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Myeloid suppressor cell (MSC) deficiencies in non-obese diabetic (NOD) mice: a role in type 1 diabetes pathogenesis?

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INTRODUCTION

CD11b+Gr-1+ natural myeloid suppressor cells (MSCs) are implicated in profound depression of T-cell functions observed during infections, tumour growth and cyclophosphamide (CyP) treatments. MSCs could trigger apoptotic death of T-cells and may be involved in the control of T-cell expansion, in a MHC-unrestricted manner, through NO secretion (inducible NO Synthase (iNOS) expression) and/or unknown cellular interactions with the targeted T-lymphocytes.

If the role of MSCs is well documented in unexpected immunosuppressions observed during tumor growth or some viral or parasitic infections, nothing is known about their potential beneficial implication in the regulation of autoimmunity. The non obese diabetic (NOD) mouse constitutes a really interesting model for investigating MSC role in autoimmunity control, as this mouse displays myeloid cell (particularly macrophage and dendritic cell) abnormalities implicated in diabetes pathogenesis.

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

RESULTS

I-MSC differentiation from HPCs in NOD mice

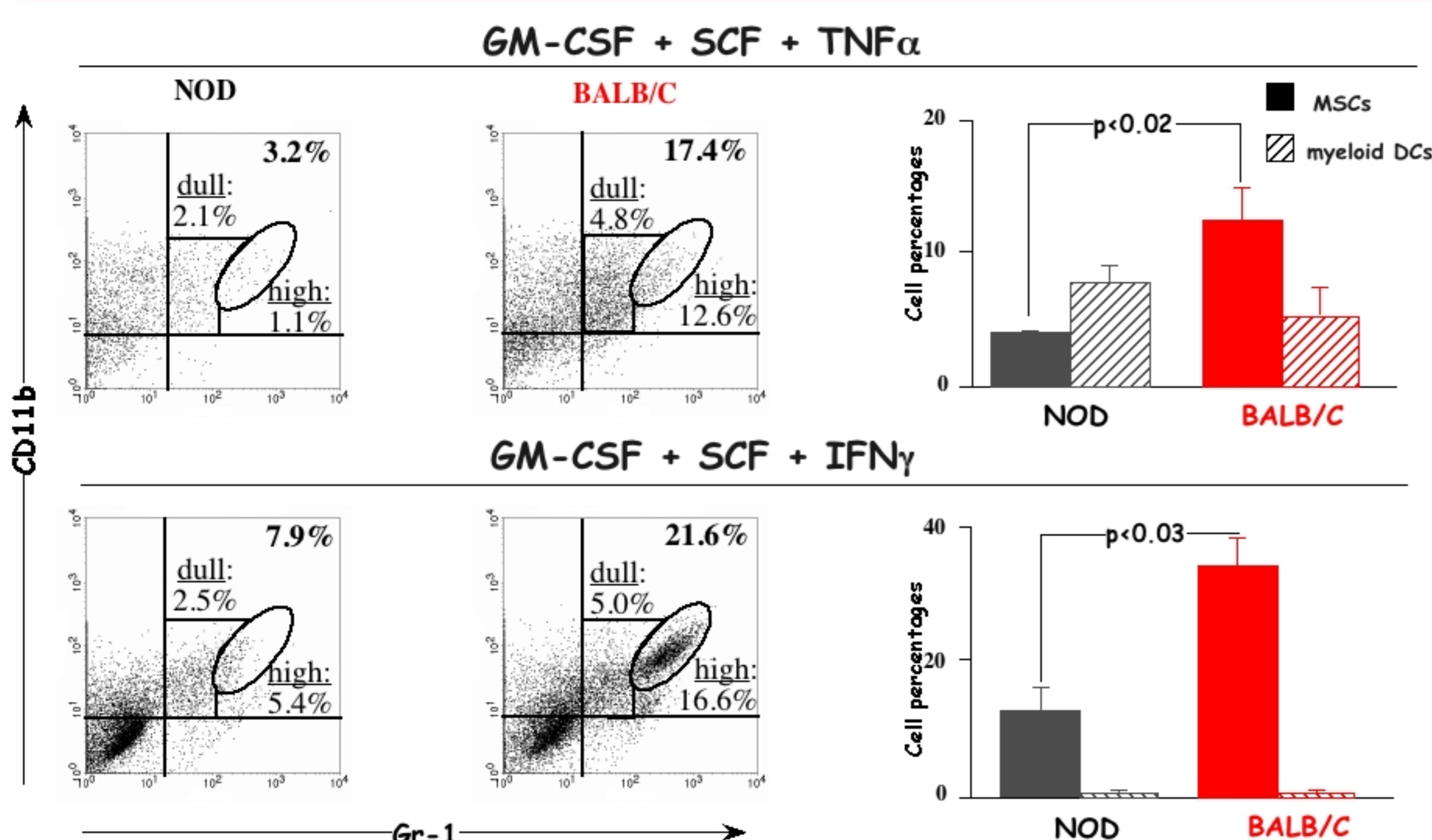


Figure 1: Purified *lin⁻c-kit⁺* HPCs from NOD and Balb/c mice were cultured with GM-CSF, SCF and TNF α or IFN γ during 6 days. MSCs proportion were then analyzed by flow cytometry. Data are means of percentages \pm SEM.

We have previously shown that CD11b+Gr-1+ MSCs secreting NO and able to inhibit T-cell proliferations, could be derived from *lin⁻cKit⁺* HPCs from bone marrow by culturing them with GM-CSF, SCF and TNF α or IFN γ for six days (S. Ferret et al, Immunol. Letters, 2004). Compared to Balb/c mice, NOD-HPCs cultured with TNF α are unable to differentiate into MSCs, whereas no difference seem to be observed between myeloid CD11b+Gr-1-CD11c+ DC obtained with the same protocol (Fig 1). With IFN γ , NOD-HPCs could be derived in MSCs but significantly lesser than in Balb/c (Fig 1).

