International Immunopharmacology 21 (2014) 255–260

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

The novel role of thymopentin in induction of maturation of bone marrow dendritic cells (BMDCs)

Yang Wang [a,*], Yan Cao [c], Yiming Meng [c], Zhenyu You [b], Xiaowei Liu [a], Zhihong Liu [a]

[a] Department of Emergency, No. 1 Affiliated Hospital, China Medical University, No. 155, North Nanjing Street, Shenyang 110001, China
[b] Department of Oncology, 202 Army Hospital, PLA, No. 5, Guangdong Street, Shenyang 110812, China
[c] Department of Immunology, School of Basic Medical Science, China Medical University, No. 92, North Second Road, Heping District, Shenyang 110001, China

ARTICLE INFO

Article history:
Received 9 May 2014
Accepted 12 May 2014
Available online 23 May 2014

Keywords:
Thymopentin
Bone-marrow-derived dendritic cells
Maturation
T-cell response

ABSTRACT

Thymopentin is an immune-modulating peptide that can stimulate cellular immune responses and has been used in many immune handicapped cases [1]. However, despite documented reports proving its efficacy in immunoregulation, there have been no reports, as yet, concerning its impact on the maturation and function of dendritic cells (DCs). In this study, we analyzed the effects of thymopentin on the detailed regulation of maturation of murine bone-marrow-derived DCs (BMDCs). The phenotypic and structural maturation of BMDCs was confirmed by transmission electron microscopy (TEM) and flow cytometry (FCM). The functional maturation was confirmed by an acid phosphatase (ACP) activity test, FITC-dextran bio-assay, test of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), labeled $CD4^+$T cell proliferation and enzyme-linked immunosorbent assay (ELISA). We determined that thymopentin up-regulated the expression of CD40, CD80, CD86, CD83, and MHC II molecules on BMDCs, down-regulated phagocytosis of BMDCs, increased BMDCs driven $CD4^+$T cell proliferation, and enhanced BMDC production of IL-12 and TNF-α. Therefore, we concluded that thymopentin highly induces BMDC maturation and intensifies DC/T-cell pathways. These data also provide direct evidence and rationale concerning the potential clinical use of thymopentin in various immune handicapped cases and suggest that thymopentin should be considered as a potent adjuvant for DC-based vaccines.

1. Introduction

Synthetic thymopentin is an immune enhancing peptide of a five-amino-acid fragment of the thymic hormone thymopoietin (residues 32 to 36) that reproduces the immunomodulatory activity of the complete hormone, with structure Arg-Lys-Asp-Val-Tyr and an approved drug in China [1,2]. It also has demonstrated efficacy in preclinical studies [3,4] and was subsequently shown to enhance response to vaccinations in human [5]. Additional data also document that thymopentin improves immunity through T-cell, NK cell, and macrophage activation [6,7,8]. Ever since its discovery, thymopentin has been used in many clinical situations, including in the treatment of patients with immune deficiencies, autoimmune diseases, cancer, and in other immune-handicapped situations [9,10,12,13,14].

Following the discovery of dendritic cells (DCs), subsequent research has changed the science of immunology and interlinked its fate with medicine [15,16,17]. These previously unknown cells are now recognized as cells that both create and curtail immunity. DCs are critical in mounting cellular immune responses. As sentinels, they patrol the body, seeking out foreign invaders, so called antigens, whether these are bacteria, viruses, or toxins [18,19]. After capturing the antigens, DCs convert them into smaller pieces and then display them on their cell surfaces to be recognized by T-cell and in this way DCs initiate T-cell responses. However, so far little data exist concerning thymopentin's possible impact on the detailed modulation of BMDCs, and we do not know whether thymopentin could have any influence on BMDCs. Due to the ever increasing importance of DC and thymopentin as an additive in cancer therapy, we performed the investigation.

