Comparison of the Accessory Activity of Murine Peritoneal Cavity Macrophage Derived Dendritic Cells and Peritoneal Cavity Macrophages in a Mixed Lymphocyte Reaction

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ABSTRACT. A detailed comparison of the accessory cell activities was carried out among murine peritoneal cavity macrophages (PEC-MΦ), peritoneal cavity macrophages stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF) plus interleukin 4 (IL-4), the most popular cytokine combination widely used to generate dendritic cells (DC) and peritoneal cavity macrophage-derived DC (PEC-DC) using a two-way mixed lymphocyte reaction (MLR). All the cell types used efficiently induced statistically significant naïve T cell proliferation at all culture time points and responder:stimulator ratios used. However, marked differences were noted in the magnitude of the proliferative responses. These variations may be attributed to the intensity of expression of MHC class II glycoproteins, as well as the actual numbers of MHC class II+ cells.

KEY WORDS: mixed lymphocyte reaction, peritoneal cavity macrophage, peritoneal cavity macrophage-derived dendritic cell.

Dendritic cells (DC) are efficient stimulators of both allogeneic and syngeneic mixed lymphocyte reaction, MLR [7]. They are the principal stimulators of primary immune responses [13, 26, 35, 38, 39]. In studies of lymphoid DC as potent stimulators of the MLR in mice, Steinman and Whitter [7] showed that a ratio of just one DC to 50–250 T cells induced a maximal reaction. Wong et al. [42] demonstrated that DC were reproducibly the most potent stimulators of T cell responses on a per cell basis when compared with macrophages even when higher levels of MHC molecules were induced on the surface of macrophages with IFN-γ [36]. Prior to this finding it was thought that B lymphocytes and macrophages were the most potent stimulators of the MLR.

In the gut, Peyer’s patches and lamina propria both contain DC, macrophages and B cells as APC [20, 21, 27, 40]. Professional APC are characterized by their constitutive high levels of major histocompatibility complex (MHC) class II expression, poor phagocytic ability, lack of sIg and Fc receptors, weak adherence to solid supports [34] and their ability to stimulate primed T cell proliferation in an antigen specific manner [29]. Studies have clearly shown that peritoneal macrophages are highly proliferating progenitors for functional DC [24, 28]. Several groups have succeeded in generating large numbers of functional DC from their proliferating progenitors located in bone marrow, spleen or blood in the presence of appropriate cytokines [2–4, 8, 10, 18, 19, 22]. The results in all these studies indicate that blood, spleen and bone marrow derived DC using growth factors can either be inhibitory or stimulatory. This, taken together with the fact that a comparison of the accessory activity of PEC derived-DC have not been done prompted us to evaluate the similarities and differences of the accessory activities of unstimulated peritoneal cavity macrophages (PEC-MΦ), peritoneal cavity macrophages stimulated with GM-CSF plus IL-4, the most popular cytokine combination widely used to generate DC and peritoneal cavity macrophage-derived DC (PEC-DC), in a two-way MLR. We used p30 (Surface antigen 1, SAG-1) transgenic mice for a two-fold purpose. Firstly, we wanted to investigate whether Toxoplasma lysate antigen (TLA), which had recently been reported to have super-antigen activity, could be used as a maturing agent for PEC-DC compared to known maturing agents, for example, lipopolysaccharide (LPS) or Staphylococcus aureus Cowan I strain (SAC). Secondly, since dissimilarity in terms of MHC class II haplotype is a prerequisite for an allogeneic MLR assay, it was presumed that stimulator cells from p30 transgenic mice would satisfy this requirement when cultured with responders from BALB/c mice. Our investigations show that all the three cell types used efficiently stimulate CD3+ T cells in a MLR. However, the allogeneic responses initiated in vitro by the generated PEC macrophage-derived DC were of higher magnitude than PEC-MΦ, consistent with their predicted potent accessory activity as DC.

