

# Establishment of B-1 cell-derived polyreactive monoclonal antibodies and expression of costimulators by B-cell to antigenic stimulation

Ju-Youn Lee<sup>1</sup>, Jeom-Il Choi<sup>1</sup>, Jo-Young Suh<sup>2\*</sup>

1. Department of Periodontology, School of Dentistry, Pusan National University
2. Department of Periodontology, School of Dentistry, Kyungpook National University

## I. INTRODUCTION

The innate immune system is comprised of the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. It is mainly directed to recognition of invariant molecules of infectious agents. Most of them are essential for pathogen survival, and are conserved and shared by groups of pathogens, such as lipopolysaccharide (LPS) present in gram-negative bacteria. It is also essential for the activation of the adaptive immune response, capable of coping with a high mutation rate and an antigen (Ag) heterogeneity of infectious agents, and of generating a long-lasting immune memory<sup>1)</sup>.

It is generally thought that the B cell com-

partment is a representative of the adaptive branch of immune defense<sup>2)</sup>. However, because of their anatomical location, B-1 and marginal zone (MZ) B cells are the first cell populations to encounter Ags<sup>3-5)</sup>. These two B cell subsets have evolved to provide a first line of defense against pathogens. With their additional properties to produce factors that can directly mediate microbial destruction and to express Toll-like receptors, B cells provide an important link between the innate and adaptive branches of the immune system<sup>6)</sup>.

B-1 cells represent a specialized subset of B cells that are distinct from the majority of recirculating conventional B cells (termed B-2) in an individual.

They are distinguished by tissue dis-

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\* Correspondence: Jo-Young Suh, Department of Periodontology, School of Dentistry, Kyungpook National University, 188-1, Samdeok 2Ga, Jung-Gu, Daegu, 702-412, Korea (E-mail: jysuh@knu.ac.kr)

tribution, cell surface phenotype, B cell receptor signal generation, capacity for self-renewal and perhaps most importantly, their role in immune defense<sup>7-11</sup>). B-1 cells, although constituting only a minor fraction of B cells in spleen and lymph nodes, represent the main B-cell population in the peritoneal and pleural cavities. B-1 cells only comprise a small percentage of all B cells, but they are the primary source of the serum Abs, known as Natural Antibodies (NAbs)<sup>8,12,13</sup>. The majority of NAbs have a low affinity and are polyreactive, with the ability to recognize autoantigens as well as Ags from bacterial and parasitic sources. B-1 cells develop *de novo* prior to weaning and persist thereafter as a self-replenishing population and produce IgM autoantibodies, including rheumatoid factor, Abs to single-strand DNA, and Abs to bacterial Ags such as LPS or phosphatidyl choline<sup>14-17</sup>).

The exact role of B-1 cells during an immune response is still under investigation. Hezenberg's group<sup>18</sup>) has provided the evidence for a mechanism of B-1 cells (NAbs). They have reported that the natural IgM Abs against viruses are present prior to infection and do not increase. Thus, B-1 cells do not respond to pathogens with an increase of IgM Abs; the increase of the antiviral IgM comes entirely from the B-2 cells.

The demonstration that B-1 cells can bind, process and present many different Ags to T cells, argues that under ordinary circumstances B-1 cells do not stimulate T cells but do so in the absence of the important

costimulators B7-1 and B7-2. Thus, B-1 cells have all the properties required for inducing and maintaining immunological tolerance and are particularly suited to pick-up the myriad of endogenous Ags to which the host might make an autoimmune response<sup>19</sup>). Thus, it is tempting to speculate, that whereas high affinity monoreactive Ag-binding B cells provide a defense against foreign invaders, low affinity polyreactive Ag-binding B-1 cells may provide a defense against autoimmune self-destruction by inducing and maintaining immunological tolerance<sup>20</sup>).

Presentation of Ags to T lymphocytes by B-1 cells is difficult to evaluate at the human level because of problems in readily obtaining normal B-1 cells and Ag-specific T cells from HLA-compatible subjects. To circumvent this problem, use of inbred mice may be suggested. The purpose of this study was to characterize B-1 cell-derived innate immunity and examine the immunoregulatory function of B cells. In the present study, we have established hybridoma producing B-1 cell-derived polyreactive monoclonal Abs. By using this hybridoma, we have characterized B-1 cell-derived monoclonal Ab responses to a panel of exogenous and endogenous Ags. Expression of costimulators by splenic and peritoneum-derived B cells to antigenic stimulation was also evaluated.