2. Materials and methods

2.1. Reagents

The thymopentin used in our experiments is a product of the Huayuan Pharmaceutical Co., Shanghai, China. We tested a sample with tachypleus amebocyte lysate to determine its pyrogen-free state, according to the China pharmacopeia. We tested a range of concentrations of thymopentin from 7.28 μg/ml to 0.5 mg/ml on the proliferation of BMDCs in vitro, and we found the most effective concentration to be

Abbreviations: BMDCs, bone-marrow-derived dendritic cells; MACS, magnetic activated cell sorting; LPS, lipopolysaccharide; TEM, transmission electron microscopy; APCs, antigen presenting cells; ACP, acidic phosphatase; FCM, flow cytometry; DAB, 3,3′-diaminobenzidine; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester.

* Corresponding author. Tel./fax: +86 24 83282934.
E-mail address: wangyangshan1959@163.com (Y. Wang).

http://dx.doi.org/10.1016/j.intimp.2014.05.011
1567-5769/

31.25 μg/ml. Based on the data, we used a concentration of 31.25 μg/ml. The monoclonal antibodies (mAbs) used in our experiments include fluorescein isothiocyanate (FITC)-conjugated anti-CD40, PE-anti-MHC-II, PE-anti-CD80, PE-anti-CD86, and PE-anti-CD83, which were all products of eBioscience (San Diego, CA) and BD Pharmingen (Franklin Lakes, NJ). The ELISA kits for IL-12 and TNF-α were purchased from eBioscience. Lipopolysaccharide (LPS) made from *Escherichia coli* (serotype 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine cytokines of interleukin 4 (IL-4) and granulocyte macrophage-colony stimulating factor (GM-CSF) were products of PeproTech Inc. (Rocky Hill, NJ). Other routine reagents and solvents in our laboratory were purchased from Sigma-Aldrich or BD Pharmingen.

2.2. The preparation of BMDCs

The animals were treated according to the Guide for the Care and Use of Laboratory Animals of China Medical University. BMDCs were prepared according to the method documented previously [11]. Bone marrow cells obtained from the femurs and tibias of female C57BL/6 mice (4–6 weeks old) were depleted of red cells with lyses buffer solution. Approximately 10^7/ml cells were grown in 24-well plates containing RPMI 1640 medium enriched with 10% fetal bovine serum (FBS), 10 ng/ml IL-4, 10 ng/ml recombinant murine GM-CSF, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. After the culture for 4 h, non-adherent cells in the culture were removed and replaced with fresh medium. On day 7 of culture, the $CD11c^+$ BMDCs were purified using anti-CD11c-coated magnetic beads and the auto-MACS system (Miltenyi Biotec, CA, USA). Purity of the sorted cells was checked with FCM finally (>90% for $CD11c^+$ cells).

2.3. BMDC structural changes shown under transmitted electron microscopy (TEM)

The $CD11c^+$ BMDCs incubated with 31.25 μg/ml thymopentin for 48 h were centrifuged down, resuspended in 0.5 ml 0.05 M pH 7.2 PBS, and fixed over-night in 2.5% glutaraldehyde, subsequently by 1% osmium tetroxide, dehydrated in ethanol and embedded in epon. Sections were cut on a Reichert–Jung Ultracut E and stained with uranyl acetate and lead citrate, and, finally, the sample was observed for structural changes under TEM (JEOL JEM-1200 EX) [15].

2.4. Changes of key membrane molecules on BMDCs

The $CD11c^+$ BMDCs co-cultured in presence of 31.25 μg/ml thymopentin for 48 h were collected in order to examine them for changes to key membrane molecules. The BMDCs were rinsed with PBS twice and combined with anti-CD40, anti-CD80, anti-CD86, anti-CD83, and anti-MHC II at 4 °C for 20 min. After thorough rinsing, the labeled BMDCs were analyzed with use of FACS Calibur (Becton Dickinson, San Diego, CA).