MATERIALS AND METHODS

Animals, culture medium and reagents: Samples were obtained from p30 (SAG-1) transgenic mice from the National Research Center for Protozoan Diseases, Obihiro University, generated by microinjection of the cloned SAG-
1 gene into pro-nuclei of embryos of mice [33], p30 negative littermates and BALB/c mice purchased from a commercial supplier (Japan Clea, Japan). All experimental mice were maintained in conventional conditions on a commercial diet (Japan Clea). They ranged from 6–10 weeks of age when used and both sexes were used. Except where stated, all cell manipulations were carried out at room temperature in Hank’s balanced salt solution (HBSS) without calcium or magnesium (Flow Labs, UK), buffered with 5 mM HEPES and 0.4 g/l sodium bicarbonate. Tissue culture medium was RPMI-1640 (Dutch modification, Gibco) supplemented with 2 mM L-glutamine; 1 mM sodium pyruvate (Flow labs); 40 μg/ml gentamicin; 100 IU/ml penicillin; 100 μg/ml streptomycin; 2% normal mouse serum; and 10 μM 2-mercaptoethanol (2-ME) (Sigma, U.S.A.) unless otherwise stated.

The following cytokines were used in this study: murine recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) (Sigma); murine recombinant interferon gamma (IFN-γ) (Sigma); and murine recombinant interleukin 4 (IL-4) (Sigma). Toxoplasma gondii lysate antigen was obtained from the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. All experiments using animals were carried out in accordance with rules and ordinances of the National Institute of Agriculture and Veterinary Medicine and are in line with acceptable international standards.

**Peritoneal macrophage and dendritic cell generation:** A peritoneal lavage was performed from mice previously stimulated with either 0.2% glycolen or 3% thioglycolate in sterile phosphate buffered saline (PBS). Immediately after slaughter the skin covering the abdomen was removed. A peritoneal lavage was carried out by flushing through the peritoneum with 5 ml of sterile PBS. Lavage cells were washed twice in HBSS and resuspended in tissue culture medium. Erythrocytes were removed by osmotic lysis with ammonium chloride solution. Macrophages/monocytes were obtained following a 2 hr or more adherence step in tis-

**Isolation of CD3+ T cells:** BALB/c mice spleens were aseptically excised, placed in a petri-dish to dissect away the serosa and fat, then cut into smaller pieces. This was followed by centrifugation of the cell suspension for 5 min at 4°C and at 600 g. In some cases 0.83% ammonium chloride was used to lyse red blood cells. Lymphocytes purified for CD3+ T cells were isolated from resulting mouse spleen leukocyte cell population using a Cellfect mouse T cell kit (Cytovax Biotechnologies, Canada) as per manufacturer’s instructions. The purity of the isolated cells from the Cellfect mouse T cell kit as determined by flow cytometry using anti-CD3 mAb was always greater than 90%.

**Mixed lymphocyte reaction (MLR) assays:** Lymphocytes purified for CD3+ T cells from BALB/c mouse spleens using the Cellfect mouse T cell Kit (Cytovax Biotechnologies) with either unstimulated PEC-macrophages; IL-4 plus GM-CSF stimulated macrophages or peritoneal macrophage-derived DC additionally prepared from P30 transgenic mice were co-cultured in 96 well flat-bottomed tissue culture plates (Falcon, U.S.A.). Individual wells contained 2 × 10^5 CD3+ T cells together with a range of concentrations of peritoneal macrophage or peritoneal macrophage-derived DC in a total volume of 100 μl. Responder CD3+ T cells were cultured with a range of concentrations of the generated DC to give final responder:stimulator ratios of 1:1, 10:1 and 100:1. Control cultures contained responder cells or stimulator cells alone. Cultures were established in triplicate wells. Cultures were incubated at 37°C in 95% air, 5% CO2 for 72, 96 and 120 hr. Four hours before the termination of cultures, cells were pulsed with a non-radio active cell counting kit-8 (Dojindo, Japan). Cells were then harvested and using an ELISA reader absorbance was determined at 450 nm respectively.

**Confocal laser scanning microscopy:** Cytospin preparations of generated peritoneal cavity macrophage-derived DC and PEC-MΦ were prefixed in acetone. Cells were stained with labeled anti-mouse MHC class II (I-A^d/) FITC (AF6-120.1) mAb (Pharmingen International, U.S.A.). All incubations were carried out in a humidity chamber at room temperature. Binding was visualized with a single step technique involving incubation with labeled mAb. After incubation cells were washed and mounted in Vecta-shield (Vector Laboratories, U.S.A.) mounting medium and observed using a Leitz TCS-SP confocal laser-scanning microscope (Leica, Switzerland).

**Flow cytometry:** Generated PEC macrophage-derived DC and PEC-MΦ were stained with labeled anti-mouse MHC class II (I-Ab)/ FITC (AF6-120.1), anti-CD11b FITC (Mac-

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**Data analysis:** Statistical analysis of the results of the antigen presentation assay was carried out as a matrix of pair-wise comparison probabilities (P) in multiple analyses of variance.

