



저작자표시 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.
- 이 저작물을 영리 목적으로 이용할 수 있습니다.

다음과 같은 조건을 따라야 합니다:



저작자표시. 귀하는 원저작자를 표시하여야 합니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 [이용허락규약\(Legal Code\)](#)을 이해하기 쉽게 요약한 것입니다.

[Disclaimer](#) 

의학석사 학위논문

**Types of antigen presentation pathways
determine the dominance of CD8 T-cell response
for minor histocompatibility antigens
after allogenic skin transplantation**

동종피부 이식에서 부조직적합항원의
면역원성의 생성에 항원제시세포의 두 종류의
항원제시경로가 관여 한다는 것에 대한 연구

2014 년 2 월

서울대학교 대학원
의과학과 의과학과전공
유 강 일

**Types of antigen presentation pathways
determine the dominance of CD8 T-cell response
for minor histocompatibility antigens after
allogenic skin transplantation**

by
Kang Il Yoo

A thesis submitted to the Department of Biomedical
Sciences in partial fulfillment of the requirements for
the Degree of Master of Science in Medicine at Seoul
National University College of Medicine

Jan 2014

Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Types of antigen presentation pathways determine the dominance of CD8 T-cell response for minor histocompatibility antigens after allogenic skin transplantation

유강일(Yoo, Kang il)

의과학과 의과학과 전공(Major in Biomedical Science

Department of Biomedical Science)

The Graduate School

Seoul National University

Immunogenicity of minor histocompatibility antigens (minor H Ags) relies on the polymorphism at the MHC-presenting peptides. Disparities at the MHC-presenting peptide between MHC-matched donor and recipient induce T cell response and are considered to be risk factor leading to failure of the transplantation. Under multiple antigen-disparity condition, such as B6 anti-BALB.B setting, T cell responses are focused on a few antigens, due to immunodominance phenomenon. H60 and H4 have been known to be dominant minor H antigens in the B6 anti-BALB transplantation

circumstance. In this study, We aimed to understand contribution of CD8 T-cell responses specific for antigens, which are ubiquitously expressed and those expressed restrictedly to hematopoietic cells, to the allo-responses after MHC-matched skin transplantation. We tracked longitudinally CD8 T-cell responses for H60 (with hematopoietic restricted expression) and H4 (with ubiquitous expression) via flow cytometry, and found that frequencies of CD8 T-cells specific for H60 increased ahead of those for H4. Even though H4-specific CD8 T-cell response was dominant over H60-specific CD8 T-cell response, preformed memory CD8 T-cells specific for H60 reduced the expansion of H4-specific CD8 T-cells, expediting the graft-rejection. H60-specific CD8 T-cell response was dominant over H4-specific CD8 T-cell response when host allo-response was induced via direct antigen presentation through donor APCs (early phase of graft rejection), while H4-specific response was dominant when allo-response was induced via indirect presentation by host APC. Altogether, it was found that H60- as well as H4-specific CD8 T-cell responses contributed to allo-graft rejection, on the contrary to previous study. And, changes in the hierarchy of immunodominance depend on origin of APCs presenting antigens to T cells.

Key word: Skin transplantation, Minor histocompatibility antigens, Hierarchy of immunodominance, Ag presentation

Student number: 2011-23804

CONTENTS

Abstract.....	I
Contents.....	IV
List of tables and figures.....	VI
Introduction.....	1
Material and Methods.....	3
Mouse.....	3
Skin transplantation.....	3
In vivo imaging.....	4
Analysis of bioluminescence data.....	4
Antibodies and flow cytometry.....	5
IFN- γ secretion analysis.....	5
Statistical analysis.....	6
Result.....	7
Lumbar LN (draining LN) are significant sites for mH-Ag-specific CD8 T-cell activation in multiple mH Ag-mismatched skin transplantation.....	7
Changing hierarchy of mH-Ag-specific CD8 ⁺ T-cell after multiple mH-Ag-mismatched skin transplantation.....	13
H60 and H4 mH-Ag affect Graft rejection and mH-Ag-specific CD8 T-cell response at mH-Ag mismatched skin transplantation.....	17
H60 specific CD8 T-cell response can influence to other mH-Ag specific CD8 T-cell response in multiple mH-Ag skin transplantation setting.....	21

Direct presentations through Donor APCs were associated with higher H60 than H4 specific CD8 T-cell response.....	26
Indirect presentations through Host APCs were associated With Changing Immune hierarchy of H60 to H4 mH Ag.....	31
Discussion.....	34
Reference.....	38
요약(국문초록).....	44

List of Figure

Figure 1. Lumbar LN (draining LN) are significant sites for mH-Ag-specific CD8 T-cell activation in multiple mH Ag-mismatched skin transplantation.

- A. Survival graph of B6 anti BALB.B skin transplantation.
- B. Experimental system of visualize the *in vivo* response of CD8 T-cells against allogeneic mH-Ag originating from the allograft BALB.B
- C. Time kinetics of BLI signal in draining LN, spleen and graft site
- D. Experimental system of visualize the *ex vivo* response of CD8 T-cells against allogeneic mH-Ag originating from the allograft BALB.B
- E. Adoptive transferred CD8 T-cell frequency in second lymphoid organ analyzed using flow cytometry.
- F. Dot-blot graph about Fig 1-E flow cytometry result.

