Prolonged expression of MHC class I - peptide expression in bone marrow derived retrovirus transfected matured dendritic cells by continuous centrifugation in the presence of IL-4

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Background & objectives: Dendritic cells (DCs) are potent antigen presenting cells which proceed from immature to a mature stage during their differentiation. There are several methods of obtaining long lasting mature antigen expressing DCs and different methods show different levels of antigen expressions. We investigated bone marrow derived DCs for the degree of maturation and genetically engineered antigen presentation in the presence of interleukin-4 (IL-4) as a maturity enhancer.

Methods: DCs and transfected retrovirus were cultured together in the presence of granulocyte-macrophage colony stimulating factor (GMCSF)-IL4, GMCSF +IL4, lipopolysaccharide (LPS). B 7.1, B7.2 and CD11c were measured by the degree of immune fluorescence using enhanced green fluorescent protein (EGFP) shuttled retrovirus transfected antigen. Degree of MHC class I molecule with antigen presentation of antigen was also evaluated by fluorescence activated cell sorting. The antigen presenting capacity of transfected DCs was investigated. Bone marrow DCs were generated in the presence of GMCSF and IL-4 *in vitro*. Dividing bone marrow cells were infected with EGFP shuttled retrovirus expressing SSP2 by prolonged centrifugation for three consecutive days from day 5, 6 and 7 and continued to culture in the presence of GMSCF and IL-4 until day 8.

Results: IL-4 as a cytokine increased the maturation of retrovirus transfected DCs by high expression of B 7-1 and B 7-2. Also, IL-4 induced DC enhanced by the prolonged centrifugation and it was shown by increased antigen presentation of these dendric cells as antigen presenting cell (APC). Cytolytic effects were significantly higher in cytotoxic T cell response (CTLs) mixed with transfected DCs than CTLs mixed with pulsed DCs.

Interpretation & conclusions: There was an enhanced antigen presentation by prolonged expression of antigen loaded MHC class I receptors in DCs in the presence of IL-4 by prolonged centrifugation.

Dendritic cells (DCs) are the most potent antigen presenting cells which play a critical role in activating T cells to initiate the adaptive immune response[1]. These cells recognize the foreign material in blood and tissues and make antigens on the surface with MHC molecules[2-4]. Though most of the studies are focused on the induction of cytotoxic T cell response cytotoxic T cell response (CTL) by DCs, the failure rate is high.

Reasons for the failure may be unstability of pulsed peptides on DCs, low number of MHC molecule expressions and loss of MHC expressions[5]. Considering these defects, we hypothesized that augmentation by any process for antigen expression in DCs and stable prolonged expression of MHC molecules will be helpful to induce long lasting expressions. We concentrated on the process of genetic engineering of MHC antigens[6], co-stiumulatory molecules[7], cytokines[8] and chemokines[9] because it is known that murine DCs retrovirally transfected with gene encoding β-galactosidase stably express, process and present it in the context of MHC class I molecules[10]. Genetic modification of DCs can be successively done with antigen engineered cells in the presence of granulocyte-macrophage colony stimulating factor (GMCSF) and interleukin-4 (IL-4) and specific CTL activity can be induced *in vitro*[10-17]. It has also been reported that IL-4 can produce significant percentages of matured DCs *in vitro* than in the presence of other cytokines[18-24].

We hypothesized that prolonged contact of antigens with DCs can lead to a higher possibility of capturing antigen and internalization and processing than just pulsing during centrifugation. We therefore, investigated the degree of DCs maturation in the presence of IL-4 and the level of MHC class I expression.

Material & Methods

The study was conducted in Nagasaki University. Male and female 6-8 wk old BALB/c mice (Jackson Laboratory, Bar harbor, Maine) were housed under pathogen free state. The study protocol was approved by the Animal Ethics Committee of the Nagasaki University.