## II. MATERIALS AND METHODS

### 1. Reagents

Cell culture media (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were obtained from GibcoBRL (Grand Island, NY, USA). Reagents purchased from Sigma (St. Louis, Missouri, USA) were cell freezing medium DMSO (serum free); hypoxanthin, aminopterin, and thymidine (HAT); hypoxanthine and thymidine (HT) medium supplements; complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), ethylene diamine tetraacetic acid (EDTA). Polyethylene glycol (PEG) 4000 was purchased from Merck (Darmstadt, Germany).

## 2. Antigens and Antibodies

We used LPS, *Porphyromonas gingivalis* hsp60 (Pghsp), PC-KLH (PC coupled to keyhole limpet hemocyanin) as exogenous Ags and Mammalian heat shock protein (Mahsp), Deoxyribonucleic acid (DNA) from calf thymus as endogenous Ags. LPS (Sigma) from *Escherichia coli* 0111:B4, Mahsp [Recombinant Mouse Hsp60 Protein (Stressgen, Victoria, BC, Canada)], DNA from calf thymus (Sigma) and PC-KLH (Biosearch Technologies, Inc., Novato, CA, USA) were used in this experiment. Recombinant Pghsp, was purified from *Escherichia coli* transformed with *P. gingivalis* GroEL<sup>21</sup>.

Peroxidase-labeled affinity purified antibodies to mouse IgM ( $\mu$ ) produced in goats were purchased from KPL (Gaithersburg MD, USA). Abs used for immunofluorescence were obtained from PharMingen (San Diego,

CA, USA): B220 (CD45R; RA3-6B2-Per cp and PE), MHC class II (2G9 and M5/114.15.2, -FITC), B7-1 (CD80, 16-10A1,-FITC) and B7-2 (CD86, GL1, -FITC)

## 3. Medium

DMEM containing 10~20% (v/v) FBS was supplemented with 0.2M glutamine and 10 mg/ml Gentamycin. The standard medium was used for the growing of myeloma and hybridoma cells.

## 4. Mice

C57BL/6 mice and RAG1 knock-out (KO) mice on the C57BL/6 background, originally obtained from Jackson Laboratories (Bar Harbor, ME, USA), were bred and reared in the Pusan National University Hospital Medical Research Institute and were used at 4~6 weeks of age.

## 5. Myeloma cell line

The HAT-sensitive Balb/c mouse myeloma cell line SP2/0-Ag14 (ATCC #CRL 1581) was used in fusion experiments.

## 6. Adoptive transfer of peritoneal B-1 cells into RAG1 knock-out mice

C57BL/6 mice were sacrificed by cervical dislocation and thoroughly cleaned with 70% ethyl alcohol. A small incision was made into the abdomen and the peritoneal cavity

(PerC) was rinsed several times through the opening with freshly prepared cold sterile phosphate-buffered saline (PBS) containing 1% FBS.

The cells collected were devoid of erythrocytes by incubating the cells with lysing buffer at 4°C for 5 min, resuspended in PBS buffer (0.5% BSA, 2mM EDTA) and centrifuged three times at 1500 rpm for 5 min. The peritoneal washing was passed through a 30µm nylon mesh (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to obtain a homogeneous cell population.

The cells were suspended with PBS buffer and a CD90 Microbead (Miltenyi) and incubated for 15 min at 4°C. PerC B-1 cells were enriched from peritoneal lavage by removing the adherent accessory cells and magnetic bead depletion of T cells. The final B-1 cells were injected into the peritoneal cavity of RAG1 KO mice.

## **7. Establishment of hybridoma producing B-1 cell-derived polyreactive monoclonal antibodies**

One week before fusion, the myeloma cell line was expanded into a complete DMEM-10/HEPES/pyruvate.

RAG1 KO mice were sacrificed by cervical dislocation 10 weeks after the adoptive transfer. After the surgical skin preparation, the spleen was exteriorized through a 1-cm left subcostal incision and isolated. Spleen cells that were collected from the mice were

fused with the myeloma cell line using PEG at a 1:1 ratio. After fusion, cells were resuspended in standard DMEM medium supplemented with 20% FBS and 1% (v/v) HAT medium supplement (DMEM-20/HAT media) to exclude non-fused cells. Next, they were distributed in 96-well culture plates at an approximate density of  $2.5 \times 10^6$  cells/well. The plates were incubated at 37°C in a CO<sub>2</sub> incubator containing 5% CO<sub>2</sub> in the air. Selective growth of the hybrid cells was carried out for fourteen days. Finally, the medium was replaced with the standard medium supplemented with 1% (v/v) HT medium supplement to neutralize the HAT medium.

