The role of hepatocyte growth factor in the differentiation of dendritic cells from peripheral blood monocytes

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ABSTRACT

Objective: To find out the effects of hepatocyte growth factor (HGF) in the development of dendritic cells (DC) from the peripheral monocytes.

Methods: The study was carried out in Black Sea Technical University Hospital, Trabzon, Turkey between 2003-2004. Seven different cytokine combinations were employed to assess phenotypical and functional differences of DCs from the peripheral monocytes in serum free culture media. Peripheral monocytes were incubated in media with cytokines for 5 days. The tumor necrosis factor-α (TNF-α) was added to the cell culture on day 5 and incubated for another 2 days. Surface and co-stimulating molecules on the cells were assessed by flowcytometry. The functional capacity of the DCs was evaluated on day 7 by purified protein derivative loading and subsequent lymphoproliferation test using methyl tetrazolium staining.

Results: On the 5th day of incubation DC development was observed in all cytokine groups, but cells were superior in cultures maintained in the presence of interleukin-4 combinations with granulocyte-macrophage colony stimulating factor (GM-CSF) or with GM-CSF+HGF. Moreover, the expression of surface and co-stimulating molecules increased significantly after incubation with TNF-α. The effect of PPD loaded-DCs on proliferation of lymphocytes was more striking in HGF containing groups.

Conclusion: It was concluded that HGF supplemented cultures exert some additive effects in relation to function of monocyte-derived DCs. But HGF alone does not seem to augment monocyte-derived DC proliferation and maturation significantly.

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Dendritic cells (DC) play an important role in the regulation of immune response and can be detected in all kind of tissues except brain, testis and eyes.[1,2] The morphology of these cells is not specific of DCs and resembles many other cells like macrophages and B lymphocytes. Therefore, not only morphological appearances but also functional characteristics await full description.[1,3]

Dendritic cells can be cultured outside the body specifically for the applications of immunotherapy. During the differentiation period, combinations of cytokines like granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), stem cell factor (SCF), fms-like tyrosine kinase 3 ligand (Flt-3L), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6) and prostaglandin E2 (PGE2), are utilized.[4] After the finding of the receptor of hepatocyte growth factor (HGF), c-met in adults, it was hypothesized that HGF not only plays an important role in the hematopoiesis of fetal period, but also in adults. In the studies thereafter, the role of HGF in the proliferation and differentiation of stem cells was demonstrated.[5,6] After the data showing HGF secretion from monocytes and monocyte precursors, it was found that HGF would play important roles in the monocyte and macrophage functions resulting in enhanced non-specific immune responses.[7,8] It has also been reported that the effect of HGF on peripheral blood and cord blood monocytes may be so that HGF increases for instance the antigen presentation efficiency of monocytes in peripheral blood but not of the monocytes from cord blood.[9,10] However, it has been reported that HGF stimulates (differentiates) the CD34+ cells into DCs and exerts even stronger stimulating effect than GM-CSF.[11] In another study, it was demonstrated that HGF receptor c-met is present in mouse DCs and that HGF has a significant effect on the functions of DCs.[12]

In this study, we planned to clarify the effect of HGF on the DC differentiation from peripheral

blood monocytes, an unknown biologic phenomenon until now.

Methods. The study was carried out in Black Sea Technical University Hospital, Trabzon, Turkey between 2003-2004. The study protocol was approved by the local institutional committee.

Isolation of mononuclear cells. A 100 ml of peripheral blood was drawn from 5 healthy consenting donors into ficoll containing tubes (BD Vacutainer CPT, NJ USA) and centrifuged for 30 minutes at 1400 rpm. The mononuclear cells (MNCs) were harvested and washed twice with RPMI 1640 (Sigma, Steinheim, Germany) for 10 minutes at 1200 rpm. The supernatant was discarded and the pellet was suspended in RPMI 1640 solution supplemented with 1% human serum albumin (HSA) (Baxter AG, Vienna, Austria) at a concentration of 5 x 10^6/ml. This suspension was then incubated in 8 T25 culture flasks (Grenier bio-one, Frickenhausen, Germany) each containing 10 ml of the medium (RPMI 1640 solution supplemented with 1% HSA) and incubated for 2 hours at 37°C in 5% CO_2. The nonadherent cells were discarded by washing 4 times with RPMI 1640 while adherent cells were cultured for 7 days in the presence of various cytokines alone or in combinations of growth factors as described below.

