

## Listeria Monocytogenes Protein Fraction Induces Dendritic Cells Maturation and T helper 1 Immune Responses

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### ABSTRACT

Fully mature dendritic cells (DCs) play pivotal role in inducing immune responses and converting naïve T lymphocytes into functional Th1 cells. We aimed to evaluate Listeria Monocytogenes-derived protein fractions to induce DC maturation and stimulating T helper (Th)1 immune responses.

In the present study, we fractionated Listeria Monocytogenes-derived proteins by adding of ammonium sulfate in a stepwise manner. DCs were also generated from C57BL/6 mice bone marrow precursor cells. Then, the effects of protein fractions on bone marrow derived DC (BMDC) maturation were evaluated. In addition, we assessed the capacity of activated DCs to induce cytokine production and proliferation of lymphocytes.

Listeria-derived protein fractions induced fully mature DCs expressing high costimulatory molecules such as CD80, CD86 and CD40. DCs that were activated by selected F3 fraction had low capacity to uptake exogenous antigens while secreted high levels of Interleukin (IL)-12. Moreover, lymphocytes cultured with activated BMDCs produced high amounts of IFN- $\gamma$  and showed higher proliferation than control. Listeria derived protein fractions differently influenced DC maturation.

In conclusion, Listeria protein activated-BMDCs can be used as a cell based vaccine to induce anti-tumor immune responses.

Keywords: Dendritic cells; Interferon gamma, Interleukin-12; Listeria Monocytogenes

### INTRODUCTION

Dendritic cells (DCs), as the professional antigen presenting cells, play critical role to induce immune

responses.<sup>1-3</sup>

DCs capture antigens (self and non-self) and present them to T cells and consequently induce various T cell subsets. Beside origin of DCs, microenvironment determines the types of immune responses induced after DC-T cells interaction.<sup>4,5</sup> The microenvironment contains danger signals and Pathogen associated molecular patterns (PAMPs) that influence DCs in

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different ways. Some PAMPs have been shown to induce production of pro-inflammatory cytokines from DCs in addition to increasing the levels of CD80, CD86, CD40 and Major Histocompatibility complex II (MHCII) while others induce only costimulatory molecule expression.<sup>6-9</sup> In most cases, PAMPs induce signaling pathways that lead to caspase activation or NF- $\kappa$ B and Interferon regulatory factors (IRFs) mediated gene expression.<sup>10,11</sup> Fully matured DCs (mDCs) convert naïve T lymphocytes into functional Th1 cells by inducing production of pro-inflammatory cytokines such as IL-12 and IL-18.

Microbes influence DCs differently.<sup>7,12,13</sup> Studies on the pathogenesis of infections showed different mechanisms used by microbes to cause damage and live within host or even escape from immune system. For example, Bordetella Pertussis-derived filamentous Hemagglutinin (FHA) induces IL-10 production from DCs and downregulates the level of costimulatory molecule expression such as CD80, CD86 or CD40 to escape from immune system.<sup>14,15</sup> On the other hand, other microbes can effectively interact with Pattern Recognition Receptors (PRRs) that are expressed by DCs and induce downstream signaling pathways leading to their maturation.<sup>16</sup> For instance, *Listeria monocytogenes* as an intracellular microorganism is able to effectively activate DCs through activating PRRs.<sup>18-20</sup> The activity of bacteria to influence DCs has been attributed to their components.<sup>21-23</sup> However, the precise effects of many of the components for activating DCs have not been studied yet. Although some researches emphasized on few bacterial components with adjuvant activity, all bacterial components were not considered in these studies and the antigens were selected because of their determined role and character. We assume, there are much information lied on the bacterial (specially related to *Listeria monocytogenes*) components to activate DCs and the adjuvant ability is not limited to the well known factors.

