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A novel phosphoprotein is induced during bone marrow commitment to dendritic cells

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Abstract

Dendritic cells (DCs) play an important role in vertebrate immunity, but little is known of the molecular events associated with their development from bone marrow (BM). This report describes induction of a signature protein marking BM commitment to DCs. Using a standard procedure, DCs were generated from BM by cultivation in vitro. Appropriate phenotypic monitoring was done primarily by immunofluorescence, and polyclonal antibody reagents were developed against immature DC lysates. Using one specific antibody reagent, we identified, purified, and sequenced a unique cytosolic phosphoprotein DP58 that occurs within 30 min during BM commitment to DCs. Its sequence matches with a computationally predicted Riken cDNA (GenBank Accession No. XP_138799), and a specific anti-DP58 peptide antibody was developed for further characterization. The study suggests that DP58 induction signals distinct pathway(s) leading to early DC progenitors that may be generated and propagated for a short period in vitro.

Keywords: Bone-marrow differentiation; Differentiation marker; DP58 protein; Novel protein; Dendritic cell progenitors; Western blot

It is now widely recognized that DCs have a remarkable ability to help induce full-blown immune responses [1,2]. This has spurred tremendous interest in DC-based vaccinology as well as in DC biology, particularly in their differentiation from bone marrow-derived progenitors [3–8].

Pathogenic antigens are known to transform immature DCs into their mature forms, and the latter interact with the lymphocytes [8]. These differentiation events have been amply demonstrated by functional and phenotypic studies and led to the discovery of various DC subpopulations [1,4]. However, neither the molecular milieu nor the progenitor precursors associated with the developmental origin of all DC subpopulations are fully understood. Controversies exist about the physiological derivation of various DC subpopulations, particularly with regard to development of myloid and lymphoid DC subtypes [3,4]. It is however, increasingly evident that the early DC progenitors are noncommittal with regards to their myloid or lymphoid orientation. del Hoyo et al. [9] have recently described a common CD11c+, MHC-II$^-$ DC-committed precursor population in murine blood that can fully reconstitute splenic DCs, but is devoid of lymphoid or myeloid differentiation potential. Amico and Wu [10] have also identified common early hematopoietic progenitors of mouse DC expressing high levels of Flt3.

This existence of a common multipotent progenitor DC (pDC) implies that the commitment to DC occurs early in response to a specific cytokine microenvironment. Cytokines, GM-CSF in particular, function as the primary growth factor promoting mouse BM differentiation into DC, whereas GM-CSF and IL4 are both required for human hematopoietic stem cell (HSC) [11]. This ability of GM-CSF to induce mouse BM

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differentiation in vitro led us to develop specific antibody reagents for identifying molecular markers associated with the early events in DC development. In this report, we describe a novel cytosolic 58 kDa phosphoprotein associated with early DC progenitors when there is no clear-cut evidence of any specific DC subtype. Since this protein is induced during BM differentiation into common uncommitted early pDC [9,10], we designated it as DP58 after DC progenitors. The sequence of DP58 matches with no known protein sequences in the NCBI database, but it has been positively identified with a Riken cDNA in the GenBank with a Z score of 2.43 using peptide mass fingerprinting. To our knowledge, this is the first report implicating a cytosolic phosphoprotein as one molecular hallmark of early DC progenitors.

Materials and methods

Mice. BALB/c mice from Harlan Sprague–Dawley (Indianapolis, IN) were bred in the animal facility of Indiana State University. The University Animal and Use Committee (ACUC) approved all animal experiments.

Cells and antibodies. Phenotyping was done using fluorescent monoclonal antibodies to CD11b, CD11c, MHCII, CD117, B220, CD86, and CD80 (all from eBioscience, USA), DEC-205 (Serotec, UK). Anti-phosphotyrosine (Zymed, USA) was used for biochemical characterization. Other materials included goat anti-rabbit-Ig HRP, rabbit anti-mouse-Ig (ICN, USA), and Western blot reagents were purchased from Pierce (USA).