II-MSC maturation from HPCs in NOD mice

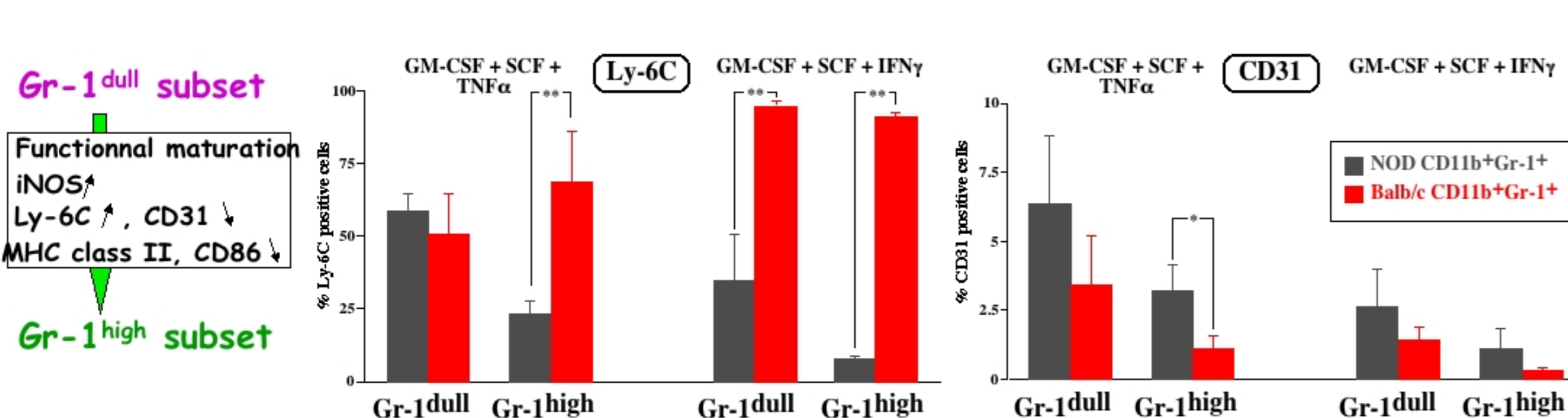


Figure 2: HPCs were cultured with GM-CSF, SCF and TNF α or IFN γ for six days and stained with anti-mouse CD11b-RPE-Cy5 and Gr-1-PE mAbs. Expression of Ly-6C and CD31 by gated CD11b+Gr-1dull and CD11b+Gr-1high populations was assessed using FITC-conjugated mAbs. Results are expressed as mean percentages \pm sem of positive cells in CD11b+Gr-1dull and CD11b+Gr-1high subsets. Results were obtained from at least three independent experiments *p<0.05, **p<0.02.

We have also previously shown that, in Balb/c mice, the majority of CD11b+Gr-1+ MSCs express high level of Gr-1 (Gr-1high subset) and the minority express low level of Gr-1 (Gr-1dull subset). Gr-1high cells are more immunosuppressive than the dull population as assessed by their iNOS expression (S. Ferret et al, Immunol. Letters, 2004). Beside, most of them exhibit a Ly-6C+ phenotype and express lesser CD31, MHC class II and costimulatory molecules which characterize immature myeloid cell precursors. In both culture situations, NOD-HPCs could not differentiate efficiently into MSCs exhibiting a functional mature phenotype (Fig 2).

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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 80 to 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 2.10^5 cells/well alone or with 2.10^5 CD11b+ cells, irradiated or not, isolated from syngeneic CyP-treated mice, in the presence or absence of stimulus. Stimuli were ConA at 1 μ g/ml or rIL-2 at 25UI/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 $^{\circ}$ C with FcBlock (BD Biosciences) and labelled with various conjugated-antibodies or isotype controls. Flow cytometric analysis was performed on FACScanTM using the CELLQuestPro software (BD Biosciences). FITC-intracellular staining detecting iNOS or MOMA-2 were performed after surface staining using Cytofix/CytopermTM 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.