2.5. Phagocytosis change confirmed with FCM

$CD11c^+$ BMDCs were treated with 31.25 μg/ml thymopentin at 37 °C for 48 h, then 100 μl FITC-dextran (40,000 D) 28–30 was added at 4 °C for 2 h, 37 °C for another 1 h, and, then, the sample underwent FACS Calibur (Becton Dickinson, San Diego, CA) to reconfirm phagocytosis.

2.6. Change of ACP activity

The number of $CD11c^+$ BMDCs treated with 31.25 μg/ml thymopentin for 48 h was adjusted to 1×10^6/ml. ACP activity inside the BMDCs was assayed by the phenol-4-AAP (amino anti-pyrine) method combined with ACP testing kit (Jiancheng Bio-engineering Institute of the South) by measuring absorbance at OD 520 nm (A_{520}).

2.7. CFSE test

To detect the proliferation of $CD4^+$T cells driven by BMDCs treated with thymopentin, 2×10^5/ml BMDCs after treatment with thymopentin for 72 h were co-cultured with CFSE labeled murine splenocytes (10^6/ml with 0.5 μl CFSE) at ratios of 1:1, 1:5, 1:10, and 1:100 for three days according to the instruction manual in CFSE testing kit (BioLegend). Finally labeled mAb to $CD4^+$T cell was added to the culture and was let to undergo FCM check.

2.8. Secretion of IL-12 and TNF-α

The $CD11c^+$ BMDCs were incubated with 31.25 μg/ml thymopentin for 96 h and then collected to detect levels of released IL-12p70 and TNF-α, with an ELISA kit by determining absorbance at 450 nm (A_{450}).

2.9. Statistical analysis

All data obtained above were processed using statistical program SPSS (Statistical Package for Social Sciences, Version 16.0) for Windows. The variables were presented as mean ± SE and $p < 0.05$, and significant differences were evaluated by ANOVA.

3. Results

3.1. Dose responses

After treatment with a range of thymopentin, the BMDCs grew into differential colonies. The result by MTS method to evaluate the expanded number demonstrated that the optimal concentration to boost cell growth was 31.25 μg/ml, as shown in Fig. 1.

3.2. Structural changes of BMDCs shown under TEM

Immature BMDCs contained more phagosomes and a strong potential to phagocyte antigen. However, when BMDCs mature, the number phagosomes will decline, and accompanying this process, a higher expression of key membrane molecules will take place. The TEM photo in Fig. 2 clearly portrays the reduction in phagosomes.

3.3. Analysis of the BMDCs' key membrane molecules by FCM

After the immature BMDCs were treated with 31.25 μg/ml thymopentin for 48 h, they matured as evidenced by the up-regulated expression of key membrane molecules of MHC-II, CD86, CD80, CD83, and CD40, which would cooperate with T-cells to initiate cellular response. Total positive cells were measured in this test and concrete changes were shown in Fig. 3 and as the following number.

For CD40, it yielded 31.25 ± 1.38 in the thymopentin group and $p < 0.01$ vs. 21.13 ± 3.26 in the RPMI 1640 group. For CD80, the yield was 38.41 ± 4.4 in the thymopentin group and $p < 0.01$ vs. 17.53 ± 1.4 in the RPMI 1640 group. CD83 yielded 31.17 ± 1.86 in the thymopentin group and $p < 0.01$ vs. 17.42 ± 2.78 in the RPMI 1640 group. For CD86, the yield was 38.16 ± 4.01 in the thymopentin group and $p < 0.01$ vs. 16.49 ± 3.19 in the RPMI 1640 group. For MHC-II, the yield was 38.22 ± 2.01 in the thymopentin group and $p < 0.01$ vs. 21.03 ± 1.

3.4. Phagocytosis study by FCM

Antigen-like particles phagocyted by BMDCs were further analyzed using a FITC-dextran test. Thymopentin down-regulated BMDC phagocytosis to the labeled dextran as compared with BMDC phagocytosis without treatment with thymopentin as shown in Fig. 4.

BMDC phagocytosis of the antigens generated the following number: 54.01 ± 3.64 in thymopentin group and $p < 0.05$ vs. 64.40 ±