Figure 2. Changing hierarchy of mH Ag-specific CD8⁺ T-cell after multiple mH Ag-mismatched skin transplantation.

- A. H60, H4 specific CD8 T-cell response analyzed using flow cytometry
- B. Dot-blot graph about Fig 2-A flow cytometry result.
- C. Result of INF-g assay

Figure 3. H60, H4 mH-Ag affect Graft rejection and mH-Ag-specific CD8 T-cell response at mH-Ag mismatched skin transplantation

- A. H60, H4 specific CD8 T-cell response analyzed using flow cytometry
- B. Mean survival time between immunized host and B6 host

Figure 4. H60 specific CD8 T-cell response can influence to other mH-Ag specific CD8 T-cell response in multiple mH-Ag skin transplantation setting.

- A. Experimental system of skin transplantation.
- B. H60, H4 specific CD8 T-cell response analyzed using flow cytometry
- C. Bar graph about Fig 4-B flow cytometry result.
- D. Result of INF-g assay

Figure 5. Direct presentations through Donor APCs were associated with higher H60 specific CD8 T-cell response than H4 specific CD8 T-cell response.

- A. Experimental system of skin transplantation.
- B. H60, H4 specific CD8 T-cell response analyzed using flow cytometry
- C. Bar graph about Fig 5-B flow cytometry result.

Figure 6. Indirect presentations through Host APCs were associated with changing immune hierarchy of H60 to H4 mH-Ag.

- A. Experimental system of skin transplantation.
- B. H60, H4 specific CD8 T-cell response analyzed using flow cytometry
- C. Bar graph about Fig 6-B flow cytometry result.

Introduction

Immune system rejection of MHC-matched but minor histocompatibility antigen (mH-Ag-) mismatched skin transplantation is induced by mH-Ag-specific cytotoxic CD8 T lymphocytes. In the case of MHC-matched but multiple mH-Ag-mismatched transplants, only a limited number of antigenic epitopes among the various background genes induce a CD8 T-cell response, resulting in oligoclonal expansion of CD8 T-cells, which is called the immunodominance phenomenon.(1-3) For example, although more than 26 mismatched mH-Ags have been defined in the MHC-matched C57BL/6 (B6) anti-BALB.B combination, CD8 T-cell responses are predominantly directed to a few of these (4). Under the multiple mH-Ag-disparity condition, H60 mH-Ag and H4 mH-Ag have been known to dominate the other mH-Ags, generating immunohierarchy with the CD8 T-cell responses on top. In general, H60 mH-Ag has been found to be a dominant mH-Ag in heart and vascularized cardiac transplantation (5). Contrarily, H4 mH-Ag has been founded to be dominant in skin transplantation (6).

The mH Ag-specific CD8 T-cell responses after transplantation are known to be induced via two pathways: one through recognition of donor-originated Antigen-presentation Cell (APC) by T-cells (direct pathway) or through

recognition of host-originated APC (indirect pathway). In the direct pathway, T-cells recognize intact donor MHC molecules on transplanted cells (7-10). The indirect pathway involves the recognition of donor peptides that have been processed and presented by host APCs (11-13). mH Ag-specific CD8 T-cell activation through the direct pathway is thought to influence acute rejection of transplants, whereas the indirect pathway is involved in chronic rejection (14). In skin transplantation, either the direct, indirect or both ways can be considered to be involved in the rejection of a skin allograft (15). However, it is not clear which pathway is more relevant in determining immunodominance.

In order to understand the underlying mechanism for the dominance of CD8 T-cell responses specific to mH-Ag in skin transplantation, I selected the B6 anti-BALB.B model and compared the magnitudes of the CD8 T-cell responses induced against the two well-known dominant mH Ag, H60 and H4. I found that immune hierarchy of mH-Ag-specific CD8 T-cell responses changed over time, with the dominance of H60 mH-Ag-specific CD8 T-cell response occurring first, then that of the H4 mH Ag-specific CD8 T-cell response. I also found that the involvement of different antigen-presentation pathways contribute to the dynamics of immunodominance between H60- and H4-specific CD8 T-cell responses in the skin transplantation model.

Materials & methods

Mice

C57BL/6 (B6: H-2b) and C.B10-H2b/LiMcdj (BALB.B: H-2b) mice were purchased from the Jackson Laboratory (USA). Luciferase transgenic mouse lines were generated after microinjection of a DNA fragment, including promoter, luciferase coding, and poly A signal sequences, into fertilized eggs from B6 mice (B6.LucTg). This strain was maintained by crossing with B6 mice at the Center for Animal Resource Development, Seoul National University College of Medicine. The transgenic mice ubiquitously express a codon-optimized firefly luciferase (Rabinovich et al., 2008) and one founder line out of 11 different founders was selected for its high level of luciferase activity.

Skin transplantation

Skin was recovered and placed in PBS solution for a maximum of 30min until used for transplantation. Full-thickness tail skin (1cm x 1cm x 0.5cm) derived from BALB.B (H-2b) donor mice was transplanted on the tail site C57BL/6J (H-2b) recipients. Recipient mice were anesthetized with evertin for the entire procedure. The skin graft was secured with a plastic adhesive

bandage for 5 days. Graft survival was evaluated by daily visual inspection. Necrosis of 75% of the transplanted skin surface was defined as rejection.