PT 67 packaging cells from Clontech, Japan, were used for retroviral stable cell transfection. For cell cultures the complete RPMI culture media was used. For the preparation of complete RPMI, RPMI1640 was supplemented with 2 mM L-glutamine, 0.1 mM non essential amino acids, sodium pyruvate, antibiotic: penicillin (50 μg/ml) streptomycin (50 μg/ml *p*H 7.4, GIBCO/BRL, Japan) and 10 per cent heat activated foetal calf serum (FCS). For adherent packaging cells used in transfections, D-MEM cell culture media (Dulbecco's modified eagle medium, *p*H 7.4, GIBCO/BRL) was supplemented with 2 mM L-glutamine, 0.1 mM non essential amino acids, sodium pyruvate, antibiotics: penicillin (50 μg/ml) streptomycin (50 μg/ml, GIBCO/BRL, Tokyo Japan) and 10 per cent heat inactivated FCS.

Identification of antigen peptides: First 303 amino acids of the surface sporozoite protein (SSP2) were selected for the immunization of BALB/c mice. To obtain the SSP2 DNA, nucleotides from 718 to 1630 were selected and synthesized using primers (USA) by polymerase chain reaction[22].

Five different peptides were recognized in the amino acid sequence which consisted of MHC class I restricted SSP2 binding motifs. Each peptide consisted of two binding domain at two sides. These five peptides were named as P1, P2, P3, P4 and P5 (Table).

Preparation of k^d restricted MHC motifs: Five peptides binding motifs, P1-P5 were purchased from Sawafy Technology Co. Ltd., Tokyo, Japan. SSP2 DNA was used for the DNA sequencing procedure and the nucleotide pattern was identified from nucleotide bank.

Peptide gene transfection to retrovirus vector: cDNA encoding SSP2 was used as the template DNA for the synthesis of the SSP2 DNA. SSP2 DNA was amplified using primers 5' AATGGATCCATGAAGCTCTTAGGATGGT and 3' ACCTTAGTTCCAGTTA and amplified SSP2 DNA was cloned to PGEM3Zf cloning vector (Promega, Japan). Insertion was confirmed by the agarose gel electrophoresis. Purified SSP2 DNA was ligated to the pMCSV puro vector at 5' EcoR1 and 3' Hpal enzyme restriction sites for the transfection for mammalian cells[22].

Retroviral gene transfection of DC cells: Retroviral gene transfer is a technique for efficient introduction of stable heritable genetic material into the genome of any dividing cell type. Current retroviral gene transfer technology was used with a co-ordinated design of packaging cell lines. Packaging cells package recombinant retroviral RNA into infectious but replication-incompetent viral particles which were

Table. Details of amino acid sequence of selected peptide antigens for the study

Peptide sequence	Starting and end amino acid	Name of peptide
KYIFVVLLL	8 -16 - k^d	P1
MYRPDAIQL	143-151- k^d	P2
VFLNGQETL	20-28- k^d	P3
GYKIAGGII	760-768 - k^d	P4
IFVVLLLCI	10-18- k^d	P5

used for the transfection. Once these packaging cells were transfected with a retroviral expression vector with desired peptides, these are packaged to cells within 48-72 h. In this experiment murine stem cell retrovirus (pMCSV puro, Clontech, Japan) was used for the introduction of SSP2 DNA. Six-well plate is 80 per cent confluent with PT67 packaging cells (instruction manual, Clontech, Japan) were transfected with 10 μg of constructed retrovirus DNA by DMRIE-C liposomal transfection reagent (LIFE technologies, GIBCO-BRL, Japan) and cultured with DMEM medium in CO_2 incubator at 37^0C for overnight. Transfected cells were selected by pulsation with 5 μM of puromycin and continued to culture for one week to obtain stable expressed cell line. This PT67 cell supernatant was used for infection of DCs used for this experiment to assess the maturation efficacy in the presence of IL-4.

DC generation and transfection: Bone marrow from 6-8 wk old BALB/c mice was flushed with complete RPMI medium and centrifuged at 1100 rpm for 5 min. Cell precipitate was re-suspended with new RPMI medium and seeded in 24 well culture plates ($1x10^6$ cells) in the presence of murine recombinant GMCSF (500 U/ml) and murine recombinant IL-4 (400 U/ml) in 5 per cent CO_2 incubator at 37^0C for 7 days. Culture medium was replaced with new medium containing GMCSF and IL-4 on every third day. Bone marrow cells ($1x10^6$/well) were infected with retrovirus expressing SSP2 (pMSCV puro-SSP2) by prolonged centrifugation (2500 rpm) for one hour at 32^0C on 5th, 6th and 7th days. Each day after transfection, medium was replaced with fresh medium containing GMCSF and IL-4.