**RESULTS**

**Characterization of isolated PEC-DC and PEC-MΦ:** The accessory function of the PEC-DC, unstimulated and IL-4 plus GM-CSF stimulated PEC-MΦ were investigated using a two-way allogeneic MLR. The antigen presenting cell (APC) and T cell interaction is dependent on the expression of MHC class II molecules [17, 43]. Therefore, as a preliminary to investigating the accessory cell capabilities of PEC-DC and PEC-MΦ, the MHC class II expression in the cell isolates were assessed as an indication of the effect of the isolation procedure on this parameter. PEC-DC were highly positive for MHC class II molecules compared to PEC-MΦ (Fig. 1). The results of the expression of MHC class II molecules in PEC-DC and PEC-MΦ as determined by confocal laser microscopy are summarized in Fig. 2. The criteria of relative size, shape and position of nucleus, cytoplasm:nucleus ratio and cytoplasmic processes were used to analyze the morphological features of PEC-DC. Subjectively, PEC-DC were generally smaller that PEC-MΦ. Flow cytometry showed that, 98.7% PEC-DC expressed MHC class II, while only 90.8% PEC-MΦ expressed MHC class II molecules (Fig. 1). The use of either 10 µg/ml TLA or 1 µg/ml LPS in combination with 250 U/ml IFN-γ did not have an effect on the expression of MHC class II molecules on PEC-DC. Moreover, data obtained using stimulators either from p30 transgenic mice, p30 negative littermates or wild type mice was the same (data not shown).

**Allostimulatory activity:** As a definitive criterion for classification as DC, PEC-DC were tested by MLR for their capacity to stimulate allo-reactive naive T cells under similar culture conditions and time simultaneously. In two experiments PEC-DC, unstimulated PEC-macrophages and IL-4 plus GM-CSF stimulated macrophages were isolated from P30 transgenic mice and co-cultured with CD3+ T cells from a BALB/c mouse over a range of stimulator:responder ratios. The results of the proliferation data (OD) are summarized in graphic form in Figs. 3, 4 and 5. Proliferation is expressed as mean optical density and where indicated with standard deviation values. For statistical analysis, data from each responder:stimulator ratio were pooled between each allogeneic experiment and each syngeneic experiment and multiple analysis of variance was applied.

There were no significant proliferative responses in syngeneic co-culture at any of the responder: stimulator ratios tested (data not shown). However, allogeneic responses were different from syngeneic responses at all responder:stimulator ratios combinations. Allogeneic responses at a 1:1 responder:stimulator ratio were significantly greater than allogeneic responses at 10:1 (P<0.00001 for PEC-DC, P<0.0003 for stimulated PEC-MΦ, P<0.0004 for un-stimulated PEC-MΦ) and 100:1 (P<0.000007 for PEC-DC, P<0.00002 for stimulated PEC-MΦ, P<0.00006 for un-stimulated PEC-MΦ). However, allogeneic responses at a 10:1 ratio were not significantly different from responses at a 100:1 ratio (P>0.7) for stimulated and un-stimulated PEC-MΦ. Interestingly, responses at a 10:1 ratio were significantly different from responses at a 100:1 ratio (P<0.0007) for PEC-DC.

Statistically significant differences were observed when allogeneic responses at 1:1 responder:stimulator ratio combination of PEC-DC where compared to un-stimulated and stimulated PEC-MΦ (P<0.0001, for un-stimulated PEC-MΦ and P<0.00005, for stimulated PEC-MΦ). A compari-
son of allogeneic responses at 100:1 responder:stimulator ratio combination between the three stimulator cell types showed that responses were not significantly different (P>0.5). However, responses at 10:1 responder:stimulator ratio PEC-DC when compared to stimulated or stimulated PEC-MΦ were statistically significant (P<0.001) respectively.

It can also be seen that the magnitude of the proliferative responses in all allogeneic MLR using the three stimulator cell-types increased with time and ratio (Figs. 2, 3 and 4). It is not clear from the results whether responses had reached an optimum at 120 hr. However, in some experiments, responses after 120 hr showed a downward trend (data not shown), indicating that they had reached their maximum at 120 hr.