For initial screening, the supernatant was examined by enzyme-linked immunosorbent assay (ELISA) for the production of Abs against a panel of exogenous and endogenous Ags used in the present experiment. Positive hybridomas were subcloned by limiting dilution (0.3 cell/well). After additional screening for monoclonality of cells, the hybridoma was selected for production of Abs by ELISA. The hybridoma cells were injected ( $5 \times 10^7$  cells/mice) in nude mice and the peritoneal ascites were collected for purification of IgM monoclonal Ab using column chromatography (ImmunoPure<sup>®</sup>, PIERCE, USA).

## **8. Measurement of monoclonal antibody titers to exogenous and endogenous antigens by ELISA**

Microtiter plates were coated with 5 differ-

ent Ags [LPS, Pghsp, PC-KLH, Mammalian heat shock protein (Mahsp), thymic DNA, 10 $\mu$ g/ml] in coating buffer and incubated overnight at 4°C. LPS, Pghsp, and PC-KLH are exogenous Ags, while Mahsp and DNA are endogenous Ags. The plates were washed and an aliquot of supernatant serially diluted was added; following this, they were incubated at room temperature. After washing the plates, peroxidase-conjugated mouse anti-goat IgM was added. After 2 hours of incubation, the plates were washed and 50 $\mu$ L of substrate solution per well was added. The reaction was stopped by adding 50 $\mu$ L of stop solution per well. Absorbance was read at 450nm in the ELISA reader (Sunrise<sup>®</sup>, XFLUOR4, version 4.51).

## 9. Immunization of Animals

To characterize the immunoregulatory function of peritoneal and splenic B cell to exogenous and endogenous Ags, 8 C57BL/6 mice were initially immunized subcutaneously with 5 $\mu$ g of each Ags suspended in complete Freund's adjuvant, followed by two subsequent injections of Ags suspended in incomplete Freund's adjuvant at 1-week intervals. The Ags used for immunization included LPS (LPS group), *Porphyromonas gingivalis* heat shock protein (Pghsp group), Mammalian hsp (Mahsp group), and thymic DNA (DNA group). Two C57BL/6 mice were included in the control group and immunized with complete and incomplete adjuvant only.