Dendritic cells generation [5]. Bone marrow cells were prepared by flushing off the femurs and tibiae of BALB/c mice. Cells were cultivated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 10 ng/ml recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF), (eBioscience and Peprotech, USA) for 6 days at 37 °C in 5% CO_2. Non-adherent cells were removed on day 2 and 4 of culture, and fresh IMDM plus GM-CSF were added. DCs generated were phenotyped by flow cytometry and on a regular basis by fluorescent microscopy.

DC6 lysates preparation for antisera development. An emulsion containing equal volume of complete Freund's adjuvant (CFA) and DCs ($1–3 \times 10^7$ cells/ml) in 0.02% SDS solution in PBS was used to immunize three rabbits intradermally. Every 10 days they were bled and boosted with DC lysates emulsified in incomplete Freund's adjuvant (IFA).

Preparation of specific rabbit antibody reagents by repeated adsorption, salt fractionation, and protein A chromatography. The sera obtained after multiple immunizations were adsorbed primarily on splenocytes, liver tissues, dendritic cells, and myeloma X63-Ag8.653 cells. Further adsorption was done, as needed, with fresh or formalin-fixed BM cells. This was followed by 50% saturated ammonium sulfate precipitation, dialysis, and protein A chromatography. One specific antibody reagent thus prepared was initially used to identify novel protein DP58 in dendritic cell progenitors.

Generation of progenitor BM cells. BM cells (1×10^7) were incubated with adsorbed rabbit antisera for 4 h on ice to generate BM4 cells, which were then treated with 1 ml of a lysis buffer [12] containing 0.5% NP40 and 0.5% MEGA9 plus 10 μl of protease inhibitor, and left on ice for 30 min before analysis by SDS–PAGE.

SDS–PAGE and Western blot. Lysates prepared according to Elvin et al. [12] were subjected to 12.5% SDS–PAGE [13], followed by Western blotting on nitrocellulose. Primary antibody was rabbit antisera (adsorbed with BM-fresh) and secondary antibody was commercially available antibody goat anti-rabbit-Ig HRP. Super Signal West Pico chemiluminescent substrate (Pierce, USA) was used to visualize proteins on films.

Immunoprecipitation. This was done as described [13,14] using protein A to isolate specific immune complex formed by mixing cell lysates with the adsorbed rabbit antibody reagent. The specific protein band obtained by SDS–PAGE band was isolated and sequenced by peptide mass fingerprinting at the Proteomics Core Laboratory of Dr. Wang Mu, Indiana University School of Medicine.

Rabbit antisera against DP58 peptide. This was done using a conjugate of keyhole limpet hemocyanin (KLH) with a peptide, KMVKYL LENSADPNIQDKSG shown in Fig. 2, as the immunogen (100 μg/injection). This was administered intradermally as an emulsion of the conjugate initially with CFA and later with IFA, and the rabbits, were bled at 10 days' interval. Purification was carried out by salt fraction using 50% saturated ammonium sulfate followed by affinity chromatography on KLH–Sepharose column. Unbound fraction was the source of anti-DP58 antibody.

Results

Fifty-eight kilodalton protein identified as DP58

To identify DP58, the freshly harvested BM cells were treated with the adsorbed rabbit anti-DC antisera reagent on ice for 4 h. This reagent was protein A-purified after repeated adsorption on BM-derived immature DCs and mouse myeloma as described in Materials and methods. BM cells obtained after 4 h incubation with the reagent, termed BM4 cells, were exposed to a lysis buffer, and the lysates were subjected to SDS–PAGE and Western blotting. We used for Western blot the same specific antisera reagent but adsorbed additionally on fixed BM cells. Controls were run using lysates of BM cells that were treated with normal rabbit serum, rabbit anti-IgG or anti-CD11c monoclonal antibodies. The results in Fig. 1A show that a 58 kDa protein (DP58) was detectable only in BM4 cell lysates using rabbit antisera reagent at 1:200,000 dilution. In contrast, the lysates of the fixed or fresh BM cells (undifferentiated) exhibited no DP58 protein, even with a higher concentration of the antibody reagent. Since DP58 protein was discerned only in the lysates of BM4 cells, but not of fresh or fixed BM cells, this suggests that this protein was induced as a cytosolic protein (Fig. 1A).