Differentiation of dendritic cells. The cells in tissue culture flasks were maintained either in just the DC growth medium (CellGro DC medium, CellGenix Technologie, Freiburg, Germany) containing 1% HSA as control or as supplemented with GM-CSF 100 ng/ml (CellGenix Technologie, Freiburg, Germany), or IL-4 50 ng/ml (CellGenix Technologie, Freiburg, Germany) or HGF 20 ng/ml (Sigma-Aldrich, Steinheim, Germany) or in medium supplemented with combinations of either as HGF+GM-CSF, or HGF+IL-4 or GM-CSF+IL-4 or HGF+GM-CSF+IL-4 with each of the factor being present at the same concentrations.

Follow-up of dendritic cells cultures. The MNCs were incubated at 37°C and in 5% CO_2 for 5 days. After 5th day of culture, 2 ml sample was taken and analyzed by flowcytometry (Epics XL Beckman Coulter, USA) using the FITC-CD14, PE-CD1a, PE-HLA-DR, PE-CD80, PE-CD83, FITC-CD86 (Immunotech Coulter Company, Marseille, France) surface markers.[13] On day 5, DC growth medium was supplemented to promote growth and TNF-α (CellGenix, Freiburg, Germany) at 20 ng/ml was also added into the DC growth media to

Table 1 - The percentage of antigen expression in the groups on the 5th day of incubation.

Groups	CD14	CD1a	HLA-DR	CD80	CD83	CD86
Control	0.5 (0.3-7)	2.21 (0.6-4.3)	69.31 (34.8-86.3)	16.72 (2.5-26.3)	9.20 (7.6-11.6)	42.45 (12-46.4)
GM-CSF	8.10* (6.1-14.1)	3.5 (1.5-4.75)	93.6* (73-98.2)	17.8 (11.2-19.5)	24.6_a** (19.5-30.1)	63** (45-79.7)
IL-4	0.7 (0.5-4.5)	2.08 (1-2.8)	95.7* (72.1-97.86)	37.63 (15.1-40.2)	14.3_b (8.6-17.1)	42.34 (31-68.7)
HGF	5.6 (1.7-19.5)	0.86 (0.2-2.79)	94.9* (71.5-96.7)	11.30 (8.86-50.4)	12.4_c (6.7-13.4)	24.83 (16.8-54.9)
GM-CSF+HGF	1.88 (0.2-8.6)	3.20 (0.6-5.53)	88.8* (76.1-94.57)	17.39 (8.10-34.2)	11.2_d (9.8-16.50)	58.3 (19-60)
IL-4+HGF	1.2 (0.20-4.4)	1.9 (0.75-5.2)	88.79* (82.3-96.7)	13.1 (11.53-30.3)	22.3_e** (20.8-29.6)	29 (24.6-73.6)
GM-CSF+IL-4	2.4 (0.4-2.8)	3.02 (0.6-9.1)	92.5* (76.6-97.4)	14.39 (13-44.5)	42.1_f** (35.6-69.4)	56.3* (45-65.5)
GM-CSF+IL-4+HGF	0.81 (0.4-1.8)	4.18 (0.4-4.4)	89.4* (70.7-95.18)	19.36 (11.20-44.8)	42.5_g** (27.2-75)	67.5** (52-75.2)

The data are represented as % of total cells in each well and indicated the median (min-max) of five independent experiments.
*p<0.05, **p<0.01 (comparison of groups with control). a-f, a-d, b-f, c-e, d-g, e-f.
Each double letter represents the intergroup comparisons like a-f as CD 83 comparison between GM-CSF and GM-CSF+IL-4 group, a-d as GM-CSF and GM-CSF+HGF groups, b-f as IL-4 and GM-CSF+IL-4 groups, c-e as HGF and IL-4+HGF groups, d-g as GM-CSF + HGF and GM-CSF+IL-4+HGF groups, e-f as IL-4+HGF and GM-CSF+IL-4 groups (p<0.01).
GM-CSF - granulocyte-macrophage colony stimulating factor, IL-4 - interleukin-4, HGF - hepatocyte growth factor.

induce maturation. On the 7th day, 2 ml sample was removed again for surface marker analysis.