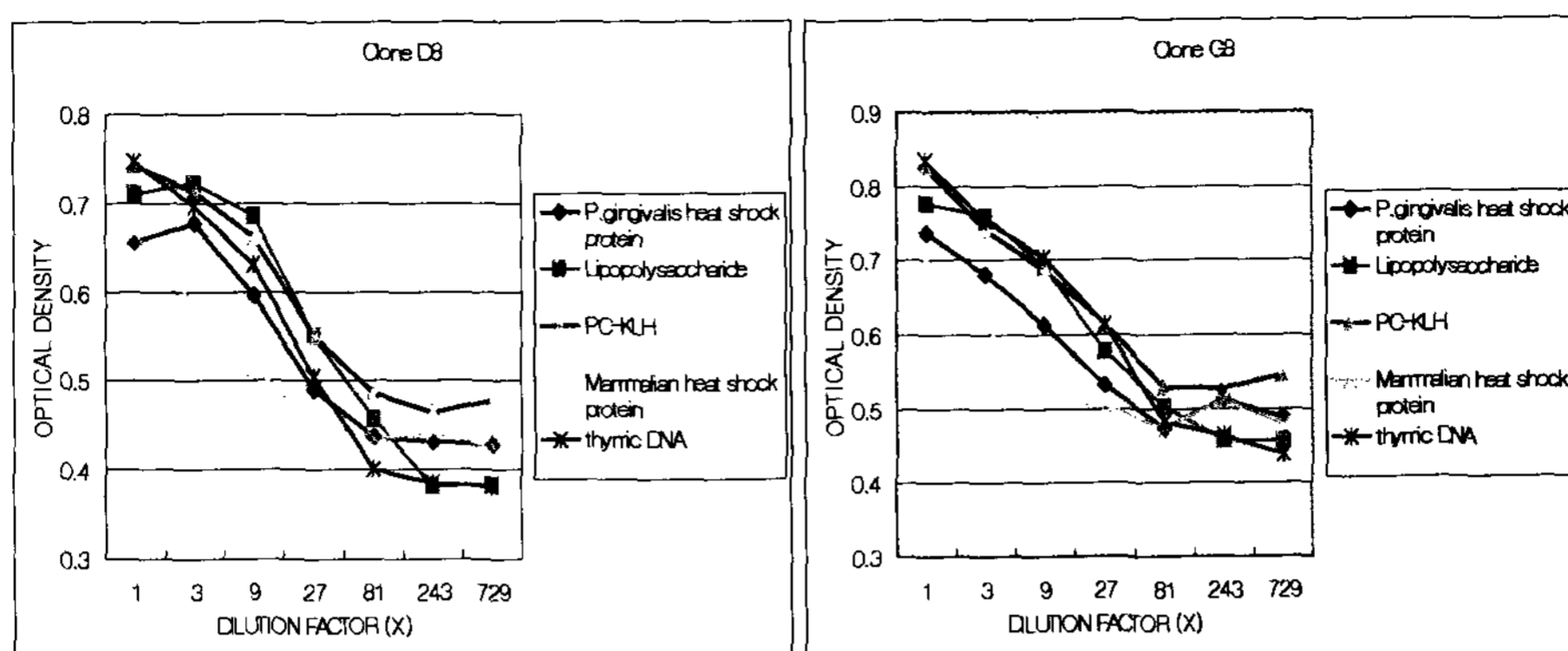
## 10. Flow cytometric analysis

At 1 week after final immunization, mice were sacrificed and their spleen cells and peritoneal cells were collected, respectively, by the method described above. All procedures were carried out according to the manufacturer's recommendation. Briefly, the cells were washed twice in PBS, counted, and resuspended in Fluorescence Activated Cell Sorting (FACS) buffer (1% BSA in PBS containing 0.01% sodium azide). For phenotypic analysis, cells (0.2-1 $\times$ 10<sup>6</sup> cells/stain) were initially incubated with CD16/CD32 (Fc block) for 20 min at 4°C. Subsequently, cells were incubated with an optimal amount of primary Abs (-FITC, MHC Class, B7-1, B7-2) followed by appropriate secondary Ab (-PE, B220). All incubation were performed on ice for 20 min and were followed by three washes with FACS buffer. Flow cytometric analysis was performed using the Cytomics FC 500 system with CXP Software (Beckman Coulter Inc.).