Detection of MHC expressing DCs by fluorescence activated cell sorting (FACS): This technique was used to identify the different types of the cells form a cell mixture using specific monoclonal antibodies. Matured dendritic cells were identified by staining with DC specific monoclonal Ab (mAb). DCs were incubated with rat anti-mouse mAb for expression of co-stimulatory molecules, B7.1/B7.2 (CD80/CD86, 1:1000 diluted, Pharmingen, Japan) for 30 min at 4^0C. Then it was followed with biotinylated anti-rat IgG antibodies (H+L. Jackson Immunoresearch Laboratories, Japan). Rat IgG (Jackson Immuno-research Laboratories, Japan) was used to avoid cross-reactivity during double labeling of B7 mAb and CD11c-FITC mAb (Becton Dickson, Japan). Thereafter, cells were washed and double labelled with (PE) conjugated avidin and FITC conjugated CD11c mAb. For detection of K^d molecules, these DCs were incubated with biotin conjugated anti-mouse K^d mAb (CTkb, Cedarline Laboratory, Japan) and followed with PE conjugated avidin (Jackson Immunoresearch).

Cytolytic activity of CTLs with pulsed DCs or transfected DCs: Spleen cells were divided into four groups for co-culture with antigen pulsed (20 μg/ml) DCs, non pulsed-negative DCs, antigen transfected DCs and with mock transfected DCs. Pulsation was done in the absence of FCS at 37^oC for 2 h. For antigen specific CTL induction, antigen was directly added to the culture plate in complete RPMI medium containing 10 per cent FCS for 7days. CTLs were restimulated with peptide pulsed mitomycin treated synergistic antigen presenting cell (APC) in the presence of 20 μ/ml recombinant IL 2 on day 7 and restimulation continued weekly. After 7 days, viable cells were harvested and tested for cytolysis assay. To assess the efficacy of antigen presentation of DCs, both pulsed and transfected DCs were used as target cells labelled with ^{51}Cr for 1 h at 37^oC and co-cultured with different types of target cells for 4 h at 37^oC in triplicate (50:1 ratio). Supernatant was used for release by gamma counter. The standard formula 100 x [(exp release-spon release)]/[(maximum release-spon release)] was used[22].

Statistical analysis: Data are presented as the mean ± SD of three experiments. Probability of significance was determined by two-tailed independent Student's t-test. $P<0.05$ was considered significant.

Results

Successful generation of bone marrow derived dendritic cells in the presence of Il-4: Bone marrow derived DCs were successfully generated in the presence of GMCSF and IL-4 *in vitro*. Cultured dendritic cells generated a consistent DC population of which 50-80 per cent of cells expressed CD11c, CD80 and CD86 the characteristic maturation surface markers (Fig. 1).