***In vivo* imaging**

In vivo bioluminescence imaging was performed using an IVIS 100 imaging system with a charge-coupled device (CCD) camera (Caliper Life Sciences, USA). Mice were kept on the imaging stage under anesthesia with 1.5% isoflurane gas in oxygen at a flow rate of 1.5 L/min and were given an i.p. injection of the substrate, D-luciferin (150 mg/kg body weight; Molecular Probes, USA). Mice were positioned supine to image the dorsal & ventral surface or on the left side to reveal the spleen or on the tail site.

Analysis of bioluminescence data

Relative intensities of emitted light were presented as pseudocolor images ranging from red (most intense) to blue (least intense). Gray-scale photographs and the corresponding pseudocolor images were superimposed with LIVINGIMAGE (ver2.12; Xenogen) and IGOR (WaveMetrics, USA) image analysis software. Signals emitted by regions of interest (ROI) were measured and data were expressed as photon flux [photon s⁻¹ cm⁻² steradian⁻¹ (sr⁻¹)], which refers to the photons emitted from a unit solid

angle of a sphere. Data are presented as the mean \pm standard error of the mean (SEM). The machine background was subtracted electronically, both from the images and from the measurements of photon flux.

Antibodies and flow cytometry

Fresh PBLs, Splenocytes and Draining LN cell from Recipient mice were incubated at 4°C for 30 min in FACs buffer (1×PBS with 0.1% bovine calf serum and 0.05% sodium azide) containing phycoerythrin (PE)-labeled H60-peptide (LTFNYRNL)/H-2K^b tetramer (H60-tetramer) and other fluorescence-labeled antibodies. The stained cells were analyzed using a FACSCalibur equipped with CellQuest software (BD Pharmingen, San Diego, CA). The H60-tetramer was obtained as described previously. The antibodies used for flow cytometry are as follows: anti-CD11a mAb (M17/4; eBioscience, San Diego, CA), anti-mCD8 mAb

IFN- γ secretion analysis

Splenocytes and Draining-LN cells derived skin transplantation host. Cells (1×10^7 per well) were activated 2h with H60, H4, VSV peptides (10 μ g/ml)

and BFA 4h for stop stimulation. Cells washed twice with phosphate-buffered saline (PBS). After washing, activated cell were fixing through 1% PFA and staining FITC-conjugated CD8 mAb, PE-conjugated H4/H-2Kb or H60/H-2Kb tetramer and APC-conjugated INF- γ mAb.

Statistical analysis

Statistical calculations and *t* tests were performed using GraphPad Prism program version 4 (GraphPad Software, San Diego, CA, USA).

Results

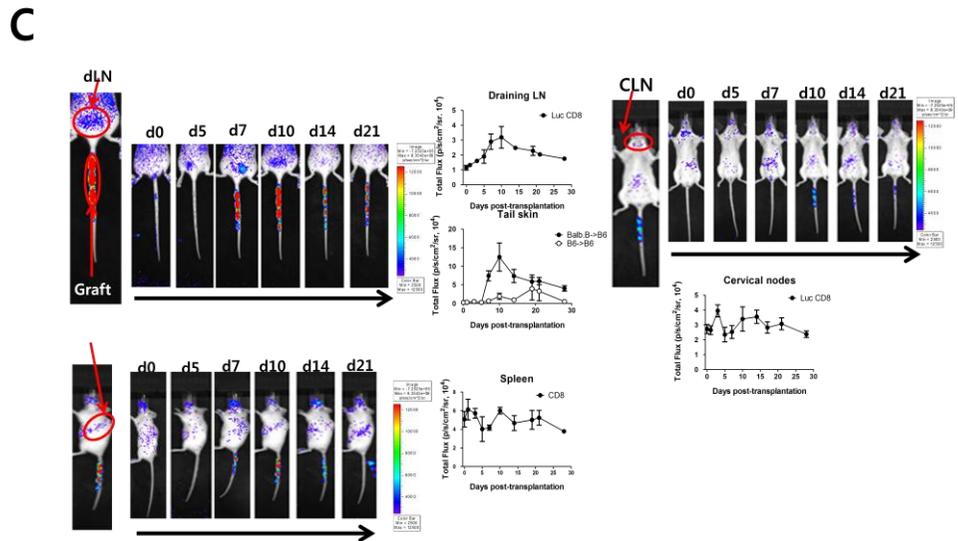
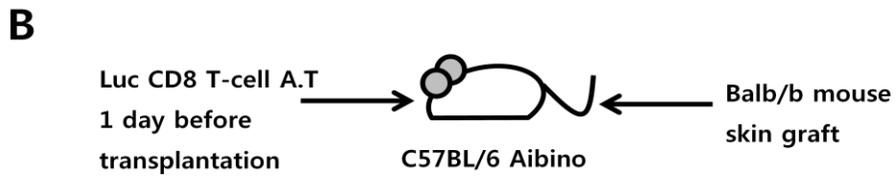
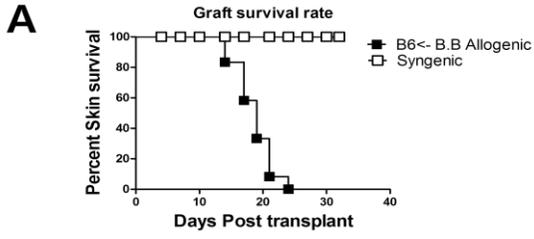
Lumbar LN (draining LN) are significant sites for mH-Ag-specific CD8 T-cell activation in multiple mH Ag-mismatched skin transplantation.