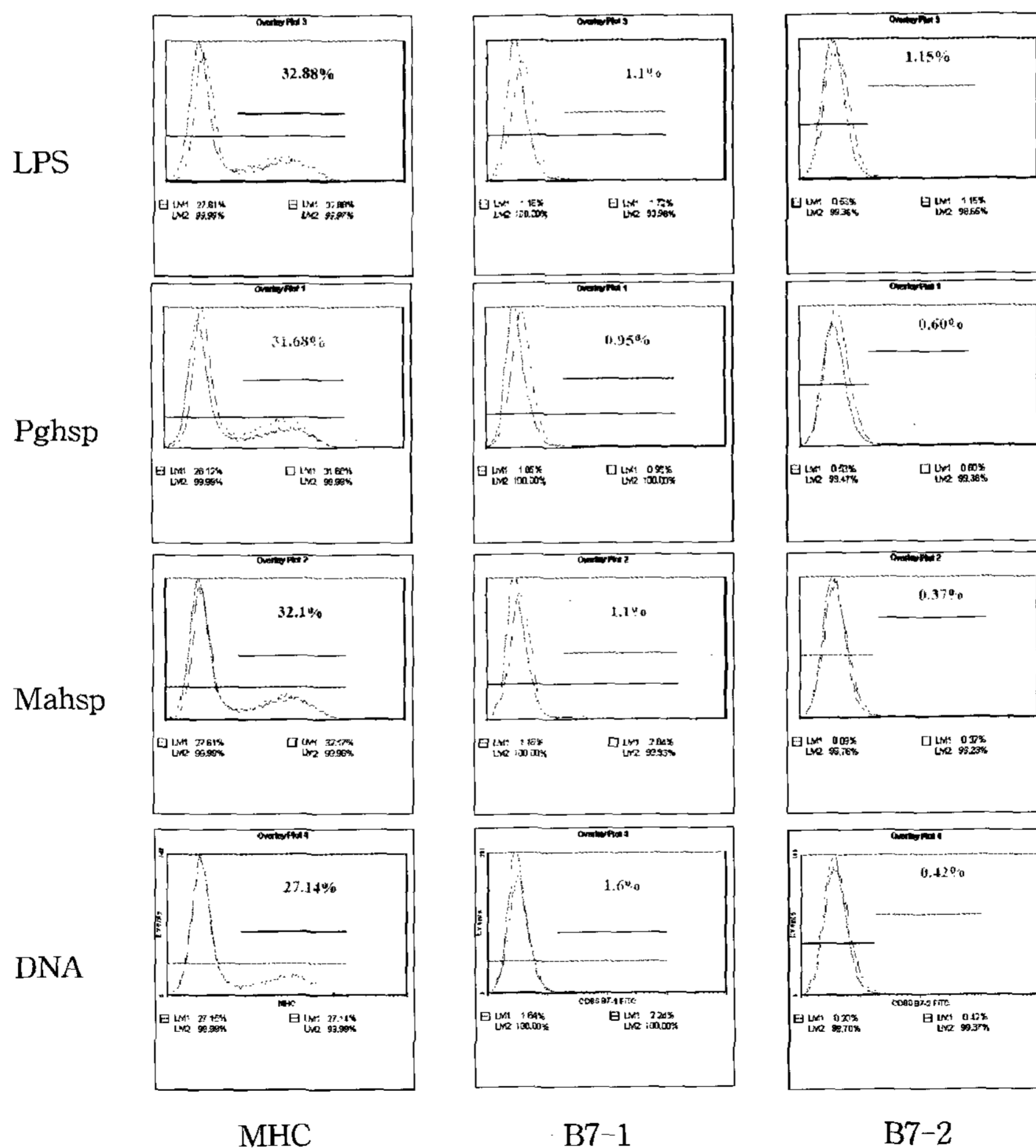
## III. RESULTS

### 1. Monoclonal antibody production against exogenous and endogenous antigens

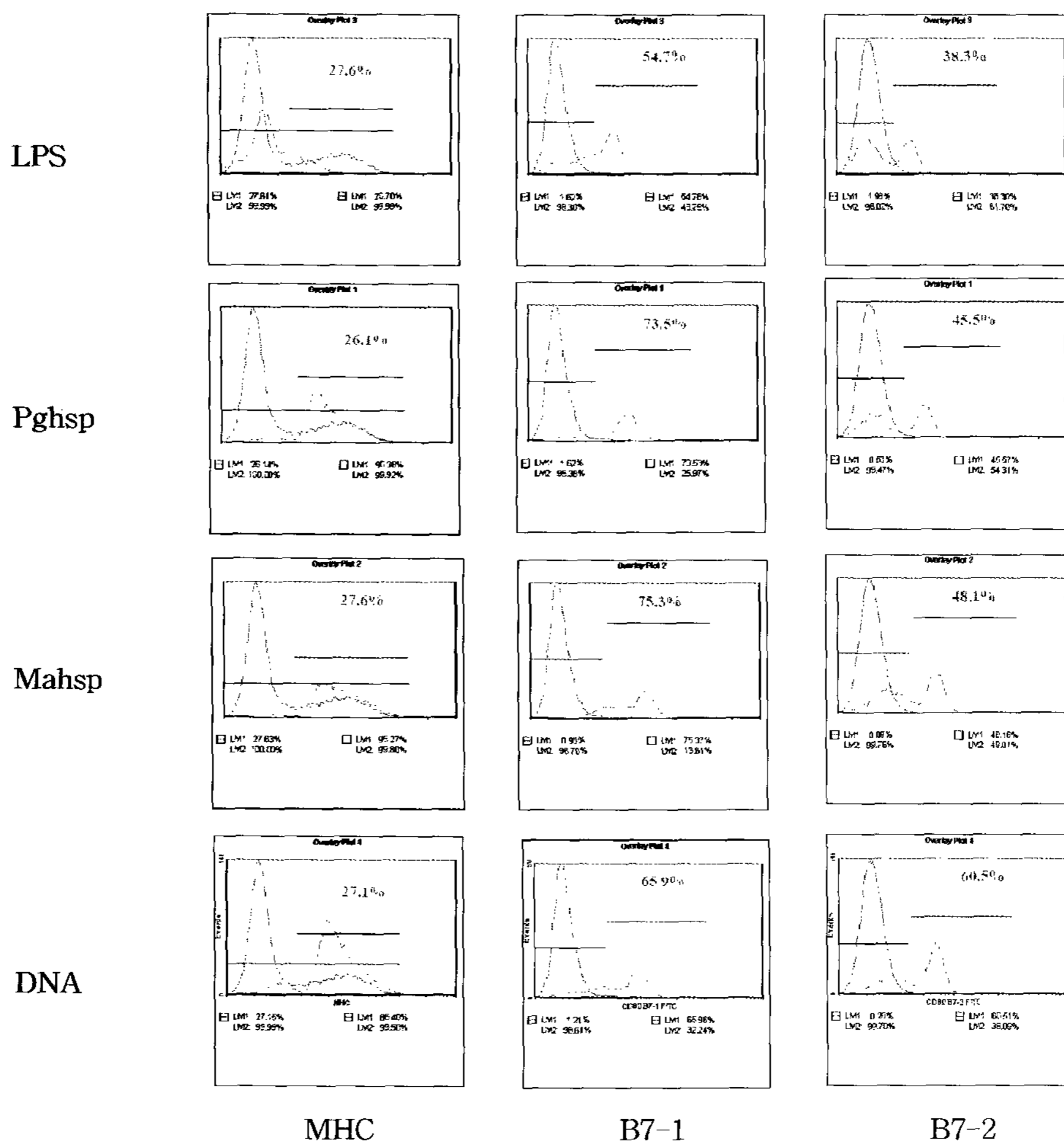
Hybridoma producing B-1 cell-derived monoclonal Ab were established. Initially, six wells out of 66 wells demonstrated the homogeneous cell clusters. After limiting dilution, 2 wells demonstrated monoclonality