The detection of the effect of dendritic cells on the proliferation and functions of lymphocytes. Fresh MNCs were prepared as described above, and incubated for 2 hours in DC growth media with 1% HSA to obtain adherent monocytes and non adherent lymphocytes. The mature DCs produced through the culture procedure as described above and adherent monocyte population obtained freshly were incubated with purified protein derivative (PPD) 20 µg/ml (BB-NCIPD-ltd. Sofia-Bulgaria) at 37°C and in 5% CO_2 for 2 hours. It is a well-described method of stimulating DC differentiation.[14] The culture flasks containing adherent PPD loaded-monocytes were then trypsinized and washed 3 times and resuspended with DC growth medium containing 1% HSA. Thereafter, the monocyte suspension and DCs preperation were irradiated at 1500cGy to use in lymphocyte stimulation tests. For this, lymphocytes obtained from MNCs as non-adherent cells were distributed into the wells of 96-well culture plates at 1 x 10^5 lymphocytes/well in 200 µl and subsequently challenged with autologous monocytes and DCs at 1 x 10^3 cells in 50 µl of the DC growth medium with 1% HSA to give a ratio of 1 stimulant and 100 target cells in a row of eight wells. Appropriate control wells were set without stimulant or target cells. The cultures were incubated at 37°C in 5% CO^2 for 48 hours. At the end of the incubation, 10 µl of MTT solution (Sigma-Aldrich, Steinheim, Germany) was added into each well to obtain a 0.5 mg/ml final concentration. The plate was incubated at 37°C for 3-4 hours to obtain formasan crystals. Afterwards, 200 µl of culture grade DMSO and ethanol mixture (v/v 1:1) was added and plates were agitated for 10-20 minutes on a rotating shaker. When the crystals were dissolved completely, optical densities of color development was measured at 545 nm using a multiwell plate reader.[15] The percent of lymphocyte proliferative efficiency of DC groups was calculated, comparing with control group including monocytes as stimulator.

Statistical analysis. All experiments were repeated at different occasions for 5 times, and the data computed as median (min-max) were compared by Mann Whitney–U test. The comparison between groups was carried out by Wilcoxon test.

Results. The results of flowcytometric analysis of surface and costimulatory molecules expression of DCs cultures on day 5 with different cytokines or with their

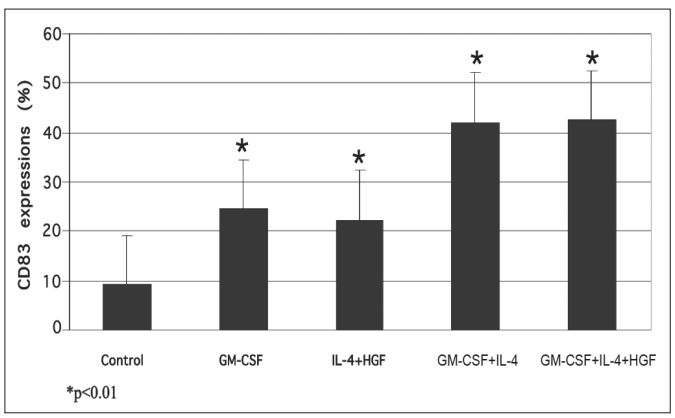

Figure 1 - The percentage of CD83 expression related to **Table 1** correlated with the aforementioned group comparisons. *p<0.01.

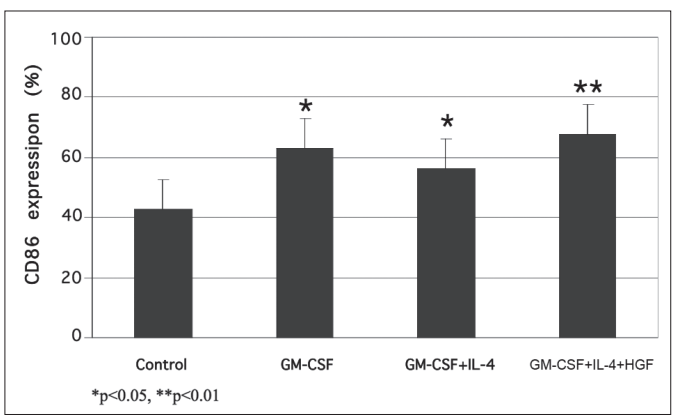

Figure 2 - The percentage of CD86 expression related to **Table 1** correlated with the aforementioned group comparisons. *p<0.05; **p<0.01.

