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Dendritic cells induced in the presence of GM-CSF and IL-5

Huanfa Yi [a,b], Lianjun Zhang [a,b], Yu Zhen [a,b], Xiaoyan He [c], Yong Zhao [a,d,*]

[a] *Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*

[b] *Graduate University, Chinese Academy of Sciences, Beijing, China*

[c] *Department of Medicine, Liverpool Hospital and the University of New South Wales, Australia*

[d] *Department of Pharmacology & Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE, USA*

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Abstract

Dendritic cells (DCs), as an important part of antigen-presenting cells, can efficiently prime naïve T cell to induce or regulate immune responses. GM-CSF, combined with other growth factors, was used to induce the differentiation of immature or mature DCs from progenitors. Here, we investigated the effect of IL-5, a Th2 cytokine, on the differentiation and function of mouse DCs induced by GM-CSF in vitro. IL-5 significantly inhibited the differentiation of DCs induced by GM-CSF, but no effects on GM-CSF/IL-4 (GM/4) induced DCs. Compared with the conventional mouse DCs developed in the presence of GM-CSF and IL-4, mouse DCs induced by GM-CSF and IL-5 (refereed as to GM/5 DCs) possessed similar cellular morphology, but they expressed high level of CD11c, but low level of MHC II molecules, CD40 and CD86, which is consistent with the immature DC phenotype. In addition, GM/5 DCs showed significantly lower immunogenicity as indicated by their poor stimulating ability to allogeneic T cells in vitro, decreased expression of pro-inflammatory IL-6 and TNF-α, as well as increased expression of TGF-β, compared with GM/4 DCs. Together, these data suggest that IL-5 could partially inhibit GM-CSF-induced DC differentiation which could be reversed by IL-4 in vitro.

Keywords: IL-5; Cytokine; Antigen presentation; Dendritic cells; Phenotype; Tolerance

1. Introduction

Dendritic cells (DCs)[1] are the most vital professional antigen-presenting cells (APCs), which have the ability to induce the immune responses of naïve T cells. According to their different phenotypes, functions and microenvironmental localizations [1–4], the DC system is comprised of several different subsets in secondary lymphoid organs, such as $CD8\alpha^+$DCs, $CD8\alpha^-$DCs and Langerhans-derived DCs and so on. In addition, DCs can be subdivided into immature DCs (imDCs) and mature DCs (mDCs) according to their developing stages [1–4], which the former can differentiate into mature stage with the effect of growth factors or environmental inflammatory or pathogen-mediated factors. The different DC subpopulations have distinct ability to regulate immune response. So far, GM-CSF and IL-4 still are the growth factors most widely used for DC generation from rodent and human precursors in vitro [5,6]. GM-CSF acts on early bone marrow (BM) precursors to enable the development of the myeloid lineage cell types of granulocytes, macrophages and DCs, while IL-4 (higher dose than GM-CSF) can significantly increase the number of mDCs [6].

IL-5, which is produced primarily by activated Th2 cells, mast cells, NK cells [7] and type I regulatory T cells (Tr1)

* Corresponding author. Address: Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beisihuan Xi Road 25, Beijing, China. Fax: +1 86 10 62659958.

E-mail address: zhaoy@ioz.ac.cn (Y. Zhao).

[1] *Abbreviations used:* BMCs, bone marrow cells; CFSE, 5, 6-carboxyfluorescein succinimidyl ester; DCs, dendritic cells; FCM, flow cytometry; FITC, fluorescein isothiocyanate; IL-5, interleukin-5; MFI, median fluorescence intensity; PE, phycoerythrin; PI, propidium iodide.

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[8], is a 45 kDa homodimeric Th2 cytokine. IL-5 receptor (IL-5R) consists of two distinct membrane proteins, namely α and β chains. The IL-5Rα alone specifically bind to IL-5 but with low-affinity [9], and the β chain is the common β chain shared by IL-5, GM-CSF and IL-3 receptors [10], which provides a molecular basis for the functional redundancy of these cytokines. IL-5Rα expression is restricted to eosinophils, basophils and some activated B cells [11]. IL-5 plays a critical role in regulating eosinophil function (differentiation, proliferation, mobilization, degranulation and activation) [7,12–14] and is a major inflammatory mediator in asthma and other allergic diseases as well as in defense against parasitic infection [11]. IL-5 is also known as a human Basophilopoietin [15]. In addition, IL-5 has the ability to induce B cell proliferation and differentiation into IgM-producing cells [16,17], to facilitate IgG isotype switching to IgG1 or IgE [11] and to substitute for IL-2 as a growth factor for cytotoxic T cell induction [18–20]. However, the physiological role of IL-5 in DC development and maturation has not been fully elucidated so far. In the present study, using the standard in vitro DC-inducing system, we have investigated the direct role of IL-5 in the differentiation and function of mouse DCs.