**Figure 1.** Dose-dependent antibody responses (expressed as optical density) of monoclonal antibodies derived from the two clones against a panel of antigens. Absorbance was read at 450 nm in the ELISA reader. They show the dose-dependent pattern and polyreactivity of monoclonal antibody responses.



**Figure 2.** Overlay plot of flow cytometric analysis of splenic B cells. MHC expression was enhanced in the test group and B7-1 and B7-2 was slightly enhanced in the test group. blue line: control group, red line: test group.



**Figure 3.** Overlay plot of flow cytometric analysis of peritoneal B cells. MHC expression was enhanced in the test group and the expression of B7-1 and B7-2 was pronounced in the test group. blue line: control group, red line: test group.

of cell clusters (clone D8 and clone G8, respectively). The monoclonal Ab produced from peritoneal ascites were tested for the Ab response by ELISA. Figure 1 shows the dose-dependent pattern of Ab responses (expressed as an optical density) of the monoclonal Ab obtained from the two clones. The two clones demonstrated a very similar pattern in terms of polyreactive reactivity to the multiple Ags tested.

## 2. Expression of costimulators by B-cells to antigenic stimulation

One week after final immunization, mice in the 4 test groups (Pghsp, LPS, Mahsp, thymic DNA) and the control group were sacrificed.

MHC expression was enhanced similarly in Splenic and PerC B cells. With regards to B7-1 and B7-2, the two factors were expressed higher in both the splenic and PerC B cells when compared with the control

group, while the expression was more pronounced in PerC B cells than in splenic B cells (Figure 2, 3). Thus, the type of Ag used in this experiment did not significantly influence the results but the origin of B cells definitely influenced the results.

#### IV. DISCUSSION

In the early 1980s, many authors demonstrated that some monoclonal Abs had the capacity to bind to a variety of different Ags<sup>22-26</sup>). By immortalizing human peripheral B lymphocytes with the Epstein-Barr Virus, Notkins<sup>20</sup>) found that 10-15% of the cells made broadly reactive Abs which are now referred to as polyreactive Abs (Natural Abs), in addition the majority of polyreactive Abs are of the IgM isotype. Consistent with a major role for B-1 cells in natural IgM Ab production, a number of natural IgM Ab specificities have been identified in the B-1 repertoire. These include specificities for phosphorylcholine<sup>27</sup>), phosphatidyl choline<sup>12,28,29</sup>), thymocytes<sup>30</sup>), LPS<sup>31</sup>), and the influenza virus<sup>32</sup>).