*Listeria monocytogenes* is a Gram positive, facultative and intracellular bacterium that particularly is known for diffusion between bystander cells by disrupting phagosomal membrane and organized actin based motility within cytoplasm.<sup>24-26</sup> These special characteristics have made this microorganism attractive for immunologists since it can be exploited as a vector to present tumor antigens via MHC I and consequently

induce antigen specific CD8+ T cells as the preferred responses against tumors.<sup>27-29</sup> Nevertheless, the bacterium has adjuvant activity that induces PRR-dependent activation of innate immune cells such as DCs, Natural Killer cells or Macrophages.<sup>30-32</sup> Heat killed *Listeria monocytogenes* (HKLM) induces Th1 responses and was introduced as an effective adjuvant to shift the responses from Th2 to Th1.<sup>33</sup> Results showed that HKLM-mediated Th1 response depends on IL-12 production. The potential to activate DCs is a valuable result to improve DC-based tumor Immunotherapy.<sup>34-35</sup> Our previous studies showed that *Listeria* lysate-activated DCs prevented tumor formation as well as decreasing established tumor growth rate.<sup>36</sup> *Listeria monocytogenes* lysate-treated BMDCs induced Th1 responses and increased survival rates in tumor challenged mice.

The present study will evaluate the effect of distinct protein fractions derived from *Listeria monocytogenes* to induce maturation of BMDCs and their effects on lymphocyte activation, proliferation and cytokine production.

## MATERIALS AND METHODS

### Animals and Tumor Cell Lines

C57BL/6 mice were purchased from the Pasture Institute of Iran and studied at 6–8 weeks of age. Mice were handled according to the guidelines for animal care and ethics. The F10 cell line was also purchased from Pasture Institute of Iran. The cell lines were maintained in RPMI-1640 (Gibco, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco, USA), 2mM L-glutamine (Sigma, Germany), 100 $\mu$ g/ml Streptomycin and 100 U/ml Penicillin.

### Generation of BMDCs

BMDCs were generated according to Inaba Protocol<sup>37</sup> with some modifications. Briefly, bone marrow cells were flushed from C57BL/6 femur and tibia and cultured for 6 days in the presence of 20 ng/ml recombinant murine GM-CSF (R&D, USA) and 10 ng/ml recombinant murine IL-4 (R&D, USA). The complete culture medium included RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2 mM L-glutamine (Sigma), 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin. The culture medium was replaced

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with fresh media in days 3 and 5. In all steps, cells had been incubated in 5% CO<sub>2</sub> incubator. Immature DCs in day 6 were stimulated by adding maturation factors and were incubated for 18 hours. BMDC purity, assessed with anti-CD11c, was typically 80 %.

### Protein Fraction Preparation

*Listeria monocytogenes* (ATCC 19115) was purchased from Iranian Research Organization for Science and Technology (IROST). *Listeria monocytogenes*-derived protein fractions were prepared briefly, bacteria were grown in BHI broth media (CONDA, Spain) to mid-logarithmic phase, pelleted and washed in PBS three times, and stored at -80 °C until use. The pellet was sonicated in the presence of protease inhibitor mini-cocktail (Sigma, Germany) and dialyzed against PBS. The sonicated suspension was fractionated stepwise by adding increasing amounts of ammonium sulfate (Merck, Germany) to obtain 4 fractions (F1, F2, F3, F4 named after 0-25%, 25-40%, 40-60%, 60-80% salt saturation, respectively). The fractions were dialyzed against Tris (0.025 mM) buffer and passed through 0.2 µm Filter. Protein concentration was measured using Bradford method by Nanodrop instrument. The fractions were stored at -70°C until use. To assess whether different fractions contained distinct proteins, SDS PAGE analysis was performed using 12% acryl amide gel. The gels were stained with commassie blue. Sample concentrations were 20 µg of protein per lane. Each fraction was tested using Limulus ameocyte lysate assay to determine LPS contents and no LPS contamination has been detected.

### Mixed Leukocyte Reaction (MLR) Analysis

To assess whether *Listeria monocytogenes*-derived protein fractions influence *Listeria* proliferation induction capacities of DCs *in vitro*, a one-way mixed leukocyte reaction (MLR) was performed with  $\gamma$ -irradiated BMDCs (3000 rad) as stimulators and primed C57BL/6 splenic T cells as responders. For priming, the mice were injected subcutaneously with the tumor cell lysate 2 weeks prior to collection of the splenocytes. Cultures were established in triplicate in 96-well, round-bottom microculture plates in the same culture medium and conditions used for BMDC generation at three different ratios of responder cells to stimulator cells (5:1, 10:1, 20:1) for 96 h. The proliferation of T cells was determined by

Flowcytometry, using CFSE staining kit (Invitrogen, USA).

### Flowcytometry and Antibodies

DC phenotype was determined using FACS analysis system (Beckton Dickinson). Mature BMDCs from 7<sup>th</sup> day of culture were stained with FITC-conjugated CD40, CD86, MHC antibodies and PE-conjugated CD11c and CD80 antibody (BD Pharmingen). In all experiments appropriate isotype control antibodies with same Ig class and isotype were used.