Matured bone marrow derived dendritic cells express co-stimulatory molecules: For the assessment of maturity of transfected DCs cells were cultured with GMCSF alone and transfected with SSP2 protein either in the presence or absence of IL-4. Cultured DCs expressed characteristic surface marker, CD11c enabling the identification of matured DCs among the bone marrow cell population. The number of CD11c positive CD80, CD86 and MHC class I expressing dendritic cells were analyzed by FACS. Data showed that bone marrow cell cultured with GMCSF alone
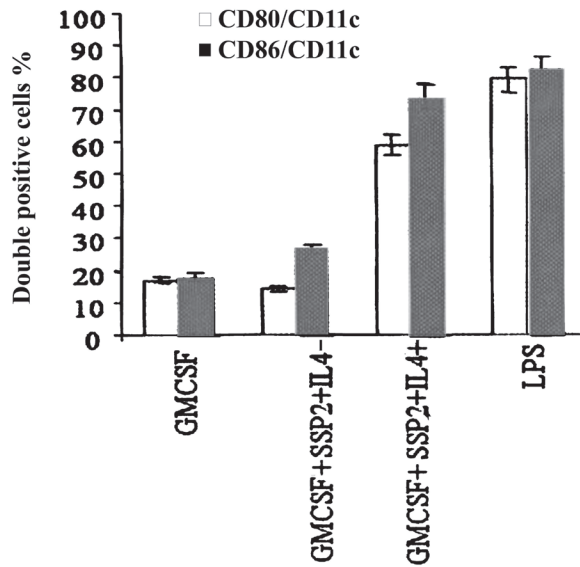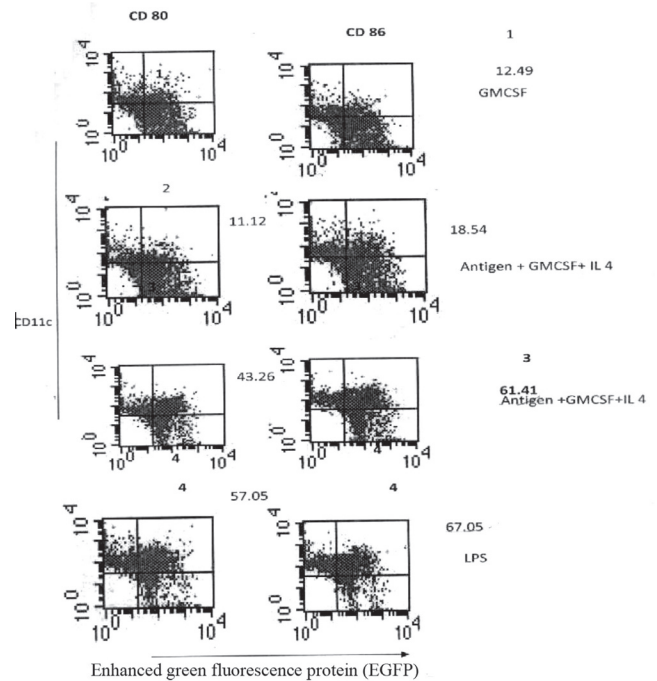
Fig. 1. For phenotype analysis, 10⁶ DCs were incubated with anti CD80 (B7.1), anti CD86 (B7.2) and double stained with CD11c directly labelled antibody. For B7-1 and B7-2 double staining, cells were cultured with GMCSF alone, (A2, B2) SSP2 transfected cells in the presence of GMCSF alone, SSP2 transfected cells in the presence of GMCSF with IL-4 and (A4 and B4) LPS (positive control) respectively. Values are mean ± SD.

Fig. 2. Shows percentage of DCs expressing B7.1 and B7.2 with CD11c positive cells in each different culture situations (GMCSF alone, SSP2 transfected, SSP2 transfected in the presence of IL-4, and LPS).

Fig. 3. Degree of MHC class I expression in CD 11c dendritic cells were seen in each different culture situations GMCSF alone, GMCSF with SSP2 transfected DCs with IL-4 and without IL-4 And positive control LPS. Mean fluorescence intensity of double stained cells is given. An istotype antibody was used as control. The experiment was repeated several times, one representative result is shown.
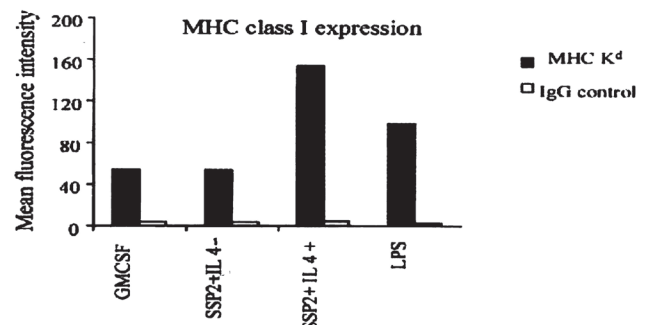
and cells transfected with retrovirus in the GMCSF without IL-4 expressed low level of CD80, CD86 and MHC on the cell surface. Expression of CD 80 and CD 86 molecules was increased in transfected cells in GMCSF in the presence of IL-4 when compared to the cells without IL-4 in culture media (Fig. 2).

Upregulation of MHC class I molecule in bone marrow derived dendritic cells in the presence of GMCSF + IL-4: To analyze the effects of IL-4 on the expression of MHC class I molecules in bone marrow derived DCs, DCs were cultured in the presence of GMCSF alone, GMCSF with IL 4, without IL-4 and positive control. Highest expression of MHC class I was shown in the presence of GMCSF and IL-4 when compared to the other culture situations (Fig. 3). It further showed that MHC class I expression was low in the absence of SSP2 transfected retrovirus culture.