2. Materials and methods

2.1. Mice

Six-to-eight-week old female C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were purchased from Beijing Vital River Animal Company (Beijing, China). All animals were kept in microisolator cages in a specific-pathogen free facility. All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

2.2. Monoclonal antibodies (mAbs) and chemical reagents

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 mAb (RM4-5), FITC-labeled rat anti-mouse CD8 mAb (53-6.7), FITC-labeled anti-mouse CD11c mAb, FITC-labeled anti-mouse CD40 mAb, FITC-labeled anti-mouse CD54 mAb, FITC-labeled anti-mouse CD86 mAb, phycoerythrin (PE)-labeled rat anti-I-A^b mAb, PE-labeled rat anti-mouse CD11c mAb.

In addition, rat anti-mouse FcR mAb (2.4G2) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory. Mitomycin C ($C_{15}H_{18}N_4O_5$) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). Mouse GM-CSF, mouse IL-4 and mouse IL-5 were obtained from R&D (USA).

2.3. Immunofluorescence staining and flow cytometry (FCM)

DCs (about 1×10^5) were washed once with FACS buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% BSA). For two-color staining, cells were stained with PE-labeled anti-mouse CD11c mAb versus FITC-labeled anti-mouse CD40, CD86 mAb or the non-specific staining control mAb, respectively. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least ten-thousand cells were assayed using a FASCalibur flow cytometry (Becton Dickinson, CA), and data were analyzed with CellQuest software. Non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAb from staining in the same dot-plot region with the anti-mouse mAbs. Certain molecule expression levels were determined as the median fluorescence intensity (MFI) of the cells positively stained with the specific mAb.

2.4. DC generation

The principle method for generating BM-DCs was adapted from previous publications [21]. Briefly, mice femurs and tibiae, sometimes including hipbones, were removed mechanically from surrounding tissues. Bone marrow cells (BMCs) was flushed with cold RPMI1640 (Hyclone, American) using a syringe with a 0.45-mm needle. Clusters within the marrow suspension were dissociated by vigorous pipetting and filtrated through a 200-Mu Nylon mesh. Erythrocytes were lysed with ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA). Afterwards, the remaining cells were cultured in complete medium (CM, RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, American), 2 mM L-glutamine, 100 U/ml penicillin,100 μg/ml streptomycin and 50 mM 2-mercaptoethanol) in the presence of either GM-CSF alone (500 u/ml; R&D), GM-CSF+IL-4 (1000 u/ml, R&D), GM-CSF+IL-5 (500 u/ml, R&D) or GM-CSF+IL-4+IL-5 in 100 mm culture dishes for 9 days. In some experiment, at day 8, the non-adherent cells were collected by gentle pipetting, and re-cultured in 10 ml fresh medium containing 500 U/ml GM-CSF with or without 1 μg/ml lipopolysacchride (LPS, Sigma) in a fresh 100 mm tissue culture plastic dish.

2.5. Cell yield and purity evaluation

Cultured cells were washed once and an aliquot volume mixed 1:1 in Trypan Blue solution (0.4%). Trypan Blue negative, large leukocytes were counted as viable under the microscope in a Neubauer chamber. The purity was assessed according to the percentage of CD11c and I-A^b double positive cells detected by FCM.

2.6. Allogeneic mixed leukocyte reactions (MLR)

Mitomycin C-treated Balb/c DCs were used as stimulator cells and seeded in triplicates in 96-well round-bottom plates at a ranged number (40–2.5×10^3/well). Freshly isolated B6 splenocytes were used as responder cells at 1×10^5/well. The total volume of each well is 200 μl. The cells cultured for 96 h. The cells were pulsed with 0.5 μCi of [^{3}H]-thymidine for the last 18 h of incubation. Cells were harvested with an automatic cell harvester (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckmon Instruments, America). Values are expressed as counts per minute (cpm) of triplicate wells.

In some experiments, responder cells were labeled with 5,6-carboxy-fluorescein succinimidyl ester (CFSE; Sigma–Aldrich) by incubation with 5 μM CFSE in 0.1 M protein-free PBS for 10 min in 37 °C water bath. Cells were immediately washed two times in RMPI1640 containing 10% FBS, then seeded 1×10^5/well in 96-well round-bottom plates, and cultured with 5×10^3 mitomycin C-treated DCs from different groups for 96 h. The cells were then harvested and stained with PE-labeled anti-mouse CD4 mAb or PE-labeled anti-mouse CD8 mAb for 30 min in 4 °C. The proliferation of $CD4^+$ or $CD8^+$ T cells was analyzed by FCM.