combinations (GM-CSF, IL-4, HGF, HGF+GM-CSF, HGF+IL-4, GM-CSF+IL-4, HGF+GM-CSF+IL-4) are summarized in **Table 1**. It shows that compared with that of control (without cytokine) group; HLA-DR expression increased in all groups, while CD14 expression increased only in GM-CSF group ($p<0.05$). The CD83 expression also increased in cultures supplemented with GM-CSF only and all IL-4 containing combination groups with or without HGF ($p<0.01$) (**Figure 1**). Moreover, CD86 expressions increased in GM-CSF, GM-CSF+IL-4 and markedly in GM-CSF+IL-4+HGF group ($p<0.05$ and 0.01) (**Figure 2**). However, CD1a and CD80 expression did not change (**Table 1**).

The results of flowcytometric analysis of surface and costimulatory molecules expression of DCs cultures on day 7, which represents the period of maturation upon addition of TNF-α at 20 ng/ml on day 5 of the culture are summarized in **Table 2**. As seen, HLA-DR

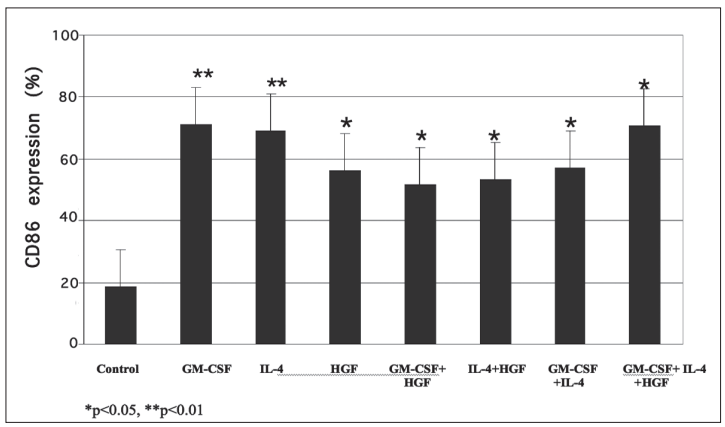

Figure 3 - The percentage of CD86 expression related to **Table 2** correlated with the aforementioned group comparisons.

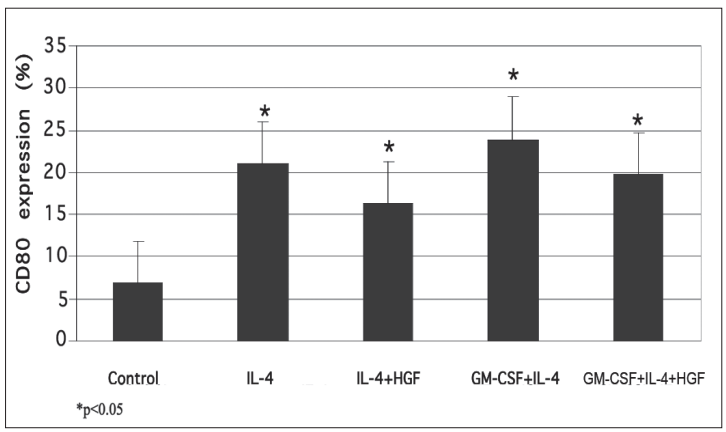

Figure 4 - The percentage of CD80 expression related to **Table 2** correlated with the aforementioned group comparisons.