To induce an mH-Ag-specific CD8 T-cell response in the MHC-matched but mH-Ag mismatched skin transplantation, we transplanted tail skin from BALB.B (H-2^b) mice on to the C57BL/6 (H-2^b) mice. The allogeneic grafts were transplanted in triplicate onto the tails of the recipient B6 mice and the fourth site was transplanted with skin from syngenic B6 female mice as control. When the grafts were examined daily, allografts were found to be rejected on average within nineteen days post-transplantation, while the syngenic grafts were not still rejected more than sixty days after transplantation, confirming the establishment of rejection after mH-Ag-specific response induction (Fig. 1A). To visualize the *in vivo* response of CD8 T-cells against allogeneic mH-Ag originating from the allograft BALB.B, I performed longitudinal bioluminescence imaging (BLI) analysis, using the tyrosinase-mutant B6 mice (B6.Albino; H-2^b) as the recipients, and CD8 T cells purified from B6.Luc^{Tg} mice (B6.Luc^{Tg}-CD8 T-cells) as the T-cells responding to the allografts. B6.Luc^{Tg}-CD8 T-cells were adoptively transferred into B6.Albino mice and were monitored for their migration and

expansion after transplantation of BALB.B skin onto the B6.Albino adoptive hosts (Fig. 1B). According to the longitudinal BLI analysis, soon after the adoptive transfer of B6.Luc^{Tg}-CD8 T-cells, injected cells were localized in the lymph-node (LN). Their proliferation was then detected in the draining-LN with a weak signal on day five post-transplantation. The signal increased in the draining-LN until day ten post-transplantation and then waned afterward. The signals were very high at the tail allo-graft sites, while the signal was rarely detected in the syngeneic graft. When comparing these dynamic signal changes in spleens draining LNs, and tail grafts, the signal change was not significant in non-draining LNs (Fig. 1C). These results confirmed that CD8 T-cells reactive to the allografts were activated in the draining LN, and then migrated to the inflamed grafted sites.

To verify the dynamics of CD8 T-cells observed during BLI analysis, I checked the dynamics of CD8 T-cells (CD45.1⁺) in the adoptive hosts that had with BALB.B allogeneic skin transplants as well as B6 control skin via flow cytometric analysis (Fig. 1D). When the CD8 T-cells in spleens, draining LNs, non-draining LNs, and tail-grafts were analyzed, similar kinetics of increase and decrease of CD45.1⁺ CD8 T cells along the response development was confirmed (Fig. 1 E and F). Together, the results from the BLI and flow cytometric analyses confirmed the kinetics of CD8 T-cell

responses against multiple mH-Ag disparate skin grafts and demonstrated that the draining LN are major sites where specific CD8 T-cells proliferate before migrating to the allografts.



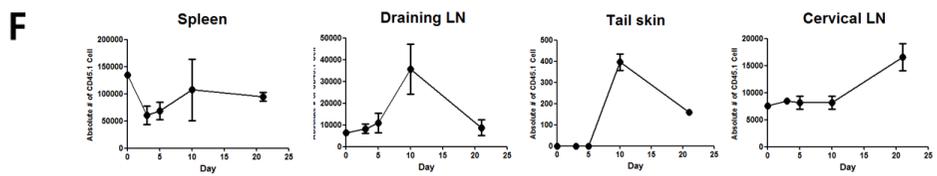
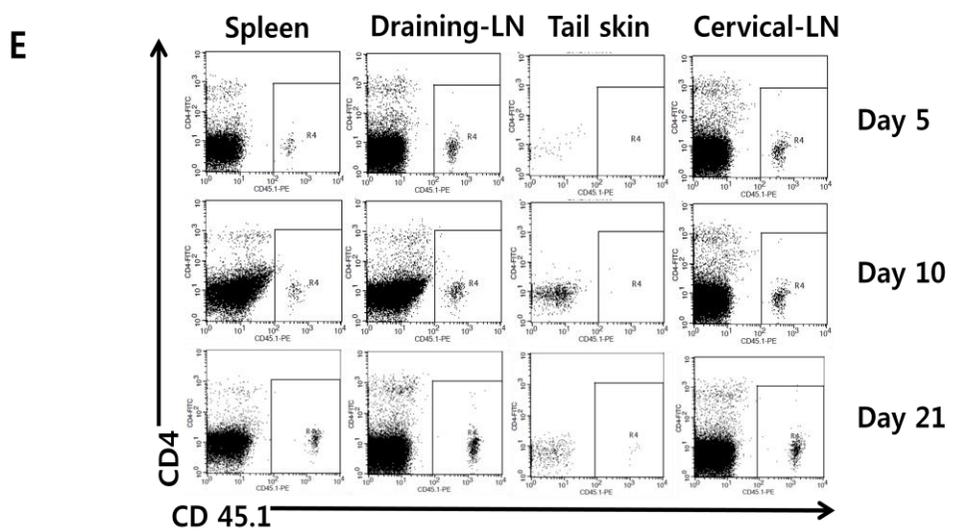
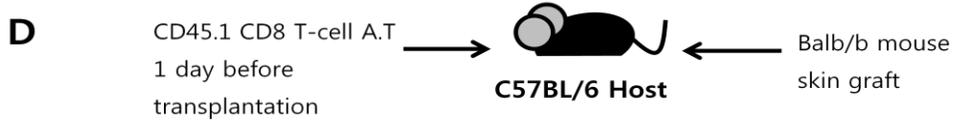


Fig 1. mH-Ag-specific CD8 T-cell activated at spleen & Draining LN in mH-Ag mismatch skin transplantation.