2.7. Reverse transcriptase PCR (RT-PCR)

The different groups ($5–10 \times 10^{6)}$ of DCs which were generated as described above were washed with PBS, and RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction, RNA concentration and quality were determined spectrophotometrically (OD260) and by denaturing agarose gel analysis, respectively. Reverse transcription (RT) was performed, and the resulting cDNA was used in the PCR. The primers used are commercially available from Invitrogen and as assays on demand. The sequences of 5′ sense and 3′ antisense primers of murine cytokines and GAPDH (input control) were as follows (5′→3′): IL-10: sense primer, ATGCCTGGCTCAGCACTGCTA; Antisense primer, CA TCACTTCTACCAGGTAAAA (PCR product 287 bp); TGF-β: sense primer, AGGGCGGGCGAGACTTTC; Antisense primer, TGCTTCAGCTTGGCCTTGTAGA (PCR product 480 bp); IL-6: sense primer, Antisense primer (PCR product 507 bp), TNF-α: sense primer, CGCAAGGTCCAACAGGTCAG; Antisense primer, TT AGGATCAAAGGTCGGCTCAG (PCR product 400 bp), GAPDH: sense primer, CGGCAAATTCAACGGCAC AG; Antisense primer, GGATGCAGGGATGATG TTCTG (PCR product 482 bp).

2.8. Statistical analysis

All data are presented as the mean ± SD. Student's unpaired *t* test for comparison of means was used to compare groups. A *p* value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Significantly reduced yield of $CD11c^+$DCs from BMCs in the presence of IL-5

We were herein interested in determining whether or not IL-5, in combination with GM-CSF, could stimulate DC generation from murine BMCs in vitro. Mouse BMCs were cultured in the presence of GM-CSF and IL-5 (called GM/5 DCs). As a control, some BMCs were cultured with GM-CSF (called GM-DCs), GM-CSF and IL-4 (called GM/4 DCs), a conventional cytokine combination that is known to generate DCs in vitro [22] , as well as GM-CSF, IL-4 and IL-5 (called GM/4,5 DCs). During day 4 and day 7, small clusters of DCs were visible, and attached to the adherent cells (macrophages and fibroblasts), then they dislodged from the surface and floated in the culture medium. By day 9, cells in all culture systems contained small lymphoid-sized cell

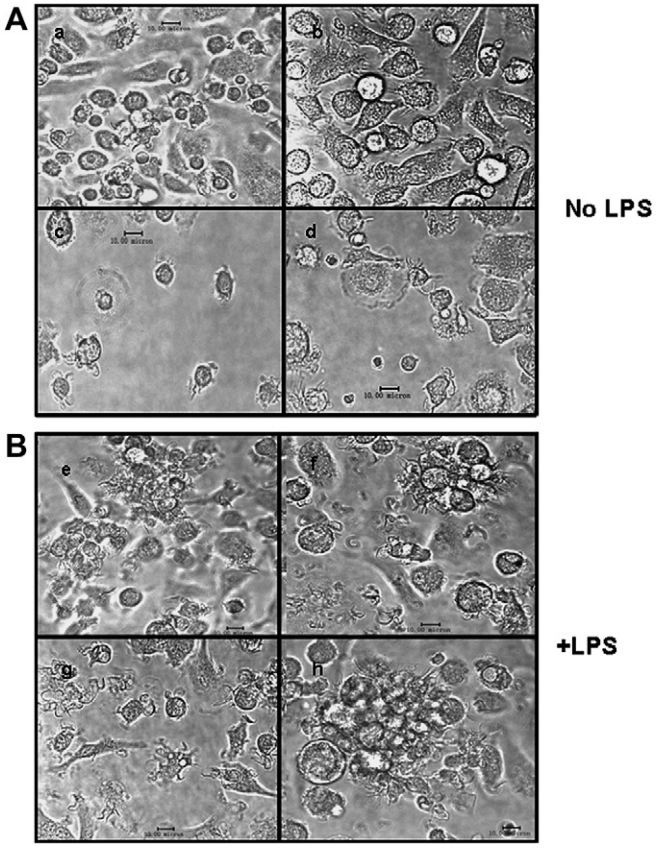

Fig. 1. The morphology of non-adherent DCs generated with different cytokines combination in the absence (A) or presence (B) of LPS. After culture for 9 days, GM/5 DCs showed a similar appearance of GM/4 DCs with dendritic morphology under phase-contrast microscopy (×400). (a and e) GM-DCs; (b and f) GM/4 DCs; (c and g) GM/5 DCs; (d and h) GM/4,5 DCs. Data were one representative of five independent experiments with similar results.

clusters with typical DC morphology (Fig. 1A, left panel). GM/5 DCs appeared to no statistically significant difference in cell size compared with other groups (data not shown). When pulsed with LPS for 24 h at day 8 of culture, DCs in all groups displayed numerous long and thin dendrites and veils, which were a sign of DC maturation (Fig. 1B, right panel). No eosinophils were detected when stained by Wright's-Giemsa (Data not shown).