To evaluate the exact role of B-1 cells without T-cell help, we have used RAG1 KO mice devoid of mature T- and B-cells, reconstituted with B-1 cells enriched from C57B/6 mice. We were able to show that B-1 cell derived monoclonal Ab was polyreactive in nature; thus, these Abs are bound to a variety of different Ags in a dose-saturable fashion (Figure 1). Notkins<sup>20</sup>) has found that several typical monoclonal poly-

reactive Abs react to different degrees with a variety of self and foreign Ags. In contrast to polyreactive Abs, monoreactive Abs bind only to the immunizing Ag. Casali and Schettino<sup>33</sup>) have shown that a major proportion of NAbs are polyreactive, displaying different affinities for different Ags. They have also shown that the binding of most natural polyreactive Abs to a given Ag can be cross-inhibited by a variety of different Ags in a dose saturable fashion. The precise mechanism underlying polyreactivity still remains unclear. One possibility is that the conformational flexibility<sup>34,35</sup>) at the Ag-binding site of polyreactive Abs is greater than that of monoreactive Abs, thereby allowing a greater number of Ags to bind. Another possibility is a diverse array of binding site structures at the combining site<sup>36</sup>).

The affinity of monoclonal Ab for most Ags is very low<sup>37-39</sup>). Because of their weak interactions with self-Ag, B-1 cell derived monoclonal Abs (NAbs) have often been neglected as the so-called background serum Abs without significant relevance. However, Kazatchkine and Kaveri<sup>40</sup>) have demonstrated that NAbs are essential components of therapeutic molecules in the form of intravenous Immunoglobulin (IVIg). IVIg has been found to be beneficial in a variety of autoimmune and inflammatory diseases. The biological role of B-1 cells during an immune response is still under investigation. Hezenberg's group<sup>18</sup>) has demonstrated that NAbs against the influenza virus are important in terms of protection against infection. Ochsenbein et



al.<sup>41)</sup> have reached the same conclusion from a study that used the lymphocytic choriomeningitis virus infection as a model. Therefore, several studies have clearly suggested that NAbs are essential for the induction of a primary immune response that protects against bacterial and viral pathogens. We demonstrated polyreactivity of B-1 cell-derived monoclonal Ab to various exogenous and endogenous Ags. Therefore, we may assume that B-1 cell-derived monoclonal Ab charge the primary defense line to infection in the fetal stage and infancy, prior to induction of the adaptive immune response with high affinity Ab.

To investigate the expression of costimulators of PerC and splenic B cells to several antigenic challenges, we have immunized the mice with 2 exogenous Ags (LPS, Pghsp) and 2 endogenous Ags (Mahsp, DNA). The results indicated that MHC expression was enhanced similarly in splenic and PerC B cells. With regards to B7-1 and B7-2, the two factors were expressed higher in both the PerC and splenic B cells when compared with the control group, while the expression was more pronounced in PerC B cells than in splenic B cells. But the expression of MHC, B7-1 and B7-2 were very similar irrespective of the kind of Ags. Thus, we can assume that the type of Ags does not significantly influence the results but the origin of B cells definitely influences the results. These results coincide with Rothestein and Kolber's study<sup>42)</sup>. They have demonstrated that PerC B cells and splenic

B cells respond differently to phorbol esters. These results have supported the possibility that residence in the peritoneal cavity influences the different responses of a B cell. It is surprising that specificity for a self-epitope does not affect all B cells uniformly<sup>43)</sup>. One possible interpretation of these results is that differentiation to signaling-incompetent B-1 occurs only in specific sites such as the peritoneum. This seems inconsistent with the findings of Liou et al.<sup>44)</sup>, who found that peritoneal B-1 and splenic B-1 cells from Ig transgenic mice were similarly resistant to experimental tolerance induction. These results suggest that B-1 cells were phenotypically and functionally identical regardless of locale.

Wang et al.<sup>45)</sup> have shown that despite their Ag-presenting ability, B-1 cells from normal mice failed to trigger the proliferation of Ag specific T cells. Analysis of the B7-1 and B7-2 showed that these molecules were not expressed on B-1 cells from normal mice. Chen et al.<sup>46)</sup> have revealed that polyreactive B-1 cells expressed high levels of MHC, but little of no B7-1 and B7-2. These findings argue that the lack of costimulators on B-1 cells is the most likely explanation for their failure to stimulate Ag-specific T cells. This suggests that B-1 cells may contribute to the induction and/or maintenance of immunological tolerance. The issue of the expression of costimulators has not fostered a consensus of opinions. Our results indicate that although B cells are similar in character to the cell surface phe-

notype, the role of B cells in immune response is different according to its origin.