### ELISA

To assess producing cytokines from DCs and lymphocytes, cell culture supernatants were collected and evaluated using IL-12p70, IL-10 ELISA kit (R&D) and IFN- $\gamma$  ELISA Kit (eBiosciences).

### FITC-Dextran Uptake Assay

Endocytosis capacity was measured by uptake of dextran (m.w: 40,000) conjugated with FITC (Molecular Probes, USA). BMDCs were incubated for 1 h at 37°C in the presence of 1 mg/ml FITC-dextran. Control cells were incubated at 4°C for 1 h. After 3 times washing, the cells were analyzed by FACS analysis system (Beckton Dickinson).

### Statistical Analysis

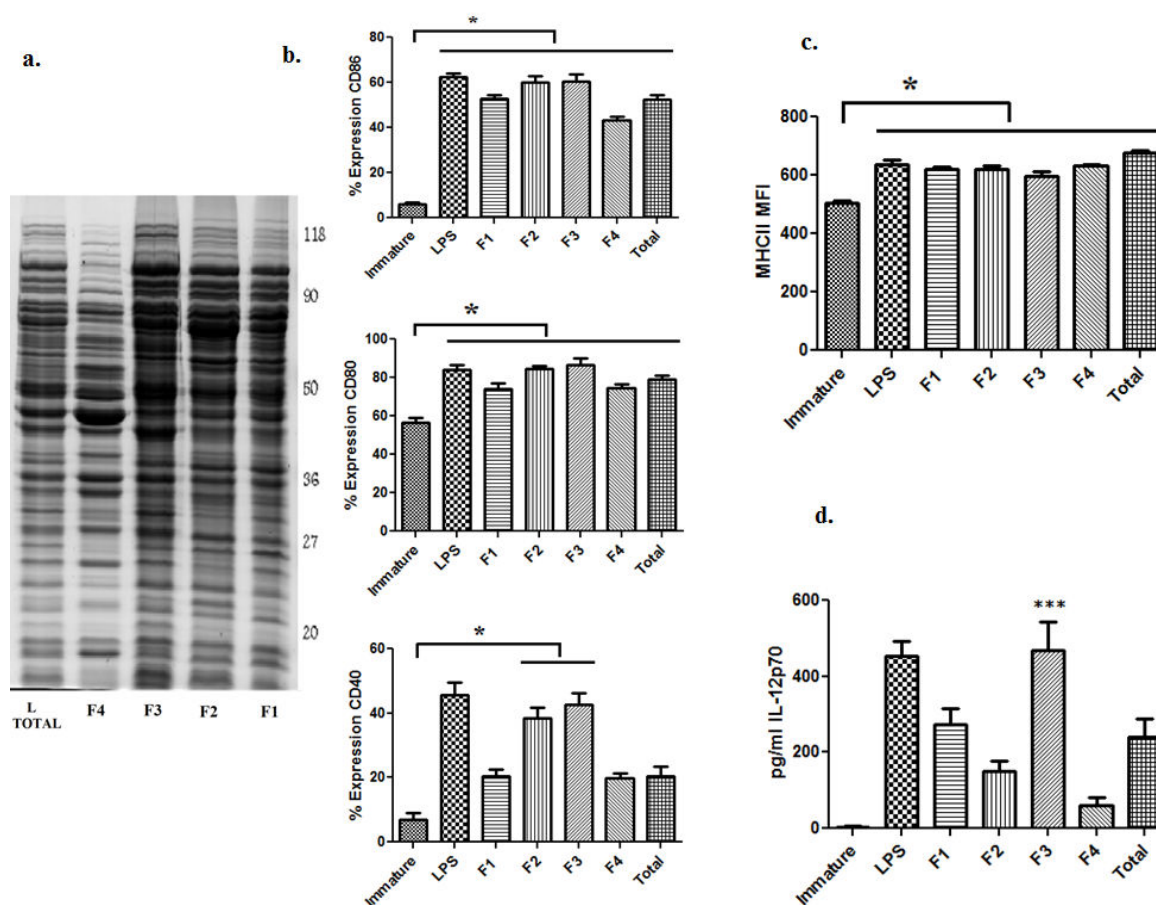
Statistical significance was evaluated using one-way ANOVA and student's T test. *P*-values less than 0.05 were considered significant. All statistical analysis was performed with SPSS, version 17 (SPSS Inc).