A. In BALB/b (H-2^b) to C57BL/6 (H-2^b), Minor MHC-mismatched combination, skin grafts were rejected around 21 days later transplantation(●: allogenic skin; n = 12; MST = 21). syngeneic (C57BL/6 to C57BL/6) combination showed no sign of rejection(○: syngenic skin; n = 4; MST = no rejection). **B.** B6 albino (H-2^b) mice for host, and B6.Luc^{Tg} CD8 T-cell A.T to host mouse, and next day proceeded BALB.B tail skin transplantation. **C.** measured for the emission of bioluminescence signals after skin transplantation. Check signal daily at first week, and twice a week after. To compare the A.T CD8 T-cell distribution in the mH-Ag mismatch skin graft, regions of interest (ROIs) were defined for the graft, spleen, draining LN, non-draining LN. (n= 6) **D.** CD45.1 C57BL/6 mice CD8 T-cell adoptive transferred to C57BL/6 mice, and next day processed BALB.B mice tail skin transplantation. **E.** Adoptive transferred CD 45.1 CD8 T-cell counted by FACS analysis. We checked spleen, draining-LN and graft at day 5, 10, 21. **F.** counted absolute number of adoptive transferred CD45.1⁺CD8⁺ cell in spleen, draining-LN and graft. (n= 4).

Changing hierarchy of mH-Ag-specific CD8⁺ T-cell after multiple mH-Ag-mismatched skin transplantation.

Immunodominance for mH-Ag in multiple mH-Ag-mismatched transplantations are known to be dependent on the types of tissue transplant. CD8 T cell response for H60 has been known to be in cardiac transplantation, while that for H4, And H4 has been in skin transplantation (6). To examine the immune hierarchy between the CD8 T cell responses for H60 and H4 in the B6 anti-BALB.B skin transplantation, I checked the frequencies of H60-tetramer- or H4-tetramer-binding CD8 T cells in the spleens, draining-LNs, and PBLs along the response development in B6 mice against BALB.B allogeneic skin grafts on days 7, 10, and 14 post-transplantation via flow cytometric analysis after staining with antibodies against CD8 and CD11a mAb and PE-conjugated H4/H-2K^b, H60/H-2K^b. At day 7, the frequencies of H60-tetramer-binding CD8 T cells are higher than those of H4-tetramer-binding CD8 T cells, in the three different organs. However, at day 10, those of H4-tetramer-binding CD8 T cells were higher than those for H6-tetramer-binding CD8 T cells, demonstrating the change in immune hierarchy along the response development (Fig 2A, B). To confirm the change in immune hierarchy, IFN- γ -secretion assay was performed using the cells from spleens and draining LNs harvested on days 7 and 10. And the results from IFN- γ -

secretion assay also showed dominance of H60-specific CD8 T cells preceded ahead of that of H4-specific CD8 T cells, coinciding with the change in dominance according to the frequencies of tetramer-binding CD8 T cells. Therefore, I concluded that even though magnitude of CD8 T cell response for H4 was bigger than and dominant over that for H60 in overall, the CD8 T cell response for H60 was found to be briefly dominant over that for H4. These results suggested that immune hierarchy is not fixed but is dynamic along immune response development.

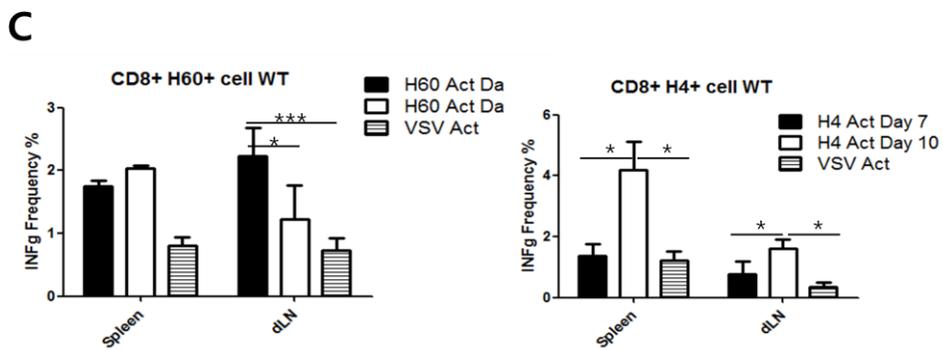
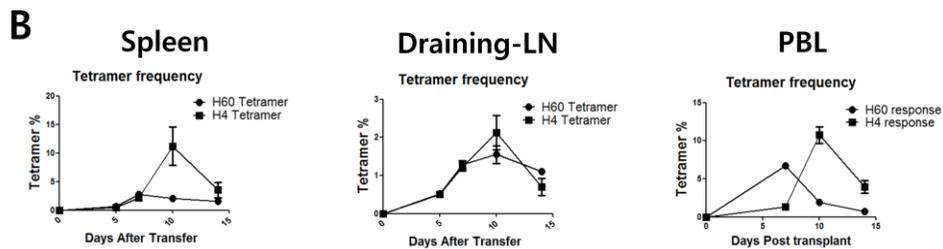
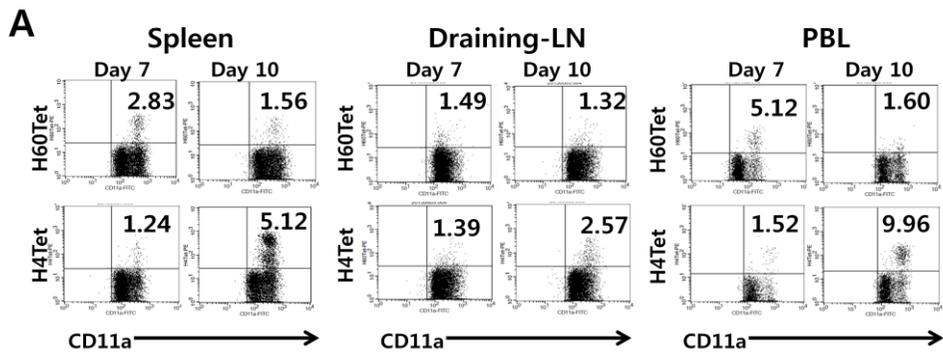