The yields and purities of DCs were assessed in all groups on day 9 of culture. The total cell numbers in all groups did not show significant differences although the cell numbers in GM/5 group was somehow lower than other groups. However, the purity of $CD11c^{+}I\text{-}A^{d+}$DCs in GM/5 group was markedly lower than that of other groups as determined by the expressions of CD11c and MHC-II ($I\text{-}A^{d}$) molecules. The percentages of $CD11c^{+}I\text{-}A^{d+}$cells in GM, GM/4, GM/4,5 and GM/5 DCs were 34%, 55%, 57%, and 20%, respectively (Fig. 2A, middle panel). The cell numbers of $CD11c^{+}I\text{-}A^{d+}$DCs in GM/5 group was about 8×10^{5}, only among 1/3 of other groups and significantly less than other groups ($p < 0.05$; Fig. 2A, right panel). Consistently, after induced by LPS, the total cell number and the percentage of $CD11c^{+}I\text{-}A^{d+}$DCs in GM/5 DCs were significantly lower than other groups (Fig. 2B).

3.2. DCs induced by GM-CSF and IL-5 displayed a more immature phenotype

The cell phenotypes of DCs induced in different conditions were detected by FCM. As expected, $CD11c^{+}$DCs induced by GM-CSF and IL-4 expressed high levels of CD40, CD54, CD80, CD86, and MHC-II ($I\text{-}A^{d}$) molecules (Fig. 3A, B, and C). However, $CD11c^{+}$DCs induced by GM-CSF and IL-5 expressed lower levels of $I\text{-}A^{d}$, CD40 and CD86 molecules than $CD11c^{+}$DCs induced by GM-CSF and IL-4 (Fig. 3). Moreover, when we stimulated DC with 1 μg/ml LPS for one day from day 8 of culture, the expressions of $I\text{-}A^{d}$ molecule and co-stimulatory molecules, including CD40, CD80, CD86, and CD54 on mouse DCs in all groups were strongly up-regulated when detected using FCM. No significant differences among these groups were observed (Fig. 4). In our culture system, some F4/80 positive cells were detected by FCM in all groups (data not shown).