## RESULTS

### Different *Listeria*-Derived Protein Fractions Induce Fully Matured DCs

Protein fractions that were precipitated by ammonium sulfate were evaluated to determine if they induce mature DCs. Firstly, to assess whether each fraction contained different components, they were loaded into 12% acrylamide gel with same concentrations (25µg) and were separated using SDS PAGE (Figure 1a). Each fraction contained different pattern of protein bands with different protein concentrations for each band.

Moreover, to assess whether *Listeria monocytogenes*-derived Protein fractions induce by



**Figure 1.** *Listeria Monocytogenes*-derived protein fractions induce phenotypic and functional maturation of DCs. **a.** SDS-PAGE analysis showed that each protein fraction contained unique pattern of proteins. The right lane demonstrated the proteins with determined molecular weight. Using salt precipitation, we prepared four distinct protein fractions (including F1,2,3,4) from whole sonicated *Listeria* lysate (L TOTAL). **b.** *Listeria monocytogenes*-derived protein fractions induced phenotypic maturation. Flowcytometry data demonstrated that all protein fraction-treated BMDCs significantly expressed higher levels of CD80 and CD86 costimulatory molecules compared to immature DCs. Also, F3 and F2 treated BMDCs significantly expressed higher CD40 costimulatory molecules than immature DCs, F1, F4 and Total-treated BMDCs. **c.** Evaluating MFI of MHCII showed higher amount for activated BMDCs than Immature DCs. **d.** F3 is the best fraction to induce IL-12p70 production from DCs. \*,  $P < 0.05$  and \*\*\*,  $P < 0.001$  means significantly different from immature BMDCs

phenotypic maturation in DCs, immature BMDCs were cultured in the presence of the protein fractions for 18 hours (Figure 1b, c). BMDCs that were stimulated all protein fractions significantly expressed higher levels of CD80 and CD86 compared to Immature DCs ( $p < 0.05$ ) while only LPS, F2 and F3 treated BMDCs expressed higher amounts of CD40 than Immature DCs ( $p < 0.05$ ). Interestingly, the same superior potential of F2 and F3 was observed for CD40 and CD86 MFI (data

not shown). Since, no difference has been observed in MHCII expression between different groups, we measured MFI for MHCII (Figure 1c). Results showed significant difference of MHCII MFI between Immature BMDCs and activated BMDCs ( $p < 0.05$ ). Generally, F2 and F3 fractions induced higher density of costimulatory molecules on DCs.

To investigate functional maturation of DCs following interaction with protein fractions, we

assessed IL-12p70 production. Immature DCs were cultured in the presence of maturation factors for 18 hours and supernatants were collected to detect IL-12p70 concentrations (Figure 1d). Immature BMDCs produced very low IL-12p70 while protein fraction-treated BMDCs significantly produced higher IL-12p70 than immature BMDCs ( $p < 0.05$ ). Among the groups, LPS and F3 significantly induced higher IL-12p70 than immature BMDCs and F2, F4 and L total-activated BMDCs ( $p < 0.05$ ). Likewise, F3 induced higher IL-12 production than F1 but the differences were not statistically significant. Also, there was no statistically significant difference between F2, F4 and immature DCs ( $p > 0.05$ ).

According to the results shown in Figure 1, F3 that was precipitated by adding 40 to 60% of ammonium sulfate was selected as the most potent among other protein fractions to induce DC maturation.