Prolonged expression of antigen loaded MHC class I in transfected DCs after prolonged centrifugation: Transfection efficacy was evaluated by infecting the DCs with EGFP shuttled retrovirus vector expressing SSP2. To analyse the duration of gene expression, transfected cells were scanned using FACs for EGFP expression from day 8 to day 12. Morphology of DCs

were not changed by microscopic viewing and the viability of DCs were assessed by stained cell counting. EGFP was expressed in DCs until day 12 since culture (Fig. 4). This data confirmed the prolonged expression of introduced gene in retrovirus transfectants. For detection of SSP2 protein, transfected DC lysates from day 8 to 12 were used for immuno-precipitation with anti SSP2 Ab. Results of Western blotting showed that
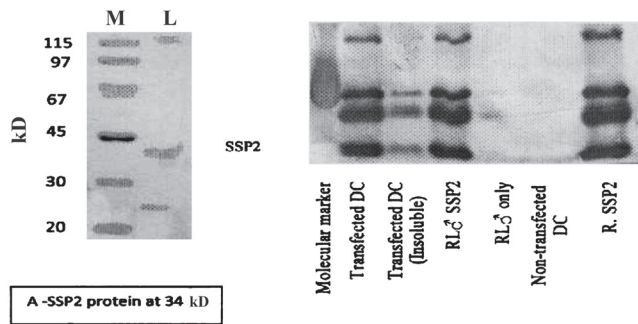
Fig. 4A. Shows the expression of recombinant SSP2 protein (34 kD protein) after PCR synthesis by agarose gel electrophoresis. It was detected as 34 kD protein. Protein expression is shown in soluble and insoluble lysates of transfected DCs, transfected RL tumour cells as a control, non-transfected DCs and non-transfected RL tumour cells. Tumour cell line is also used in this experiment for the comparison among different types of antigen presenting cells. Synthesized SSP2 protein was used as the positive control. M, Marker; L, SSP2 injected line.
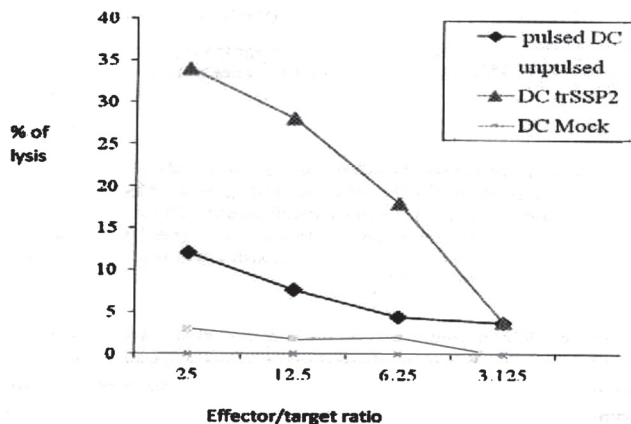
Fig. 4B. CTLs from BALB/c mice were analyzed for its cytolytic activity of the matched antigen-pulsed target cells by ^{51}Cr-release assay. For antigen specific CTL induction, CTLs were cultured with A and C peptides for 7 days. For cytolytic assay, CTLs were re-cultured with peptide-pulsed and transfected DCs and transfected P815 tumour target cells and mock transfected for the analysis for Cr-release. These antigens specific CTLs identified and killed significantly higher number of peptide transfected targets than the pulsed targets. Cytolytic activity of transfected target cells is significantly more than the RL♂ mock cells. Data represent one of the three experiments.

SSP2 was expressed from day 8 to day 12 (Fig. 5, data not shown).