Next we determined the time course for induction of DP58. The results (Fig. 1B) indicate that the induction was detectable in BM cells within 30 min, although the protein band was most discernible after 4 h in BM4 cells. Clearly, this adsorbed rabbit anti-DC reagent facilitates differentiation of BM cells, possibly through cross-linking of a cell surface protein. To explore this possibility and to remove any antibody directed to any putative cell surface protein, we further adsorbed the purified anti-DP58 reagent repeatedly on formalin-fixed freshly isolated BM cells, and then used it to expose fresh and live BM cells for 4 h. Interestingly, this adsorption of anti-DC reagent with fixed BM cells totally prevented

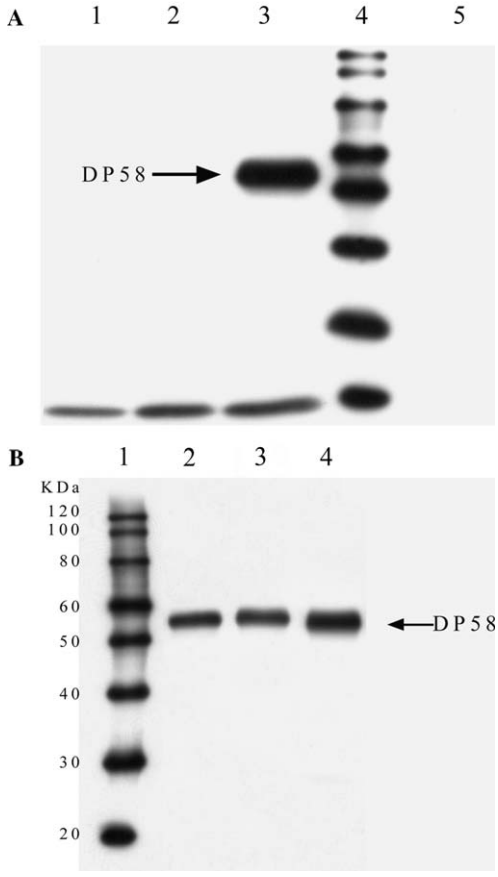

Fig. 1. (A) Expression of DP58 protein in differentiating BM cell lysates. Lane 1, BM fresh; lane 2, BM after 4-h treatment with normal rabbit serum; lane 3, BM after 4-h treatment with the rabbit specific antibody reagent; lane 4, protein standard; and lane 5, formalin-fixed BM after treatment with the rabbit specific antibody reagent. (B) Temporal expression of DP58 protein in BM cell lysates. Lane 1, protein standard; lane 2, BM after 30 min treatment with the rabbit specific antibody reagent; lane 3, BM after 60 min treatment with rabbit specific antibody reagent; and lane 4, BM after 4-h treatment with the rabbit specific antibody reagent.

fresh BM from differentiating into BM4 cells, but it still was capable of detecting DP58 protein in the lysates of existing BM4 cells (Fig. 1B). This indicates the presence of a cell surface molecule on fresh BM cells, which is needed for cytosolic DP58 induction. This observation was corroborated using anti-DP58 peptide antibody as described later (Fig. 3B). We are currently focusing on identification of this putative surface protein.