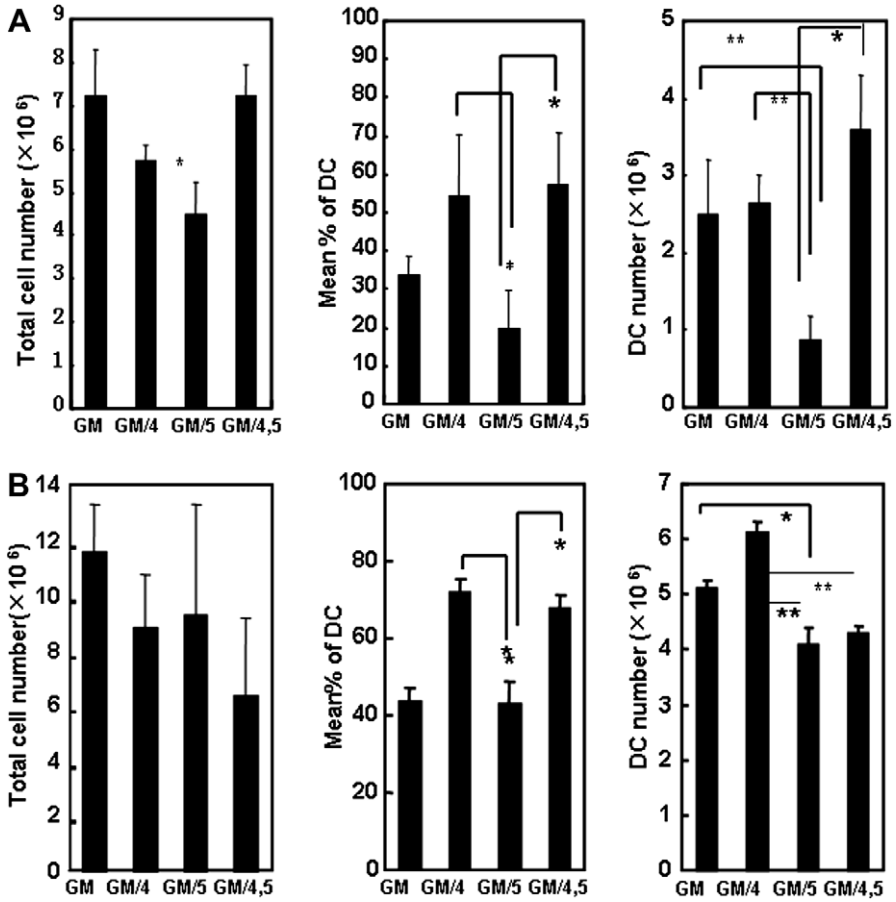

Fig. 2. The yield and purity of non-adherent DCs generated with different cytokine combination. (A) The cell numbers of non-adherent cells and percentages of DCs in all groups without LPS treatment. (B) The cell numbers of non-adherent cell and percentages of DCs in all groups after LPS treatment for 1 day. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the indicated groups.

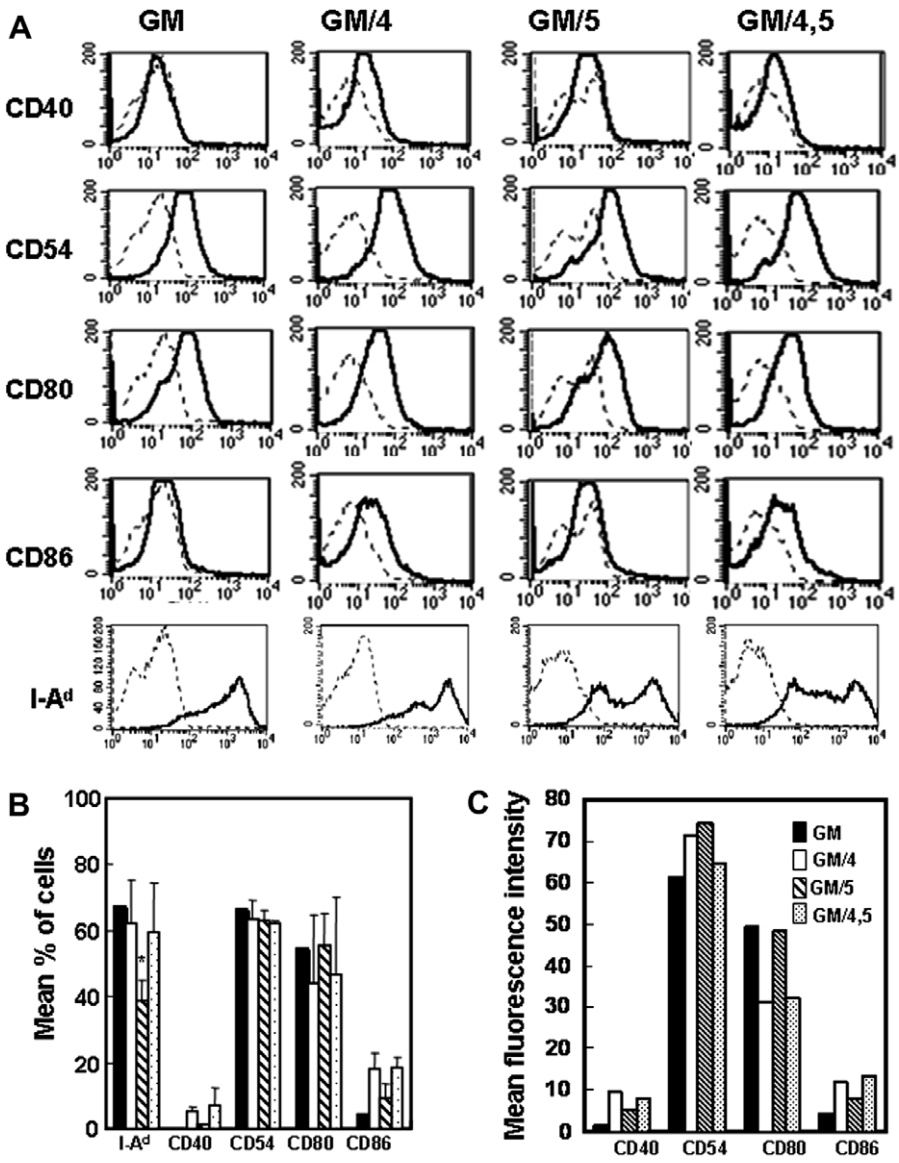

Fig. 3. GM/5 DCs exhibit lower expressions of I-A^d, CD40, CD86 than conventional GM- or GM/4- DCs before LPS treatment. (A) One representative of the expression of co-molecules on gated CD11c$^+$DCs when detected by FCM. The percentages (B) and the mean fluorescence intensities (C) of CD40, CD86 and I-A^d on CD11c$^+$DCs before LPS stimulation. $^*p < 0.05$, $^{**}p < 0.01$ compared with the indicated groups.