**DISCUSSION**

Allo-reactivity in *in vitro* MLR is thought to be a function of the T cell receptor (TCR) recognition of allogeneic MHC class II as a foreign peptide complex [25]. The most prominent if not exclusive function of DC is providing accessory function. This study has demonstrated that both PEC-MΦ and PEC-DC can efficiently stimulate an allogeneic MLR. However, allogeneic responses initiated *in vitro* by PEC-DC were of a higher magnitude than PEC-MΦ, demonstrating that the generated PEC-DC have potent MLR activity, consistent with their predicted activity as DC.

There is now extensive evidence that DC are principal stimulators of allogeneic lymphocytes, for example, in the mouse and rat [12, 14, 36, 42]. In these and several other studies DC were reproducibly the most potent stimulators of allogeneic MLR when compared to macrophages. The results of this study are in agreement with those of Spalding *et al.* [34], who demonstrated the stimulatory ability of mouse splenic DC in allogeneic MLR. Similar work on DC from mouse intestinal lamina propria and pig Peyer’s patches [21, 20, 27, 40], as well as studies of DC from the human colonic lamina propria demonstrated the presence of cells with potent stimulatory activity in the allogeneic MLR.

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*Fig. 2. Expression of MHC class II membrane antigens as determined by confocal laser scanning microscopy on cytospin preparations. (A) Peritoneal cavity macrophage-derived dendritic cells (PEC-DC). (B) Peritoneal cavity macrophages (PEC-MΦ). PEC-DC were highly positive for MHC class II antigens. C and D are the actual cells (A and B respectively) without fluorescence as determined by confocal laser scanning microscopy. Note that, Subjectively, PEC-DC were generally smaller that PEC-MΦ (×670).*
In contrast, Langerhans’ cells are weak stimulators of the allogeneic MLR upon initial isolation [9], but in the presence of GM-CSF and IL-1, in vitro rapidly mature into potent MLR stimulators [5, 41]. Variations in levels of cytokine production in the epidermis and the intestine may account for the differences in maturity of DC at the two sites. Both IL-1 and GM-CSF, which have been reported to amplify DC function [15] are produced by cells of the human intestinal tissues [30], but levels have not been compared with those of the epidermis.

Peritoneal cavity macrophage derived-DC from p30 transgenic mice demonstrated greater allogeneic MLR stimulatory activity when incubated with T cells from inbred BALB/c mice at all three responder:stimulator ratios and time points. Significant proliferation was observed in our study using stimulators from p30 transgenic mice and responders from BALB/c demonstrating that the used stimulator and responder cells were different at specific MHC class II loci. This is in agreement with previous reports that MHC class II molecules are involved in lymphocyte transformation and proliferation [31, 37] and that T cell proliferation occurs when cells of different MHC haplotype are mixed in a MLR culture [32]. We have also shown that clustering with dendritic cells or other professional or non-professional APC precedes and is essential for T-cell proliferation [1].

Despite the demonstration that PEC-DC had a potent MLR activity which was higher compared to PEC-MΦ we can speculate that the variations observed in the kinetics between MLR experiments using different stimulator cell types may have been due to the fairly complex isolation procedure. The actual time taken to set up the experiments introduced limitations as T cells tended to clump. An effect as T cell clumping would be expected to produce variations within the experiments. The apparent variations in the representative MLR may also be an in vitro artifact caused by suppressive factors secreted during cell isolation or due to damage the cells are subjected to during the long isolation technique, chemical, cytokine and antigen treatment. Prostaglandin producing cells have been implicated in immunosuppression in the lung [6]. Studies have also demonstrated opposing actions of DC and macrophages in vivo. This may be a possible explanation in the case of possible contamination of the PEC-DC population by macrophages or T cells. In contrast, other studies have shown that macrophages can increase the sensitivity of MLR to Peyer’s patch cells [27].
It is most likely that this effect may be mediated by macrophages production of IL-1 which is not produced by DC [16], but which amplifies DC function [15] and all these factors require further experimental analysis. Although it has been reported in previous studies that an allogeneic MLR can occur without the involvement of IL-1 [23], it is likely that all T cell responses require a number of co-stimulatory factors [11]. Finally, this study has demonstrated that PEC macrophage-derived DC have a potent MLR stimulatory activity which was statistically significant and of consistent amplitude, thus demonstrating their most important APC function for T cell proliferation. This stimulatory activity was higher than that observed in both stimulated and unstimulated PEC-MΦ. Moreover, PEC-DC were shown to exhibit more class II expression than PEC-MΦ. Further studies need to be carried out to determine the exact factors that may be involved in this stimulatory activity variation between the APC cell types used in this study.

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REFERENCES