DP58 protein is induced during BM differentiation into DCs

To determine whether the BM4 cells (generated following incubation with rabbit anti-DC antisera) could differentiate into DCs, we cultivated them in GM-CSF for 6 days. No other cytokine such as IL4 displayed the ability to generate mouse DCs, as was also shown by others [11], and BM4 cells did not differentiate into DCs in the absence of GM-CSF. Phenotypic analyses (Table 1) revealed that BM4 cells differentiated into DCs that closely resembled $CD8\alpha^-$ DCs generated from fresh BM using only GM-CSF. However, while neither DCs expressed DEC-205, the DCs generated from BM4 cells had a very low expression of B220 marker. Since it was difficult to categorize these cells into either lymphoid or myeloid lineages, we consider BM4 cells as undifferentiated pDCs.

Isolation and sequencing of DP58 from BM-derived cell lysates

To determine if DP58 was a novel protein induced during BM differentiation into DCs, we purified it by immunoprecipitation and isolated the protein band following SDS–PAGE. This was then subjected to controlled tryptic digestion and the sequence of the peptides generated was positively identified with a computationally predicted Riken cDNA (NCBI database Accession No. XP_138799) (Fig. 2).

Next we addressed whether this novel cytosolically induced differentiation-related protein is a glyco- or phosphoprotein. While the periodic acid-Schiff staining for glycoprotein proved negative, the Western blotting (Fig. 3A) using a commercial anti-phospho-tyrosine antibody reagent indicated that DP58 was a phosphoprotein induced during BM differentiation.

Table 1
Phenotypic characteristics of different lysates

Cells	Anti-CD11c	Anti-CD11b	Anti-CD8α	Anti-MHCII	Anti-CD80	Anti-CD86	Anti-CD117	Anti-B220	Anti-DEC-205
BM-F	–	±	–	–	–	–	–	±	–
DCs	++	++++	–	++++	–	–	++	++	–
BM4[a]	–	++	–	–	–	–	–	+	–
BM-24[b]	–	++	–	–	–	–	–	+	–
DCs (derived from BM4)[c]	++	++++	–	++++	–	–	++	±	N/d
Spleen	++	++	++	++	–	–	++	++	–
2C3	–	–	–	–	–	N/d	N/d	N/d	N/d

BM-F stands for fresh bone marrow cells.
[a] BM after 4-h treatment with the rabbit specific antibody reagent.
[b] BM after 24-h treatment with the rabbit specific antibody reagent.
[c] BM4 cells cultivated in GM-CSF for 6 days. 2C3 [13], tumor cell line used as a negative control and spleen cells used as the positive control.

MDEGSEVSTDGNSLIKAVHQSRLRLTRLLLEGGAYINESNDRGETPLMIACKTKH
VDQQSVGRA**KMVKYLLLENSADPNIQDKSG**KSALMHACLERAGPEVVSLLLKS
GADLSLQDHSGYSALVYAINAEDRDTLKVLLSACQAKGKEVIIITTAKSPSGRHT
TQHHLNMPPADMDGSHPPATPSEIDIKTASLPLSYSSETDLTLFGFKDKELCGGSD
NTWDPDSPPRKPVIATNGPKLSQAPAWIKSTPSLKHQARVASLQEELQDITPEEEI
AYKTNALALSKRFITRHQSIDVKDTAHLLRAFDQVNSRKMSYDEINYHSLFPEGS
QTSVEIPTDRDPDSNQIFASTLKSIVQKRNSGANHYSSDSQLAEGVTPPTVEDGKA
AKKKIFAPSPSLLSGSKELVEPAPPGPLSRRNHAVLERRGSGAFPLDHSLAQSRPG
FLPPLNVNPHPPITDIGVNNKICGLLSCGQKALMPTAPIFPKEFKTKKMLLRRQSL
QTEQIKQLVNF

Fig. 2. Amino acid sequence of DP58 determined by peptide mass fingerprinting. The underlined sequence corresponds to the DP58 peptide, which was to raise specific antibody.