3.3. DCs induced by GM-CSF and IL-5 had reduced immunogenicity to allogeneic T cells

The ability to stimulate allogeneic T cells of DCs has been used as a benchmark by a number of investigators [21,23]. The capacity of mouse DCs induced in different conditions to stimulate the proliferation of allogeneic T cells was examined. MLRs were prepared in 96-well plates, and cultured for 4 days. As few as 2000 GM/4 DCs were able to stimulate a strong allogeneic T cell response with a dose-dependent fashion (Fig. 5A), GM/5 DCs stimulated allogeneic T cells proliferation less than GM/4 and GM/4,5 DCs ($p < 0.05$) but had a comparable proliferation level with GM-DC group. And the intension of MLR become significantly higher with the DC maturation by LPS (Fig. 5B), but no significant differences were detected among all the DC groups. After allogeneic splenocytes were labeled with CFSE, The cell division of CD4$^+$T cells stimulated with different DCs was determined by FCM. Identical results were observed as determined in MLR assays (data not shown).

3.4. GM/5. DCs expressed less IL-6 and TNF-α, and more TGF-β than GM/4 DCs

After we observed the phenotypical and functional difference of GM/5 DCs compared with GM/4 DCs, we then investigated the profile of cytokine secretion by these DCs. As shown in Fig. 6, GM DCs, GM/4 DCs and GM/4,5 DCs expressed high levels of IL-6 and TNF-α as detected by RT-PCR. GM DCs expressed higher levels of TGF-β than GM/4 DCs or GM/4,5 DCs. Interestingly, GM/5