### F3-Treated BMDCs Induced IFN- $\gamma$ Production and Proliferation in Lymphocytes

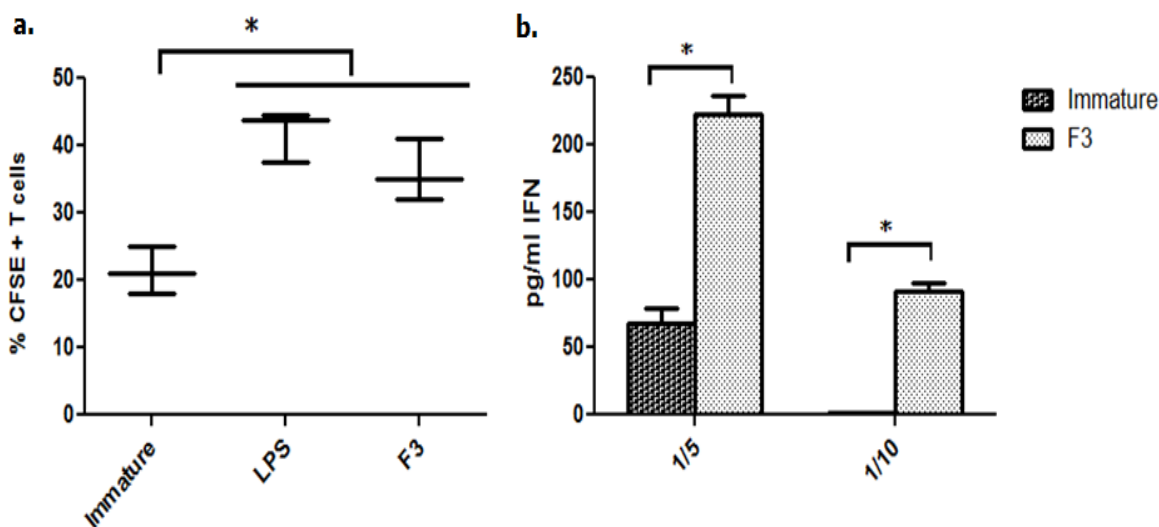
T cell proliferation has been recognized as the hallmark of increasing immune responses and mature DCs generally are potent inducers of T cell proliferation. F3 and LPS-matured DCs and immature DCs as stimulators were co-cultured with CFSE stained-lymphocytes (as responders) for 5 days in 3

ratios of 1/5, 1/10 and 1/20 (stimulator/responders). Figure 2a shows the ratio of 1/5. BMDCs matured with F3 significantly induced higher proliferation in primed stained lymphocytes than immature BMDCs ( $p < 0.05$ ). LPS also was assessed (as a positive control) to determine T cell proliferation. It also induced higher proliferation than immature-DCs ( $p < 0.05$ ).

In addition lymphocytes cultured with F3-treated BMDCs produced higher IFN- $\gamma$  in comparison with immature BMDCs as control ( $p < 0.05$ ). IFN- $\gamma$  is recognized as hallmark of Th1 response as well as NK cell activation. Lymphocytes were cultured with irradiated F3-treated or immature BMDCs for 48 hours and IFN- $\gamma$  production was measured using ELISA kit after incubation time (Figure 2b). BMDCs that were stimulated by F3, induced higher IFN- $\gamma$  production than immature BMDCs ( $p < 0.05$ ).

### F3-Treated BMDCs Showed Less Endocytic Capacity than Immature DCs.

Immature DCs capture antigens more efficiently compared to mature DCs. This characteristic is in coordination with their functions. In other words, Immature DCs in peripheral sites have been organized to capture foreign antigens immediately while mature DCs should only present processed antigens to naïve T cells in lymph nodes to efficiently mount immune



**Figure 2.** F3-treated BMDCs strongly induced IFN- $\gamma$  production and proliferation in lymphocytes. This figure demonstrates the significantly higher potential of F3-treated BMDCs than immature-DCs to induce (a) proliferation and (b) IFN- $\gamma$  production (in 1/5 and 1/10 ratio) from splenic cells. LPS-treated DCs were also assessed as positive control for proliferation (ratio: Stimulator/ responders).\*,  $P < 0.05$ , means significantly different from immature DCs.

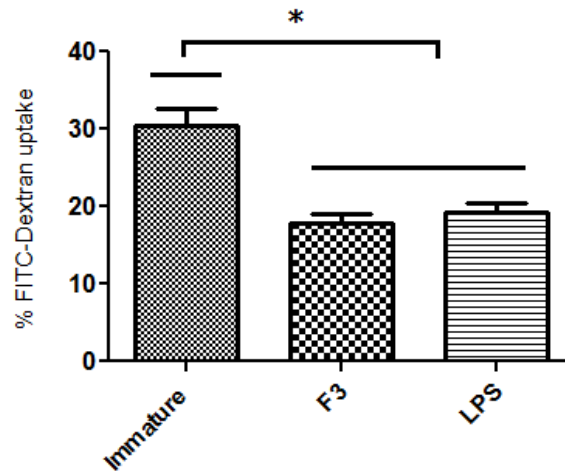


Figure 3. F3-treated BMDCs show low endocytic capacity. This figure shows F3-treated BMDCs had lower capacity to phagocytose antigens in comparison to immature DCs. LPS-treated DCs also showed significantly lower potential to phagocytose antigens. \*  $P < 0.05$ , significantly different from immature DCs.