III- MSC functional recruitment after a CyP treatment in spleen of NOD mice

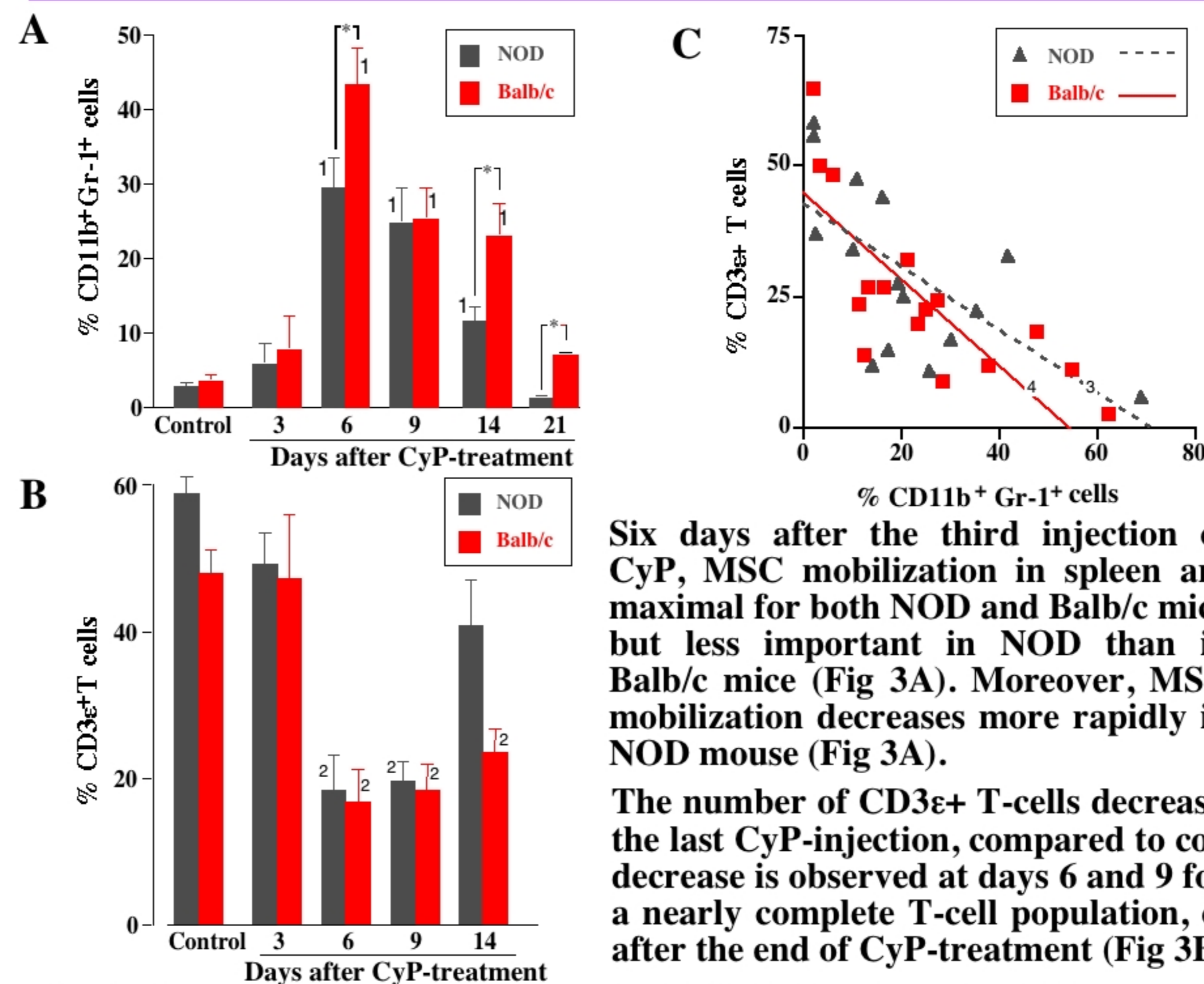


Figure 3: Female NOD and Balb/c mice were simultaneously injected ip with either CyP or physiological saline (control mice) 3 times at 72-hour intervals. Spleen cells were analyzed 3, 6, 9, 14, and 21 days after the end of CyP delivery by flow cytometry in order to quantify CD11b+Gr-1+ cells (CD11b-RPE-Cy5 and Gr-1-PE mAbs, panel A) and CD3 ϵ T-cells (CD3 ϵ -PE mAb, day 21 not determined, panel B). For both analyses, treated and control mice were sacrificed at the same time. Results are expressed as mean percentages \pm sem of CD11b+Gr-1+ or CD3 ϵ cells relative to total spleen cells. Results were obtained from at least three mice from both strain and for each time point in three independent experiments. *p<0.05, 1: p<10 $^{-4}$ (CD11b+Gr-1+ cells in spleen of CyP-treated NOD or Balb/c mice versus syngeneic control mice at day 6, 9, and 14), 2: p<0.03 (CD3 ϵ cells in spleen of CyP-treated NOD (day 6 and 9) or Balb/c (day 6, 9 and 14) mice versus syngeneic control mice). Panel C represents correlations of percentages of CD3 ϵ cells and CD11b+Gr-1+ cells in spleen of NOD and Balb/c mice. Spleen cell analyses at day 3, 6, 9, and 14 following the third CyP injection were taken into account. 3Rho Spearman = - 0.69 (p<10 $^{-4}$), 4Rho Spearman = - 0.85 (p<10 $^{-4}$).

Six days after the third injection of CyP, MSC mobilization in spleen are maximal for both NOD and Balb/c mice but less important in NOD than in Balb/c mice (Fig 3A). Moreover, MSC mobilization decreases more rapidly in NOD mouse (Fig 3A).