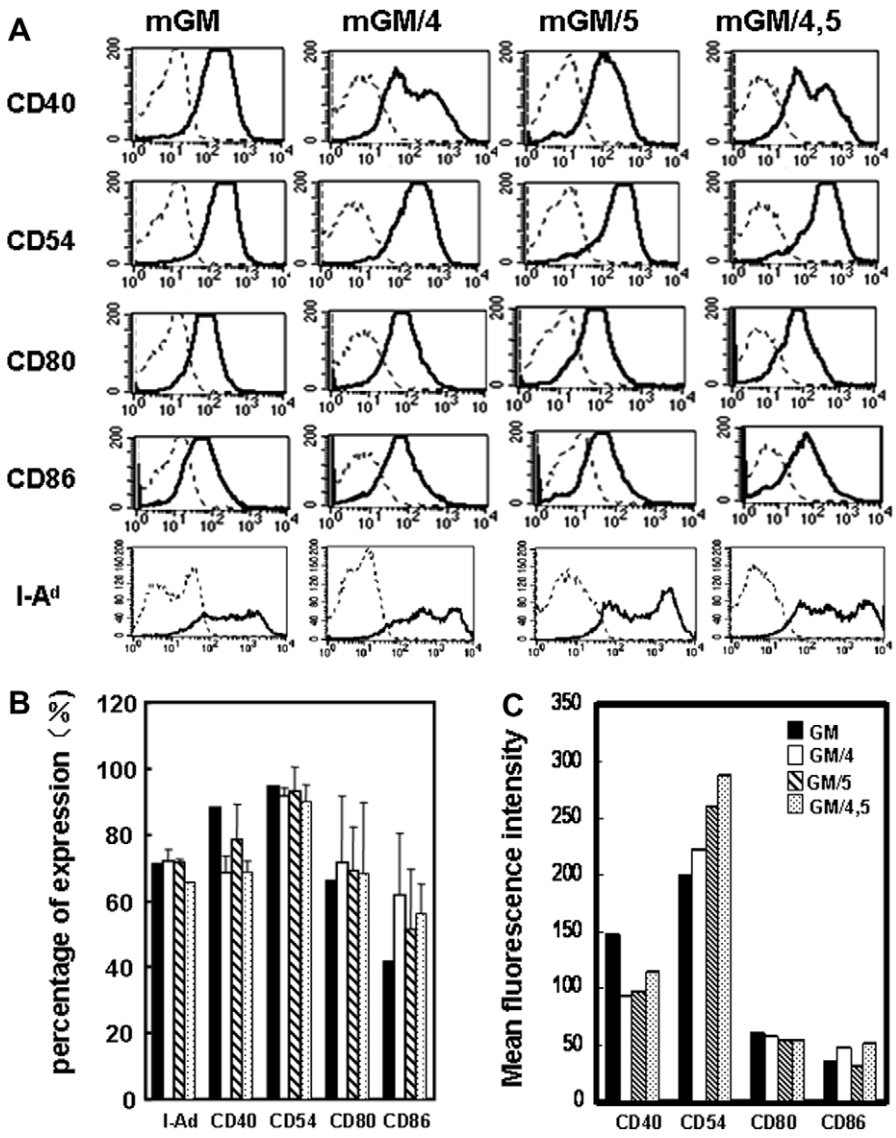

Fig. 4. The expressions of co-molecules on CD11c^{+}DCs after LPS treatment. All DCs were stimulated with 1 μg/ml of LPS for 1 day from day 8. (A) One representative of the expression of co-molecules on gated CD11c^{+}DCs when detected by FCM. The percentages (B) and the mean fluorescence intensities (C) of CD40, CD86 and I-A^{d} expression on CD11c^{+}DCs after LPS stimulation.

DCs expressed lower levels of IL-6 and TNF-α than other DCs, and more TGF-β than GM/4 DCs and GM/4,5 DCs. These preliminary data support the poor immunogenicity of GM/5 DCs.

4. Discussions

DCs are the most potent APCs of the immune system compared with B cells and macrophages. DCs are derived from stem cells in bone marrow, and distribute in different tissues with immature or mature status. DCs are greatly rare in most tissues, which once impeded the study of the ontogeny, phenotype and function of DCs. Since the establishment of the expansion method of DCs [27], many cytokines were combined with GM-CSF to induce DCs from bone marrow or blood precursors in vitro, such as TNF-α, IL-4 [6,28], stem cell factor [29], Flt-3 ligand [30,31], TGF-β [32], IL-3 [33,34], CD40L [35], IL-15 [36] et al. DCs generated in different cytokine combinations exhibit potential differences in their ability to modulate immune response [31,36,37].

IL-10, a Th2 cytokine, can be used to block DCs maturation and skews the Th1/Th2 balance to Th2 [38] or induce Tr1 cells [26,39]. Furthermore, recently, He et al. found that rat IL-5 treatment delayed fully allogeneic heart graft rejection and facilitated tolerance induction with anti-T cell mAb therapy [40]. Our experiments were focused on the potential direct role of mouse IL-5 in DC maturation and differentiation. In our cultures, DCs were generated with various cytokine combinations from mouse bone marrow progenitors, in that not eosinophil was detected in the system. Our finding wasn't consistent with previous reports

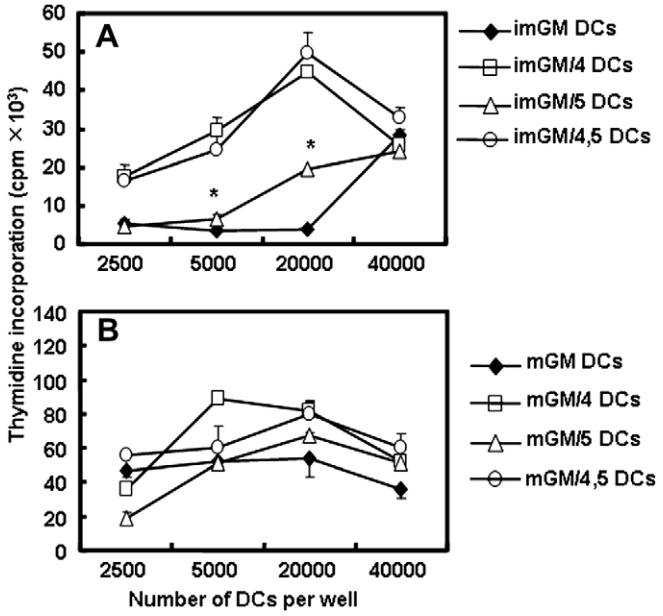

Fig. 5. GM/5 DCs are less potent than conventional GM/4 and GM/4,5 DCs in the stimulation of allogeneic T cell proliferation. Allogeneic MLR was performed with CD11c$^+$GM/5 DCs, GM DCs, GM/4 DCs and GM/4,5 DCs without (A) or with (B) LPS-prestimulation as described in the Materials and Methods. The data are shown as the means ± SD. $^*p < 0.05$ vs. groups of GM/4 DCs and GM/4,5 DCs, respectively. Data were one representative of three independent experiments with similar results.