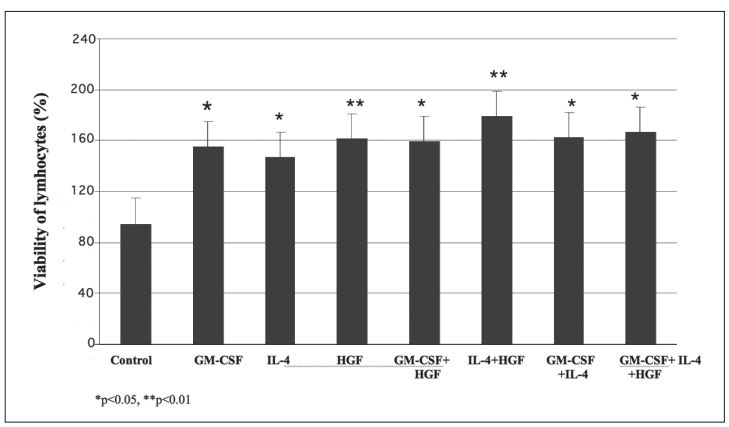

Figure 5 - The percentage of lymphocyte proliferation related to **Table 3** in correlation with the group comparisons.

and CD86 expressions increased in all groups (**Figure 3**), while CD14 expression increased only in GM-CSF group compared to control (p<0.05, p<0.01). Besides, CD83 expression increased in GM-CSF (p<0.05), IL-4 (p<0.05) groups and in cultures with IL-4+HGF (p<0.05) and more clearly in GM-CSF+IL-4, GM-CSF+IL-4+HGF groups (p<0.01). The CD80 expression increased in all IL-4 containing groups (**Figure 4**). However, CD1a expression increased only in GM-CSF+IL-4 and GM-CSF+IL-4+HGF groups as compared to control (p<0.01) (**Table 2**).

When the results were compared with regard to before and after the addition of TNF-α, it was noted that CD1a expression in GM-CSF+IL-4 and GM-CSF+IL-4+HGF groups (p<0.05) and CD83 expression in IL-4 group increased comparably when TNF-α was added into the culture medium (p<0.05). However, no significant changes were found in other surface or costimulatory molecules expressions before and after TNF-α stimulation.

Both the 5th and 7th day results showed that the DC description markers increased in cultures conditioned with various stimulants alone or in combinations among which the most efficient one being the GM-CSF+IL-4+TNF-α and its HGF supplemented combinations.

The results of lymphocyte stimulation tests with PPD-loaded DCs obtained on day 7 are presented in **Table 3**, and shows that lymphocyte proliferation was induced significantly in all groups as compared to control group (p<0.01, p<0.001) (**Figure 5**). Overall it was shown that HGF supplemented cultures exert some additive effects in relation to function of monocyte-

Table 2 - The percentage of antigen expressions in the groups on the 7th day after the addition of tumor necrosis factor-α.

Groups	CD14	CD1a	HLA-DR	CD80	CD83	CD86
Control	2.42 (0.3-3.7)	1.5 (0.15-2.8)	46.1 (45.1-68.4)	6.9 (3.1-12.8)	14.8 (9.6-15.6)	18.4 (14.5-48.3)
GM-CSF	7** (4.5-11)	1.3 (0.1-2.3)	97.5** (81-97.7)	15.1 (6.2-23.1)	26.5_a** (20.4-31.6)	71** (55-78)
IL-4	1.4 (0.5-2.8)	1.9 (0.4-19.4)	93.7** (79-97.4)	21* (12.1-50.4)	28.2_b** (21.6-33.1)	69** (54-71.5)
HGF	1.5 (1-15)	0.6 (0.4-2.7)	98** (78.6-98)	9.1 (3.5-22)	13.6_c (7-15.2)	56* (35.5-57)
GM-CSF+HGF	4.3 (0.15-5.1)	1.1 (0.15-9.6)	93.3** (76.4-96.2)	20.8 (5.5-23.5)	9_d (6.2-16)	51.8* (30.4-79)
IL-4+HGF	1.6 (0.2-2.9)	1.6 (0.3-6.8)	93.1** (71.8-96.8)	16.3* (8.9-22.7)	27.6_e** (21.2-48.6)	53.2* (44.5-63.2)
GM-CSF+IL-4	2.1 (0.2-3.7)	9** (4.25-13)	97.1** (76-97.9)	23.9* (12.3-32)	38.4_f** (27-53)	57* (31.2-67)
GM-CSF+IL-4+HGF	1.8 (0.6-4.9)	5.7** (3-12.6)	88.6** (86-97.3)	19.7* (9.1-40.1)	42.1_g** (26.2-63)	70.5* (37.8-71)