Fig 2. Immune dominance of mH-Ag-specific CD8 T-cell response was changed according to the flow of time.

A. Frequency of mH-Ag-specific CD8 T-cell in skin transplantation was changed H60 mH-Ag to H4 mH-Ag at day 10. PBL, Splenocytes and draining LN cells were recovered on post transplant day 7 or day 10 from skin graft recipients and stained with CD11 mAb, CD8 mAb and PE-conjugated H4/H-2K^b, H60/H-2K^b tetramer. Gates in the figures represent the percentages of tetramer-positive cells within CD8⁺ T, CD11^{high} cells. In repeat experiments, similar data were obtained. **B.** Comparison of H60 mH-Ag-specific CD8 T-cell response and H4 mH-Ag-specific CD8 T-cell response at PBL, spleen and draining-LN at 4, 7, 10, 14 days after skin grafts. **C.** Spleen cell and draining-LN cells derived skin transplantation host. Cells (1×10^7 per well) were activated 2h with H60, H4, VSV peptide (10 μ g/ml) and BFA for 4h to stop stimulation. Activated cell were fixing through 1% PFA and stained FITC-conjugated CD8 mAb, PE-conjugated H4/H-2K^b or H60/H-2K^b tetramer and APC-conjugated INF- γ mAb. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Student T test.

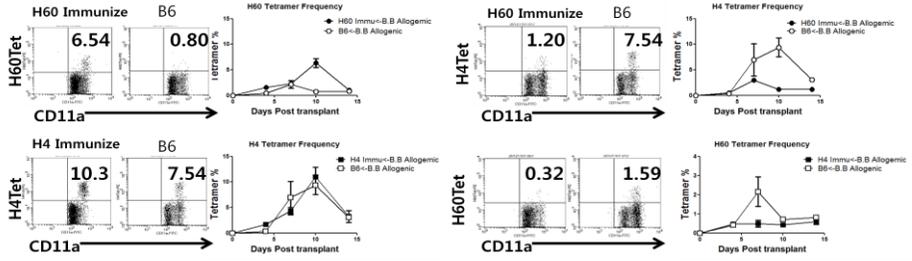
H60 and H4 mH-Ag affect Graft rejection and mH-Ag-specific CD8 T-cell response at mH-Ag mismatched skin transplantation

Since the H60-specific CD8 T cell response was briefly dominant over H4-specific response, I wondered whether there might exist an influence from the CD8 T cell response specific for H60 on that for H4, during the B6 anti-BALB.B response. To examine this, I generated H60-specific or H4-specific CD8 memory T cells in the B6 host by injecting splenocytes from H60 congenic or H4 congenic mice, respectively, and then each group of immunized mice were transplanted with skin from BALB.B mice 30 days later, after which the frequencies of H4-tetramer or H60-tetramer binding CD8 T cells in the blood from the immunized and grafted B6 mice were longitudinally checked via flow cytometric analysis. In addition, the survival of the BALB.B allo-grafts was checked. The results from the flow cytometric analysis showed that the generation of H60-specific or H4-specific memory CD8 T cells has suppressed expansion of H4- or H60-specific CD8 T cells in the hosts after the allogeneic skin transplantation, respectively (Fig. 3A right panel). Furthermore, the survival time of BALB.B allograft was reduced in the hosts in which H60 or H4-specific CD8 memory T cells had been established.

These results demonstrated that the dominance of H60-specific CD8 T cells

during the brief period of time has influenced the immune response for H4 and survival time of allograft.

A



B

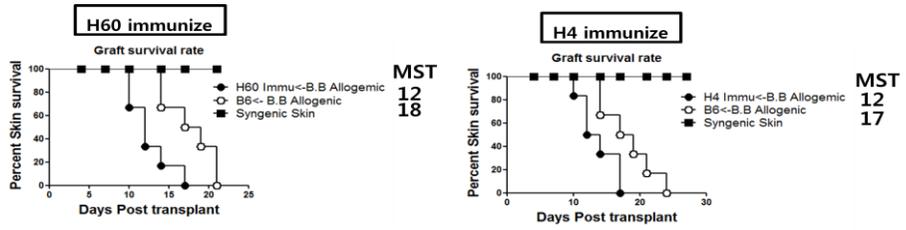


Fig 3. H60 mH-Ag and H4 mH-Ag-specific CD8 T-cell were affecting in mH-Ag mismatch skin graft rejection process.