The relationship between infectious disease and B-1 cells has been investigated by several authors. Interleukin-10 (IL-10), an anti-inflammatory Th2 cytokine, is known to down-regulate the functions of antigen-presenting cells and Th1 development. Sasaki<sup>47)</sup> has demonstrated that IL-10 deficiency is associated with a higher susceptibility to alveolar bone loss. B-1 cells both produce and utilize IL-10 as an autocrine growth factor<sup>48)</sup>. Therefore, we may hypothesize that B-1 cells have a preventive role in alveolar bone loss due to periodontitis. On the other hand, renal ischemia reperfusion injury (IRI) is a leading cause of acute renal failure in both allografts and native kidneys. B-1 cells also play a critical role in renal IRI<sup>49)</sup>.

B-1 cells had two-sided effects for inflammatory disease and their exact role was not clearly determined. Therefore, to identify the role of B-1 cells, further studies concerning clinical disease were carried out. However, within the context of our experiment, we concluded that B-1 cell derived monoclonal Abs are polyreactive to a variety of Ags in a dose-saturable pattern. In addition, innate immune response elicited by PerC or splenic B cells is a complex process in nature. It requires the expression of different multiple costimulators by these antigen presenting cells in response to different Ags. Based on our findings, it is suggested that PerC and splenic B cells share distinc-

tive characteristics in their modes of immunoregulatory function.

## V. CONCLUSION

We have utilized hybridoma producing B-1 cell-derived monoclonal antibodies to characterize B-1 cell-derived monoclonal antibody responses to the exogenous and endogenous antigens. Expression of costimulators (MHC Class, B7-1, 7-2) of peritoneum-derived and splenic B cells was also evaluated by the flow cytometric analysis (FACS). After the limiting dilution, we established two clones demonstrating similarity in their manner of polyreactivity and in terms of their dose-saturable response to the multiple antigens tested. PerC and splenic B cells revealed a distinctive pattern in their mode of expressing costimulators when challenged by exogenous or endogenous antigens.

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## B-1 세포 유래 다중반응성 단클론 항체의 형성과 항원 자극에 대한 B 세포의 동시자극자 발현

이주연<sup>1</sup>, 최점일<sup>1</sup>, 서조영<sup>2\*</sup>

1. 부산대학교 치과대학 치주과학교실
2. 경북대학교 치과대학 치주과학교실

**연구 목적** : 면역 반응에서 B-1 세포의 정확한 역할은 아직 명확히 규명되지 않았으나 최근 B-1 세포가 면역의 내성을 야기하고 유지하는데 필요한 특성들을 가지고 있음이 밝혀지고 있다. 이에 본 연구에서는 B-1 세포 유래 단클론 항체의 특성과 항원 자극에 대한 B 세포의 동시자극자 (MHC Class, B7-2, 7-2) 발현을 평가하여 B 세포의 면역조절 기능을 알아보고자 한다.

**연구 대상 및 방법** : B-1 세포 유래 단클론 항체를 형성하는 잡종세포주를 이용하여 다양한 내, 외인성 항원에 대한 단클론 항체의 반응 양상을 평가하였다. 여러 내, 외인성 항원으로 면역한 쥐의 복강과 비장 B 세포의 동시자극자의 발현을 Fluorescence Activated Cell Sorting (FACS)을 이용하여 평가하였다.

**결과** : 최종적으로 단클론을 형성하는 2개의 클론을 형성하였고, 이 B-1 세포 유래 단클론 항체는 dose-saturable pattern을 띄는 다중 반응성을 나타내었다. FACS를 이용한 동시자극자의 발현 검사에서는 MHC 발현은 복강과 비장의 B 세포가 유사하였으나, B7-1 과 7-2는 복강의 B 세포에서 더 뚜렷한 발현을 보여주었다.

**결론** : B-1 세포 유래 단클론 항체는 다양한 내, 외인성 항원 자극에 대해 dose-saturable 한 다중반응성을 나타낸다. 복강과 비장의 B 세포는 내, 외인성 항원의 면역에 있어서 동시자극자 발현이 명확히 다른 양상을 나타냄을 확인할 수 있었다.