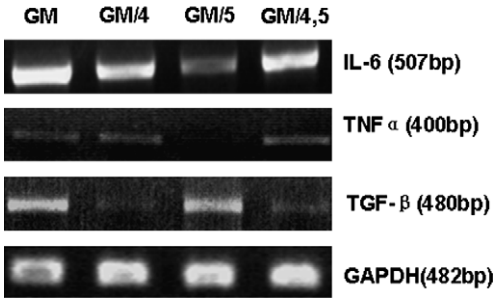

Fig. 6. The mRNA expression of IL-6, TNF-α and TGF-β in GM DCs, GM/4 DCs, GM/5 DCs and GM/4,5 DCs. All DCs were generated as described in Section 2. RT-PCR was performed as described in Section 2. Data were one of representative of three independent experiments with similar results.

which revealed that GM-CSF plus IL-5 can induce eosinophils from FDCP-Mix cells, a cytokine-dependent, karyotypically normal, multipotent cell line [41]. The possible reason was that the concentration of IL-5 in our culture was higher than that of the former. This concentration of IL-5 was gotten from our preliminary experiments and He's experiment (personal communication).

The size and shape of DCs from all groups showed no significant differences among them, but the purity and number from GM/5 DCs were significantly lower than that of GM/4 DCs. It is of note that IL-5-specific signaling can lead to a switch toward up-regulation of functional IL-5Rα in human CD34$^+$ cell [42], increased numbers of CD34$^+$ cells expressing TM-IL-5Rα were detected in the bone marrow as early as 24 h after allergen inhalation challenge of asthmatics [43]. In addition, CD34$^+$/ TM-IL-5Rα mRNA1 cells were recently detected in airway biopsies from atopic asthmatic subjects [44]. The IL-5 binding to the receptor is competed with either IL-3 or GM-CSF in humans, probably same in as well [45]. So it is possible that the increase in the number of IL-5 receptor α chain leads to a decreased GM-CSF binding to GM-CSF receptor, which results in the reduction of DC generation. However, the different effects of IL-5 on GM-CSF-induced or GM-CSF+IL-4-induced DCs indicate that the inhibitory function of IL-5 on DCs may be not so simple and its suppression may be achieved by other pathways. It needs to be addressed.

By examining DC surface phenotype markers, we found that GM/5 DCs appear to be less mature than GM/4 DCs. The GM/5 DCs expressed significantly less MHC II molecules, CD40, and CD86, though CD80 expression on DCs had no significant difference among all groups. Our present results failed to offer evidence for the potential mechanisms which need to be investigated in the future.

Consistent with the phenotype of DCs, GM/5 DCs had a less potent capability of stimulatory allogenic T cell proliferation than GM/4 and GM/4,5 DCs, but there was no statistical difference compared with GM DC. The cell proliferation of GM/5 DC showed a dose-dependent manner. The proliferation of allogeneic T cells induced by GM/4 and GM/4,5 DCs reached their peak proliferation at 20,000 cells/well, then the proliferation cut down. However, GM/5 DCs in that density was still in an increasing curve until the number reached 40,000 cells/well, which also indicates that GM/5 DCs had a poor stimulatory ability to allogeneic T cells. When LPS was employed to stimulate DC maturation, the allo-stimulation capability of GM/5 DCs become accentuated, which was similar to the rest of the groups with no significant differences detected. However, in those groups adding IL-4, when stimulated with LPS, the expression of MHC, adhesion and costimulatory molecules didn't up-regulated significantly, perhaps because IL-4 can increase the number of mature DCs [6], which leaded to DC insensitive or resistant to LPS stimulation.

To further determine the characteristics of IL-5-induced DCs, we investigated their cytokine profiles. It has been reported that mature DCs are IL-6$^+$TNFα$^+$ [24,46], while the absence of proinflammatory cytokines is considered one of the critical factors in determining the tolerogenic potential of DCs [24,47]. Here, our preliminary data showed that GM/4 DCs expressed large amount of IL-6 and medium level of TNF-α. It is striking that GM/5 DCs expressed lower levels of TNF-α and IL-6 and higher levels of TGF-β mRNA compared with GM/4 DCs. this cytokine profile of GM/5 DCs is fully in keeping with their immature characteristics, IL-5 may have the ability to suppress TNF-α and IL-6 production and enhance TGF-β expression. However, adding IL-4 can reverse the inhibitory effect of IL-5 (Fig. 6), which was consistent with the phenotype and MLR results. In addition, several lines of

evidence have demonstrated that DCs producing higher amount of IL-10 could induce Tr1 cells [25,48,49]. In our present model, IL-10 mRNA was undetectable in any group, but GM/5 DCs expressed a certain level of TGF-β which is another tolerogenic cytokine.

In summary, IL-5 could directly inhibit the differentiation and function of mouse DCs mediated by GM-CSF in vitro as indicated by the decreased cell yield, low level expression of co-molecules, lowered expression of pro-inflammatory cytokines (IL-6 and TNF-α), expression of tolerogenic cytokine TGF-β and poor immunogenicity to allogeneic T cells. The role of IL-5 as a regulatory factor in DC generation and maturation may help us to understand the biological function of IL-5 in the regulation of immune responses.

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