CTL assay comparison between peptide pulsed and antigen transfected DCs: The efficacy of CTL lysis was compared by antigen pulsed DCs and antigen-SSP2 transfected DCs by Cr release assay. To analyze the efficacy of antigen presentation of transfected DCs
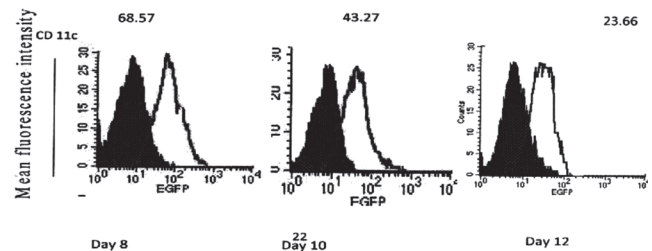
Fig. 5. Enhanced transfection efficacy of retrovirus transfected dendritic cells by prolonged centrifugation. DCs were infected with EGFP shuttled retrovirus vector and green fluorescence was measured by FACS. Cells were compensated for each staining (CD11c and EGFP) and number of CD11c positive EGFP expressing cells is given. Kinetic study of CD11c expressing DCs was done from day 8 to day 12. Data show the mean fluorescence intensity with time.

and pulsed DCs, these transfected DCs after prolonged centrifugation, were injected *i.p* for three times at weekly interval into BALB/c mice. The spleen cells were collected on day 7 since last immunization. Fig. 4B shows that cytolytic effects are significantly higher in CTLs mixed with transfected DCs than CTLs mixed with pulsed DCs.

Discussion

DCs are the most potent stimulators of primary immune responses and have been recognized as potential tools for vaccine productions[10-16]. DCs are usually stimulated and matured by pulsation of antigens[13]. This leads to the dendritic cell maturation and these are usually stable only for hours[10]. It has been shown the induction of immunity by DC vaccines largely depends on maturation stages because it determines the functional stages. To induce cytotoxic T cell response, DCs should be matured and capable of expressing MHC molecules[10,13,23]. Because of this observation we hypothesized that DCs can be better stimulated and matured and can be made stable using viruses because virus can directly transfer antigen peptides to the ER-MHC molecule via proteosome. Our results confirmed that antigen transfected retrovirus vector stimulated immature dendritic cells to express MHC molecules and co-stimulatory molecules as earlier as day 6 of culture. These DCs expressed the MHC class I molecule until day 6 while some researchers found that pulsation only expressed for few hours[10]. MHC expression of pulsed DCs and transfected DCs in the presence of IL 4 could not be compared. It is a known fact that pulsation of antigen is not an endogenous engineered process and this might have led to high dissociation power and low dissociation time of antigen from MHC class I

molecule[10]. In addition, pulsation of antigen to DCs has shown very low binding efficacy[10]. This can be explained by the process of natural antigen processing technique in the body. Our results corroborated with that of Liu *et al*[23] showed that vaccination of mice with engineered fusion hybrid vaccine enhanced tumour protection. This explains that primary DCs can be matured for CTL induction by genetically modified retrovirus transfection effectively.

Maturation kinetics are not well studied and maturation of DCs even in the presence of GMSCF was low when compared with the presence culture of IL-4 in culture media[22,23]. In contrast, we showed that retrovirus transfected DCs were more matured in the presence of IL-4 in addition to the GMCSF. This explained that IL-4 facilitated the upregulation of MHC class I molecule from the inside the DC cell. In addition, it might have increased the processing of antigens into peptides through proteosome. Our results are similar to those of Liu *et al*[23] who found that IL-4 was helpful in DC maturation when there was a blockage from tumour cells. IL-4 has been shown to be more effective in differentiation of DCs from peripheral blood cells than IL-13[24]. IL-4 induced DCs exhibit greater endocytic capacity at low antigen concentrations and these DCs were better stimulators of allogeneic T cells than IL-13[14]. Our results further showed gentle upregulation of co- stimulatory molecules like B7.1 and B7.2 in the presence of IL-4 when compared to other positive control.

Our study had some limitations. We could not compare with the other interleukins like IL 2 IL 6 and IL 12 which are important for the DCs and CD8 T cell maturation.

In conclusion, results of our study indicate that murine DCS can be retrovirally transfected by using special technique - prolonged centrifugation for the enhanced expression of antigen in the surface of the cells. Antigen transfected by retrovirus by this technique in the presence of IL-4 generated functionally matured DCs expressing co-stimulatory molecules which showed readiness for priming of T cells. These cells expressed the transfected gene in the DCs at a higher level and IL -4 enhanced the processing and presenting the antigen into the internal MHC molecule. This technique can be applied in the process of induction of CD 8 T cell immunity using DCs as vaccine targets. DCs can be made more stable and more expressive with prolonged centrifugation without any damage to the DC morphology.

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