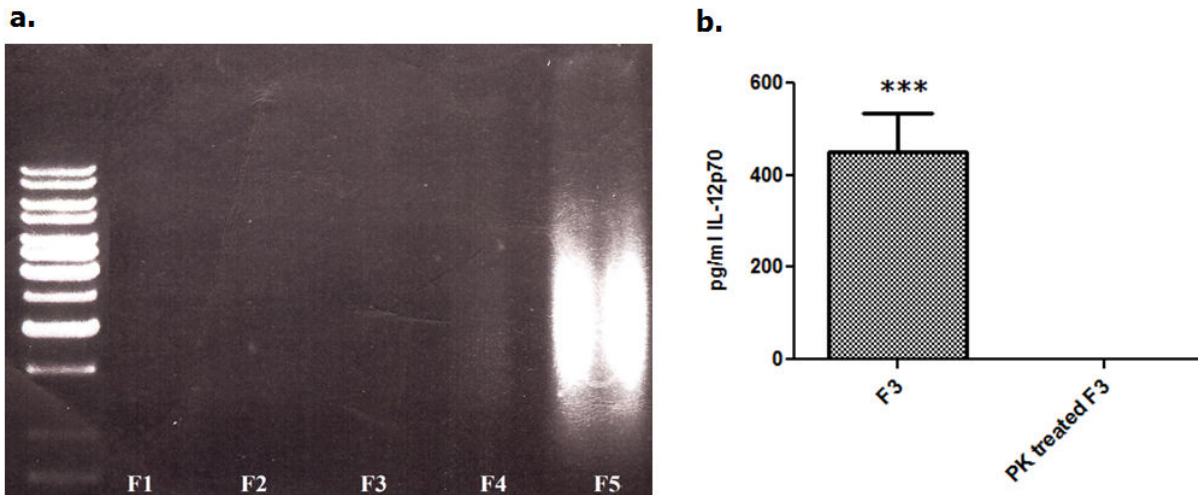


Figure 4. The effect of F3 on DCs was mediated by protein not DNA. a. This figure demonstrates that there was no DNA contamination (detectable by 1% agarose gel) in F1, F2, F3 and F4 while DNA contents were observed as a smear in the last fraction (F5). b. F3-treated Proteinase K is not able to induce IL-12p70 production in DCs ( $P < 0.001$ ).

responses. Therefore, reducing endocytic capacity happens as a result of maturation. Using FITC-dextran (Figure 3), we showed that immature BMDCs had much more endocytic capacity than F3 treated-DCs or LPS-treated BMDCs ( $p < 0.05$ ). This observation emphasizes on the F3 potential to induce DCs maturation.

#### The Contributing Maturation Factor in F3 Is Protein, not DNA

Protein fractions were evaluated to determine whether they contained any DNA or other active components, which were able to induce cytokine production by DCs. To confirm that there was not any DNA contamination in protein fractions, we analyzed each fraction using one dimensional gel electrophoresis

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(1%). After staining with Ethidium Bromide, we observed no DNA contamination in fractions (F1=0-25, F2=25-40, F3=40-60 and F4=60-80) while a DNA smear was observed in the F5=80-100% fraction (Figure 4a). In addition, by using Proteinase K that effectively digested protein components, we confirmed that exclusively protein content of F3 (not any other parts) caused functional maturation in DCs (Figure 4b). Digested F3-treated BMDCs produced no IL-12p70 ( $p < 0.001$ ).

### DISCUSSION

DCs have been recognized as prominent regulators of immune responses.<sup>1,2</sup> Because of their capacity to express diverse surface and intracellular PRRs, they can sample microenvironment and respond to surrounding milieu by changing their genes expression pattern.<sup>40,41</sup> Therefore, the dual role of DCs to induce both immunogenic and tolerogenic responses was determined by the PRR ligands in the microenvironment.<sup>3,42,43</sup> We showed that killed *Listeria monocytogenes* contained protein components that could potentially induce phenotypic and functional maturation in DCs. Also, activated DCs could strongly induce IFN- $\gamma$  production and proliferation in lymphocytes.