Furthermore, to identify pDCs and study DC differentiation, we developed an anti-DP58-peptide reagent using the sequence underlined in Fig. 2. We used this antibody reagent to detect the presence of DP58 in unstimulated and stimulated BM cells as well as in DCs. The results show that DP58 was undetectable in DCs (lane 2, Fig. 3B), possibly because of low levels of pDCs cells expressing of DP58. It is also evident (Fig. 3B) that anti-peptide antibody recognized DP58 only in differentiating BM cells such as BM4 cell lysates (lane 4), and it could not stimulate fresh BM cells to differentiate (lane 3). This was expected in view of the results in Fig. 1B, leading us to conclude that DP58 is the cytosolic marker of early progenitor DCs, possibly involved in signal transduction.

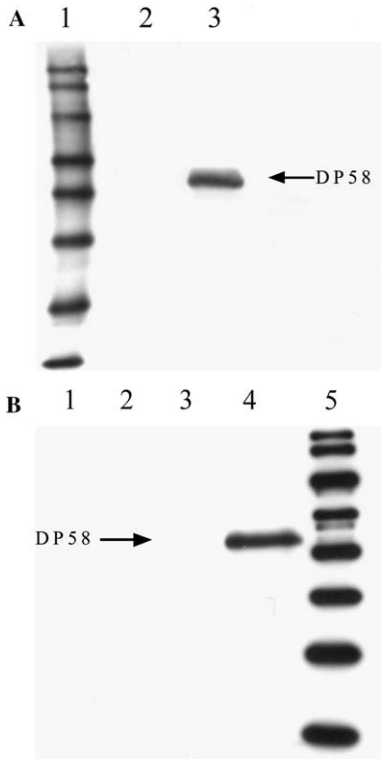

Fig. 3. (A) DP58 protein as phosphoprotein. Lane 1, protein standard; lane 2, BM fresh lysates; lane 3, lysates of BM after 4-h treatment with the rabbit specific antibody reagent. (B) Detection of DP58 using anti-DP58 peptide antibody on Western blot. Lane 1, BM fresh lysates; lane 2, DC lysates; lane 3, lysates of BM after 4-h treatment with the rabbit anti-DP58 peptide antibody; lane 4, BM after 4-h treatment with the rabbit specific antibody reagent; and lane 5, protein standard.

Discussion

This study is the first report on a novel protein DP58 that was identified using a polyclonal anti-DC antibody reagent. The reagent was specifically prepared from antisera raised against the lysates of immature DCs that were generated following 6-day cultivation of mouse BM cells in GM-CSF [5]. Before use, DCs were carefully phenotyped using fluorescent anti-CD11c, anti-CD11b, anti-CD8α, anti-MHC-II, anti-CD80, anti-CD86, anti-CD117, anti-B220, and anti-DEC205 monoclonal antibodies (Table 1).

We reasoned that this cultivation of BM cells in GM-CSF should yield not only DCs, but also some undifferentiated BM cells that are at intermediate stages of development. The cell lysates from such heterogeneous DC-enriched (over 95%) population would likely contain various immunogenic molecules in a pecking order of immunogenicity derived from all cell types. This explains why our polyclonal antisera recognize not only the antigenic components of mouse DCs but also others associated with the differentiating BM cells. Importantly, the specific reagent prepared from these antisera readily detects DP58 protein only in differentiating BM cells within 30 min, and these differentiating BM cells develop into DCs when they are exposed to GM-CSF. Moreover, this induction of DP58 happens long before DCs emerge from BM cells following 6-day cultivation in GM-CSF. It suggests that DP58 occurs primarily in pre-DC population, and is highly immunogenic since the contribution of pre-DC population would be minimal in the DC lysate immunogen used to raise the antisera.