The number of CD3 ϵ + T-cells decreases in spleen of Balb/c mice, 6 and 9 days after the last CyP-injection, compared to control syngeneic mice (Fig 3B). While a similar decrease is observed at days 6 and 9 for CyP-treated NOD mice, NOD mice recovers a nearly complete T-cell population, contrary to CyP-treated Balb/c mice, 14 days after the end of CyP-treatment (Fig 3B).

For both mouse strains, recruitment of CD11b+Gr-1+ cells is highly positively correlated with T-cell decrease in spleen (Fig 3C). However, a similar proportion of CD11b+Gr-1+ cells is associated with a lower CD3 ϵ + T-cell depletion in CyP-treated NOD mice compared to CyP-treated Balb/c mice (Fig 3C), strongly suggesting that NOD-MSCs are less effective for T-lymphocyte inhibition.

IV- MSC maturation and function in spleen of NOD mice after a CyP treatment

Markers	NOD	Balb/c
Gr-1 ^{high} *	79.2 \pm 3.0%	91.7 \pm 1.6%
CD31	3.5 \pm 1.6%	1.6 \pm 0.5%
Ly-6C***	6.0 \pm 1.4%	86.4 \pm 3.6%
MOMA-2	98.3 \pm 1.0%	97.8 \pm 1.0%
CMHII	3.9 \pm 0.8%	2.0 \pm 0.5%
CMHI	28.0 \pm 5.9%	30.1 \pm 5.4%
F4/80**	5.5 \pm 1.7%	2.7 \pm 0.6%
CD86	0.4 \pm 0.2%	0.2 \pm 0.0%
CD40	4.2 \pm 1.2%	3.0 \pm 0.9%

Table 1: Results are expressed as mean % \pm sem. *p<0.01, **p<0.002, ***p<10 $^{-4}$.

As the MSC differentiation defect observed in CyP-treated NOD mice concerned exclusively the immunosuppressive Gr-1high cell population (Table 1) and regarding the lack of Ly-6C expression by NOD-MSCs (Table 1), NOD mouse splenic MSCs, CD11b+Gr-1+ populations from CyP-treated NOD mice might contain a smaller number of suppressive cells, than those from CyP-treated Balb/c mice.

MSCs are known to inhibit activated T-cell proliferations. NOD MSCs do not exhibit a defect in NO production, secreting similar quantities of NO in response to activated T-cells (Fig 5A). Moreover, no difference was observed between NOD and Balb/c MSC expression of iNOS (Fig 5B). When irradiated, these cells do not secrete NO and could not express iNOS. In this context, ie in absence of NO, contrary to Balb/c-MSCs, irradiated NOD-MSCs could not inhibit efficiently activated T-cell expansion (Fig 5C).