Distinct *Listeria*-derived protein fractions induced full maturation in DCs. BMDCs treated with the fractions showed higher levels of costimulatory molecules (CD80, CD86 and CD40) than immature BMDCs. Nevertheless, F2 and F3 showed higher capacity to induce phenotypic maturation in comparison with F1 and F4. It is worth noting that MHCII level was not different in mature and immature DCs. Evaluating MFI for MHCII showed that *Listeria*-derived-protein-treated DCs expressed higher levels of MHCII molecules per cell in comparison with immature DCs. In general F2 and F3 were superior to other fractions (F1, F4) to induce phenotypic maturation. In addition, mature BMDCs produced higher IL-12p70 than immature DCs. F3 was recognized as the best maturation factor in comparison with other fractions to induce IL-12p70. Interestingly, F2 that strongly induced phenotypic maturation was unable to produce high IL-12p70. This characteristic can be explained by stating that F2 contained tolerogenic ligands that induced high levels of costimulatory molecules but low levels of

proinflammatory cytokines, the state which is defined as semi-mature DCs.<sup>44,45</sup> There are many studies that determined microbial components as tolerogenic factors.<sup>46</sup> However, further studies to evaluate IL-10 production from F2-treated BMDCs are required to confirm the point. Interestingly, it has been observed that *Listeria monocytogenes* (LM) promote tumor growth through TLR-2 signalling.<sup>47</sup>

Moreover, we showed that *Listeria*-derived-protein-treated BMDCs strongly induced IFN- $\gamma$  production and proliferation in lymphocytes. Presumably, binding costimulatory molecules such as B7 family members to CD28 induce IL-2 production.<sup>48</sup> This cytokine induces proliferation in an autocrine manner.<sup>48,49</sup> Also, F3-treated DCs induced IFN- $\gamma$  production from lymphocytes. Different splenic cells produce IFN- $\gamma$  cytokine including T cells and Natural killer cells as prominent cells to eliminate tumor cells and pathogens.<sup>50-52</sup> Although we showed that high levels of IFN- $\gamma$  were produced from lymphocytes, we have not specifically determined type of the cells that produced this cytokine and the issue has remained to be discovered. However, due to the well known immunogenic activity of IFN- $\gamma$  in immune system, in settings such as tumor immunotherapy or treatments to eliminate pathogens, IFN- $\gamma$  production itself is very important. In accordance with our results, different studies showed that interactions between surface molecules on DCs such as B7:H3 and lymphocytes lead to IFN- $\gamma$  production.<sup>48,53</sup> Therefore, inflammatory signals induce proliferation by increasing B7 family members.

In addition, uptake assay showed that immature DCs were more efficient than F3-treated DCs to uptake FITC-dextran. Generally, Immature DCs are located in peripheral sites to capture and prepare antigens to present to T cells.<sup>54,55</sup> After encountering with antigens and during migration toward secondary lymphoid tissues, DCs will reduce their endocytic capacity while increasing phenotypic maturation. Clearly, DCs should have a stable membrane to maintain MHC-peptide complexes in longer periods to raise the possibility of recognition of antigenic peptides by TCRs.<sup>56,57</sup> Therefore, activated DCs reduce the recycling process while increasing the costimulatory molecules and MHCII complex expression. Various studies investigated, the effect of maturation and PRR stimulation on membrane stability and preventing antigen capture.<sup>56,58</sup>

The activity of F3 is restricted to its protein components. The results of gel electrophoresis clearly showed that there was no DNA contamination in *Listeria*-derived-protein fractions except in F5. This result confirmed two points: First, it showed that salting out using ammonium sulfate was effective to exclusively precipitate proteins not nucleotide components; Second, it clearly showed that logically we should not use the last fraction (F5) due to its contamination, as we took it into account. In addition, by using Proteinase K, we specifically showed that protein components, not any other component, were responsible for IL-12p70 production from treated BMDCs. Similarly, prior studies demonstrated that mostly stimulating activity was restricted to microorganisms'-derived-protein components as was clearly shown that *Toxoplasma*-derived-protein component, not its DNA or RNA were responsible to activate NK Cells or induce protective Th1 immune responses.<sup>59</sup>

In general, finding superior maturation factors is required to improve DC-based cancer therapy and adjuvant therapy against infections. Particular *Listeria* derived protein fraction (F3) strongly activates dendritic cells and may be very effective to use in cancer immunotherapy. However, the effects of this protein cocktail should be evaluated by further studies.

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