The data are represented as % of total cells in each well and indicates the median (min-max) of 5 independent experiments.
*p<0.05, **p<0.01 (comparison of groups with control). a-f, b-f, a-g, b-g, a-d, c-e, c-g, d-g.
Each double letter represents the intergroup comparisons like a-f as CD 83 comparison between GM-CSF and GM-CSF+IL-4 group, b-f as IL-4 and GM-CSF+IL-4 groups, a-g as GM-CSF and GM-CSF+IL-4+HGF groups, b-g as IL-4 and GM-CSF+IL-4+HGF groups (p<0.05), a-d as GM-CSF and GM-CSF+HGF groups, c-e as HGF and IL-4+HGF groups, c-g as HGF and GM-CSF+IL-4+HGF groups, d-g as GM-CSF+HGF and GM-CSF+IL-4+HGF groups (p<0.01). GM-CSF - granulocyte-macrophage colony stimulating factor, IL-4 - interleukin-4, HGF - hepatocyte growth factor.

Table 3 - The effects of purified protein derivative-loaded dendritic cell groups stimulated with various cytokines and their combinations on the percentage of lymphocyte proliferation on 7th day (MTT test , % proliferation of the lymphocytes).

Control %	GM-CSF	IL4	HGF	GM-CSF+HGF	IL4+HGF	GM-CSF+IL4	GM-CSF+IL4+HGF
94.5 (68-121)	155* (113-191)	147* (81-191)	161** (137-199)	159** (132-305)	178.5** (141-244)	162** (138-212)	166.5** (138-262)

*p<0.01, **p< 0.001 (groups compared with control). The figures are the median (min-max) of data from 5 independent experiments
GM-CSF - granulocyte-macrophage colony stimulating factor, IL-4 - interleukin-4,
HGF - hepatocyte growth factor, MTT - methyl tetrazolium

derived DCs. But HGF alone does not seem to augment monocyte-derived DC proliferation and maturation significantly (**Table 3**).

Discussion. Dendritic cells can be cultured from bone marrow, peripheral blood and cord blood CD34+ cells, but can also be generated from peripheral blood monocytes. The latter cell type can convert to DCs in appropriate culture conditions supported with a variety of cytokines among which GM-CSF, IL-4 and TNF-α combinations are known to be the most basic ingredients to use individually in combinations in the DC proliferation and differentiation.[16-19]

In our study, the culture of monocytes in the presence of GM-CSF resulted in HLA-DR, CD83 and CD86 surface molecule expressions indicating DC differentiation. However, it was noted that along with these markers CD14 expression also increased, suggesting that GM-CSF induces both the monocytic and DC colonies. The GM-CSF is reported to induce monocytes to antigen presenting macrophage-like cells alone,[20] and also is efficient on the proliferation and survival of DC precursors.[21,22] In addition to GM-CSF, IL-4 is also a very important cytokine in the differentiation of DCs from monocytes, acting as a suppressing factor for the overproliferation of macrophages, and thus, resulting in DC differentiation and maturation. With the addition of IL-4 into monocyte cultures, CD14 expression decreases as a sign of DC conversion of monocyte precursors instead of differentiation of macrophages.[23] We also observed that IL-4 alone increased HLA-DR expression compared to the control group and it also augmented CD83 expression when combined with GM-CSF, but decreased CD14 expression as compared to GM-CSF alone. Moreover, combination of IL-4 with HGF alone or its combination with GM-CSF+HGF increased CD83 expressions. Overall, these findings suggested the importance of IL-4 in DC differentiation from monocytes.