Identification of DP58 with a Riken cDNA in the database certainly advances our ability to identify many hypothetical proteins. The complex process of bone marrow differentiation into specific cell lineages such as dendritic cells involves numerous molecular interactions. Specific cytokines such as GM-CSF is known to drive mouse BM-associated HSC to DC-specific development and give rise to committed progenitor stem cells. Since GM-CSF or our specific

antibody reagent can both cause BM cells to differentiate via induction of DP58 phosphoprotein, and since the antibody reagent in particular detects DP58 in cell lysates within 30 min of BM cultivation as mentioned earlier, this is suggestive of ongoing intracellular events. Furthermore, the fact that anti-DP58 peptide antibody generated based on the sequence of DP58 also detects this protein only in lysates of differentiating but not fresh undifferentiated BM cells or DCs lends further support to this contention. This also suggests that this protein is induced as a result of activation and that this activation is possibly coordinated through a phosphorylation event. Studies are underway to assess the role of DP58 in DC development.

Furthermore, to our knowledge, there is no specific method to generate and propagate early progenitors. On the basis of phenotypic studies, the BM4 cells reported here certainly fit the description of some of the early DC progenitors [3,4]. Since these cells can be cultivated for a short period in the absence of GM-CSF, it provides us the ability to clone or enrich and functionally characterize these DC progenitors from these cells. Further investigation is in progress to elucidate the physiological role and significance of BM4 cells in the context of DP58 induction during BM differentiation.

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References

[1] J. Banchereau, F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, K. Palucka, Immunobiology of dendritic cells, Annu. Rev. Immunol. 18 (2000) 767–811.
[2] M.F. Lipscomb, B.J. Masten, Dendritic cells: immune regulators in health and disease, Physiol. Rev. 82 (2002) 97–130.
[3] M.G. Manz, D. Traver, T. Miyamoto, I.L. Weissman, K. Akashi, Dendritic cell potentials of early myloid and lymphoid progenitors, Blood 97 (2001) 3333–3341.
[4] C. Ardavin, Origin, precursors and differentiation of mouse dendritic cells, Nat. Rev. Immunobiol. 3 (2003) 582–591.
[5] L. Krishnan, S. Sad, G.B. Patel, G.D. Sportt, The potent adjutant activity of archaeosomes correlates to the recruitment and activation of macrophages and dendritic cells in vitro, J. Immunol. 166 (2001) 1885–1893.
[6] S. Grabbe, S. Beissert, T. Schwartz, R.D. Granstein, Dendritic cells as initiators of tumor immune responses: a possible strategy for tumor immunotherapy? Immunol. Today 16 (1995) 116–120.
[7] J. Bubenik, Genetically engineered dendritic cells based cancer vaccine, Int. J. Immunol. 18 (2001) 475–478.
[8] G. Schuler, B. Schuler-Thuner, R.M. Steinman, The use of dendritic cells in cancer immunotherapy, Curr. Opin. Immunol. 15 (2003) 138–147.
[9] G.M. del Hoyo, P. Martin, H.H. Vargas, S. Ruiz, C.F. Arias, C. Ardavin, Characterization of a common precursor population for dendritic cells, Nature 415 (2002) 1043–1047.
[10] A.D. Amico, L. Wu, The early progenitors of mouse dendritic cells plasmacytoid predendritic cells are within the bone marrow hematopoietic precursors expressing Flt-3, J. Exp. Med. 198 (2003) 293–303.
[11] J.I. Mayordomo, T. Zorina, W.J. Storkus, et al., Bone marrow-derived dendritic cells serve as adjuvants for peptide-based antitumor vaccines, Stem cells 15 (1997) 94–103.
[12] J. Elvin, C. Potter, T. Elliott, V. Cerundolo, A. Townsend, A method to quantify binding of unlabeled peptides to class I MHC molecules and detect their allele specificity, J. Immunol. Methods 158 (1993) 161–171.
[13] S.K. Ghosh, P. Patnaik, R.B. Bankert, Expression of μ and γ membrane forms of immunoglobulin segregate in somatic cell hybrids, Mol. Immunol. 24 (1987) 1335–1343.
[14] S. Gallagher, S.E. Winston, A. Steven, J.G.R. Hurrell, Isolation and analysis of proteins, Curr. Protoc. Immunol. 4 (2000) 1–3.