomalies in cellular contact signals between iMacS and activated T-cells. Currently, we are testing the role *in vivo* of these deficiencies in diabetes progression, by testing co-transfer of iMacS from NOD or Balb/c with T-lymphocytes isolated from diabetic NOD (spontaneously or treated with CyP) in NOD/SCID mice.

The authors are grateful to Mrs M. Allard and M. Ouary and Mr C. Chevalier for their kind assistance. These results were presented at the International Symposium on Dendritic Cells in Bamberg on september 2002. * poster CFC2002 N°C67.

DEFICIENCE DES *i*MACS CHEZ LA SOURIS NOD : IMPLICATION DANS LE DIABETE AUTOIMMUN ?

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Le rôle des cellules dendritiques (DCs) et des macrophages dans la destruction des cellules β au cours du diabète de type 1 (T1D) n'est pas clair. La souris NOD, modèle privilégié du T1D, présente des anomalies fonctionnelles en DCs potentiellement impliquées dans la rupture de tolérance vis-à-vis d'autoantigènes β insulaires. Les *i*Macs (macrophages inhibiteurs $CD11b^+Gr-1^+CD11c^-$) peuvent se différencier en DCs et sont responsables du déficit fonctionnel des lymphocytes T observé lors de traitements par le cyclophosphamide (CyP) et au cours d'infections virales. Les *i*Macs induisent l'apoptose des lymphocytes T *via* la sécrétion de NO et pourraient être impliqués dans le contrôle de l'expansion lymphocytaire. Le but de notre étude est de caractériser le phénotype des *i*Macs chez la souris NOD et d'étudier leur rôle éventuel dans la pathogénie du T1D. Le traitement par CyP (3 fois 100 mg/kg à 3 jours d'intervalle) augmente l'incidence du T1D chez les NOD-CyP ($p < 0,003$). Il induit aussi un recrutement splénique de *i*Macs, 6 jours après la dernière injection, de façon moins importante chez les NOD-CyP que chez les Balb/c-CyP ($p < 0,05$). De plus, le nombre d'*i*Macs spléniques exprimant le phénotype mature $Ly-6C^+$ est beaucoup plus faible chez les NOD-CyP ($p < 10^{-4}$). Concernant leur fonction suppressive, les *i*Macs de NOD-CyP sécrètent des quantités similaires de NO en réponse à des lymphocytes activés. Cependant, contrairement aux *i*Macs de Balb/c-CyP, les *i*Macs de NOD-CyP sont incapables d'inhiber la prolifération T en l'absence de sécrétion de NO ($p < 0,04$). Enfin, nous observons aussi un déficit de la différenciation *in vitro* en présence de GM-CSF, SCF et $TNF\alpha$ des progéniteurs hématopoïétiques lin^-kit^+ en *i*Macs ($p < 0,02$) chez les NOD *versus* Balb/c. Ce déficit correspond essentiellement à une absence de maturation en *i*Macs $CD11b^+Gr-1^{high}Ly-6C^+CD31^{low}$ ($p < 0,04$). En conclusion, les *i*Macs de souris NOD présentent des déficiences de maturation et de fonction. Ces anomalies pourraient être impliquées dans la progression du T1D induit par CyP. Nous testons actuellement cette hypothèse et le rôle éventuel des *i*Macs dans le T1D spontané.

Mots-clés : *i*Macs, cellules dendritiques, autoimmunité, tolérance périphérique, lymphocytes T.

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