Given that the monocyte cultures with HGF alone increased HLA-DR expression without any effect on CD14 expression but cells with DC-like morphology may suggest HGF as an effective agent for DC differentiation. However, weak expressions of CD1a, CD80, CD83 and CD86 may imply that HGF alone is not so important in the monocytic DC proliferation. In other studies, with a short period of peripheral monocyte cultures with HGF, it was reported that the antigen presenting efficiency as well as HLA class I expression increased, but expressions of other specific costimulatory molecules such as CD80 and CD86 did not change.[9,10]

In our study, the CD1a expression was lower in comparison to other DC specific costimulatory molecules (CD80, CD83, and CD86) in all cytokine groups. In similar studies, it was shown that the existence or nonexistence of CD1a expression is strictly related to culture medium. Moldenhauer et al[24] compared the effect of autologous plasma or fetal bovine serum inclusion in 2 different culture mediums on the CD1a expression during DC proliferation from peripheral monocytes. In the fetal bovine serum supplemented medium group CD1a expression increased, and this was probably due to bovine antigenic stimulation. On the other hand, the culture medium supplemented with autologous plasma decreased CD1a expression. However, from the point of antigen presenting capacity, no difference was seen between the medium supplemented with bovine serum or autologous plasma.[24] Conversely, Xia et al[25] report that CD1a+ DCs could stimulate CD4 T lymphocytes more efficiently. These reports support our data showing that low expression levels of CD1a in cultures maintained in the presence of all cytokine groups, probably related to serum free culture medium, which contained no extrinsic antigenic stimulation.

Overall, our results and other studies suggest that the most important cytokine during DC formation on day 5 was IL-4, and the most appropriate combination was GM-CSF+IL-4 and GM-CSF+IL-4+HGF. HGF alone did not have significant effect on DC formation.

With regard to the role of TNF-α in DC cultures, it was seen that as expected CD80, CD83, CD86 and especially CD1a expressions were augmented in the groups showing DC maturation. Thus, it was evident that TNF-α plays an additive role in the presence of IL-4. In some studies, CD80 is reported to be more specific for DC, and unlike CD80 the expression of CD86 is expressed by GM-CSF induction.[26,27] Chen et al[28] reported that the expressions of costimulatory molecules on DCs increase in the presence of TNF-α during maturation, whereas the phagocytic capacity of DCs as an additional marker of maturation declines. Büchler et al[29] reported that a variety of cytokine combinations had similar effects on DC development, but DC development from peripheral blood monocytes was strongly related to TNF-α added IL-4+GM-CSF combination on 5th day culture.

Taken together, the data presented above suggested to us that the most efficient cytokine combination in the DC development and maturation in our model was obtainable in culture conditions supported with a cocktail of GM-CSF+IL-4+ TNF-α or GM-CSF+IL-4+HGF+TNF-α. As part of our ongoing studies, we reported previously that HGF in comparison with GM-CSF is a better stimulant for DC differentiation of CD34+ cells harvested from human bone marrow samples,[11] however, our current findings suggest that HGF may not be so effective on DC differentiation from peripheral blood monocytes.

In the second step of the present study, we investigated the antigen presentation capacity of DCs loaded with PPD, and showed that PPD-loaded DCs exerted significantly higher lymphocyte proliferation effect compared to the control group. It was of note that lymphocyte proliferation seemed to be augmented whenever HGF was added in the cultures alone or in combination with other cytokines. We believe that the functional capacity of DCs generated in the presence of HGF is well improved. Using an IL-3 dependent murine myeloid cell line transferred with HGF receptor c-met, it was shown in a study that the combination of IL-4 and HGF had a synergistic effect on the proliferation capacity of the cells.[30] In other studies, the antigen presenting capacity of peripheral blood monocytes but not cord blood monocytes was shown to increase in short time cultures in the presence of HGF.[9,10] Kurz et al[12] reported that bone marrow DCs obtained from C57BL/6 or BALB/c mice carried HGF receptor c-met, and that activation of c-met receptor resulted in an increase in the adhesion of DCs to laminin, but antigen presenting functions were not affected. However, in contrast to our findings, Okunishi et al[31] recently reported that treatment with HGF both in vitro and in vivo potently suppressed DC functions such as Ag-presenting capacity, leading to down-regulation of Ag-induced Th1 and Th2 type immune responses. Rutella et al[32] in a very recent paper, reported that monocytes cultured with HGF differentiated into accessory cells with DC features and secondary stimulation of HGF-DC-primed CD+ cells with immunogenic DCs differentiated with GM-CSF and IL-4 from monocytes resulted in T cell proliferation.

In summary, our findings suggested that HGF supplemented cultures exert some additive effects in relation to function of monocyte-derived DCs. However, HGF alone does not seem to augment monocyte-derived DC proliferation and maturation significantly.

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