A. For the Formation of H60 mH-Ag or H4 mH-Ag-specific memory CD8 T-cell, Male H60c mouse splenocyte (2×10^6 per mice) or Male H4c mouse splenocyte (2×10^6 per mice) adoptive transferred to C57BL/6 mouse. After 4weeks later A.T, Balb.B tail skin transplantation. PBL were recovered on post transplant day 4, 7, 10 and 14 from skin graft recipients and stained with CD11 mAb, CD8 mAb and PE-conjugated H4/H-2K^b, H60/H-2K^b tetramer. FACS analysis data represent day 10 after skin graft. **B.** Graft rejection time was increased recipient have mH-Ag-specific memory CD8 T-cell. Recipient have H60 or H4 mH-Ag-specific memory CD8 T-cell were grafts were rejected around day 12 (●: H60 or H4 immunize host allogenic skin; n = 12; MST = 12). No treat C57BL/6 host allograft were rejected around day 18 (○: No treat host allogenic skin; n = 12; MST = 18). Four to six mice were tested in each

H60 specific CD8 T-cell response can influence to other mH-Ag specific CD8 T-cell response in multiple mH-Ag skin transplantation setting.

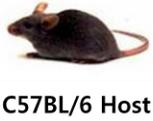
I thought that the brief dominance of H60-specific CD8 T cell response after the allogeneic BALB.B-skin transplantation might be ascribed to the hematopoietic cell lineage-restricted expression of H60 in congenic strain. Then, I compared the kinetics of CD8 T cell responses specific for H60 between when the H60 is expressed restrictedly in hematopoietic cells and in every type of cells ubiquitously by transplanting the skins from the H60 congenic mice (B6.CH60) or H60-transgenic mice (Act-H60 Tg) which express H60 under the control of actin. In addition, female B6 mice were transplanted with skin from H4 congenic mice (B6.CH4) or BALB.B as control. (Fig 4A) And the PBLs from the transplantation B6 hosts were analyzed via flow cytometry after staining with H60- or H4-tetramers. The results from the flow cytometric analyses showed that responses for H60 and H4 were delayed when the B6 mice were transplanted w from congenic or transgenic it skin mice, compared with when they were transplanted with skin from BALB.B mice. And it was found that the kinetics of CD8 T cell response for H60 expressed ubiquitously was similar to that for H4, in that the frequencies of tetramer-binding CD8 T cells peaked on day 10 and continuously high even on day 14 post-transplantation, even though the

frequencies of H4-specific CD8 T cells were higher than those of H60-specific CD8 T cells. Also in the case of the frequencies of CD8 T cells reactive to hematopoietic cell-restricted H60 peaked on day 10 and little waned on day 14 post-priming. (Fig. 4B.C) These results demonstrated that CD8 T cell response for H60, which expressed restrictedly on hematopoietic cells, could also last long. Therefore, the brief dominancy of H60-specific CD8 T cell response during the B6 anti-BALB.B response was considered to be the result of competition between the responses for H60 and H4.

When the IFN- γ secretion assay was performed with the splenocytes harvested from the transplanted hosts, the frequencies of the IFN- γ -secreting cells in response to stimulation with H60 or H4-peptide supported the dynamics of H60 or H4-specific CD8 T cells observed with flow cytometric analysis (Fig. 4D).

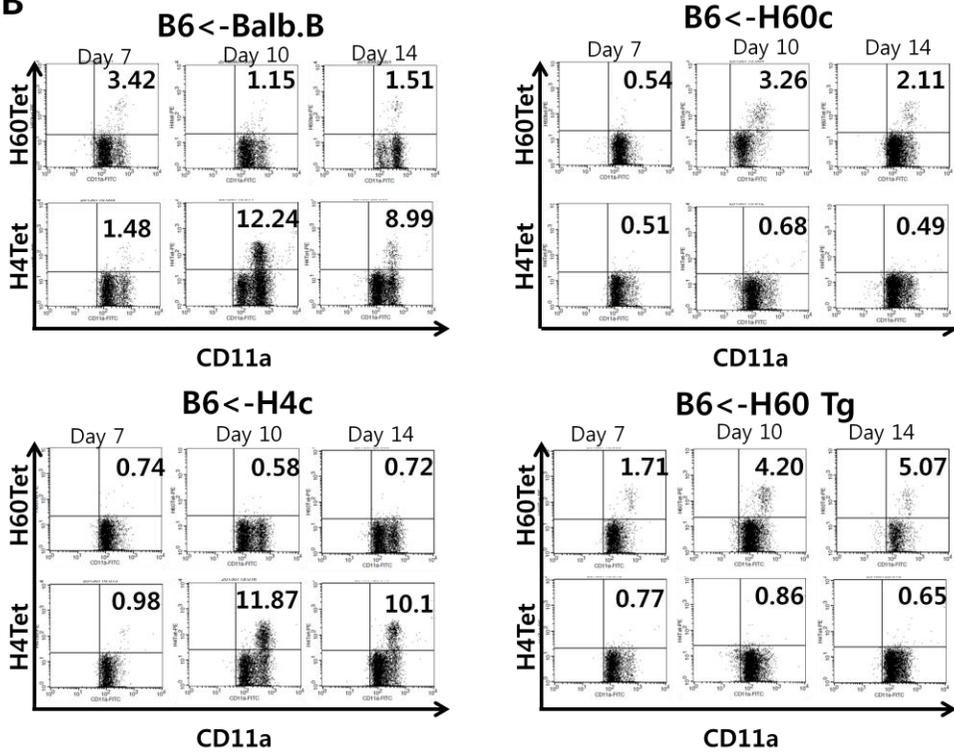
Altogether, the results obtained with single antigen-disparate transplantation demonstrated that no matter whether expression of H60 was restricted to hematopoietic cells or ubiquitous, H60-specific CD8 T cells could be induced and lasted relatively long, compared with that under multi-antigen-disparate transplantation condition.