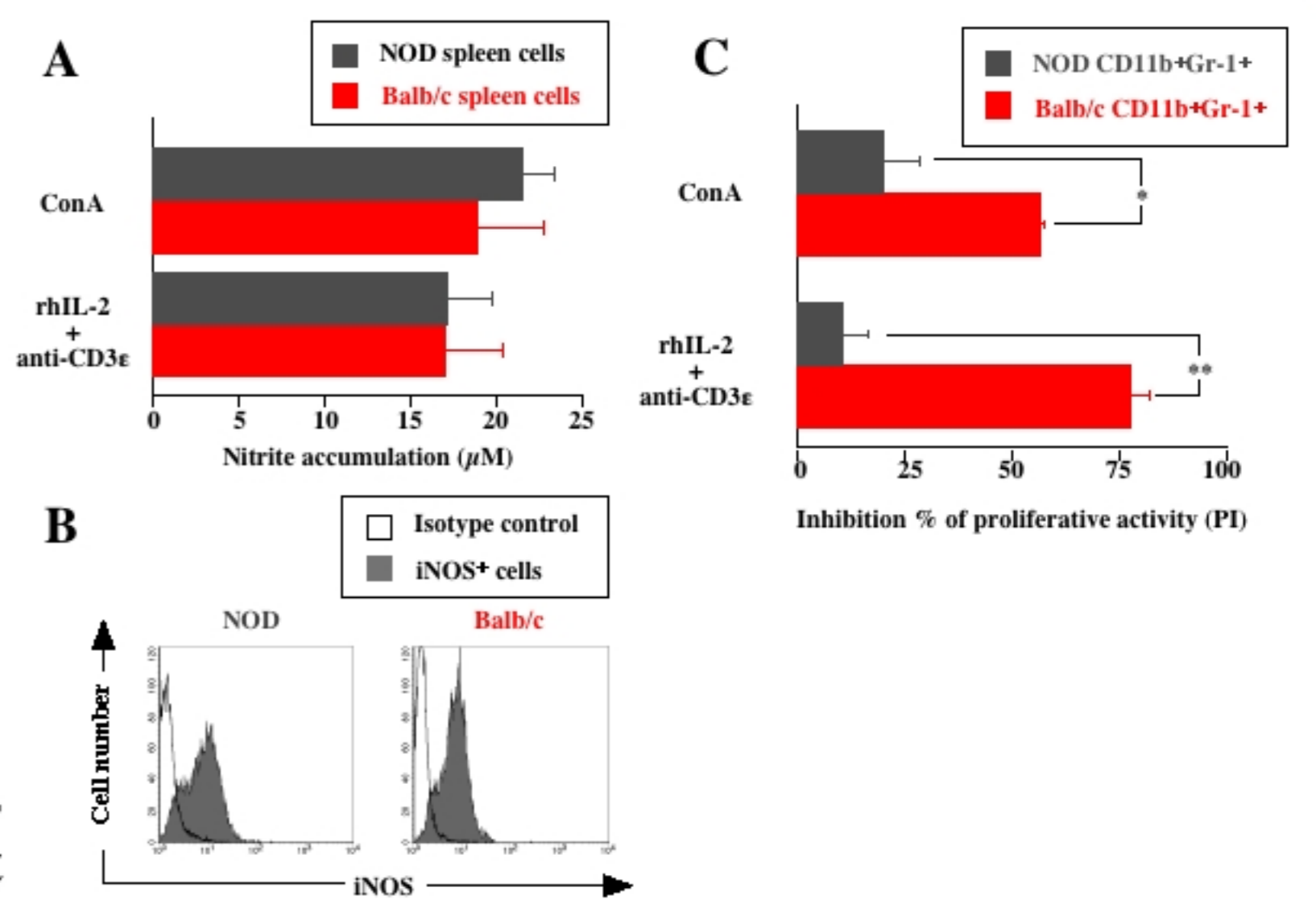


Figure 5: A/ No secretion of CD11b+ spleen cells from CyP-treated mice assessed by the quantification of nitrite in supernatants of 48h *in vitro* culture in presence of activated T-cells. B/ Intracellular expression of iNOS by iMacs. Splenic CD11b+, recovered 6 days after the last injection of CyP, 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. C/ Inhibition of syngeneic splenocyte proliferative activity by splenic irradiated CD11b+Gr-1+ cells isolated from CyP-treated mice 6 days after the last CyP injection. The % of inhibition was calculated as followed: 100 - (SI (splenocytes + CD11b+) / SI (splenocytes) *100). SI: stimulation indices. *p<0.04, **p<0.03.

SUMMARY and VIEWPOINT

MSC differentiation from NOD HPC is very ineffective and this defect mainly affects the Gr-1high subset representing the most functional MSC population. In comparison with CyP-treated Balb/c mice, MSCs are less recruited in spleen of CyP-treated NOD mice and exhibits a profound maturation defect regarding their expression of Ly-6C. Moreover, they do not fully inhibit activated T-cell proliferations in absence of NO secretion. MSC from CyP-treated NOD mice are less able to induce T-cell depletion in spleen after CyP-treatment suggesting that MSC may be unable to inhibit self-reactive T-cells in NOD mouse. We are presently testing this hypothesis in the CL4-TCR/INS-HA double transgenic model in collaboration with R. LIBLAU (INSERM, Toulouse, France).