A

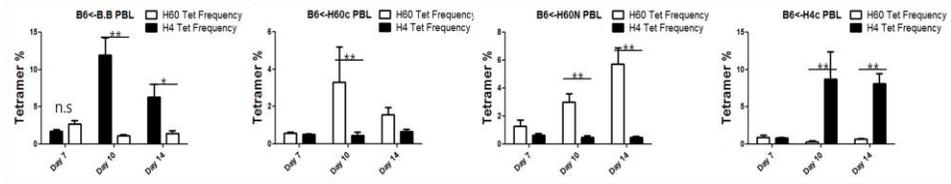


1. Balb/b
 2. H60 congenic
 3. H60 tg
 4. H4 congenic
- ← Mouse tail skin transplantation

B



C



D

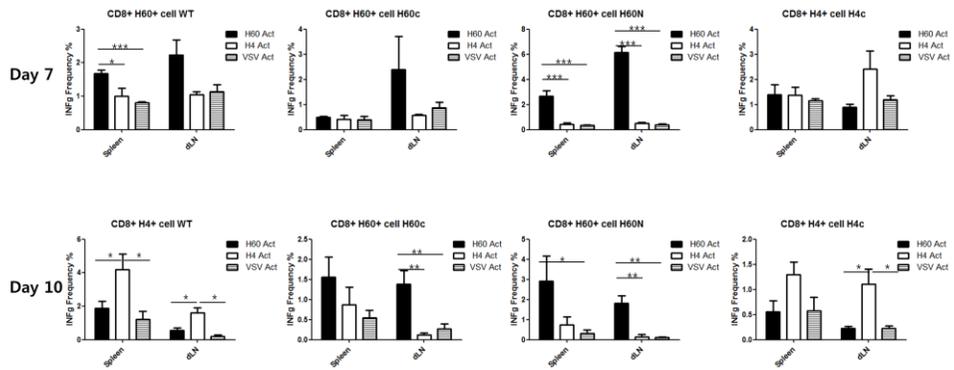


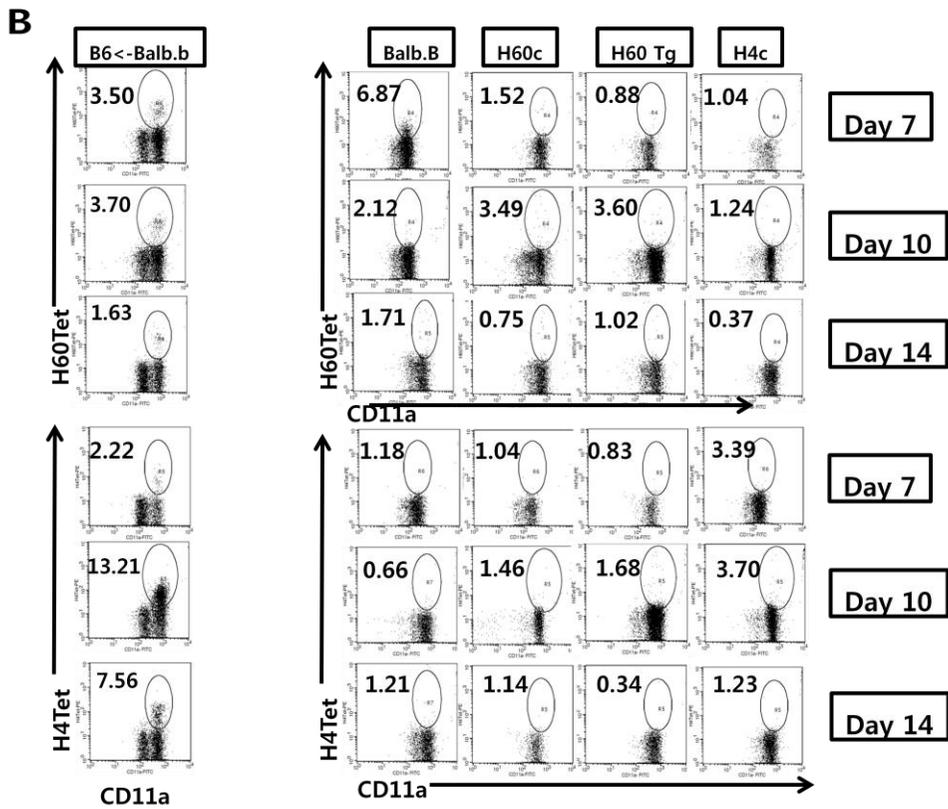
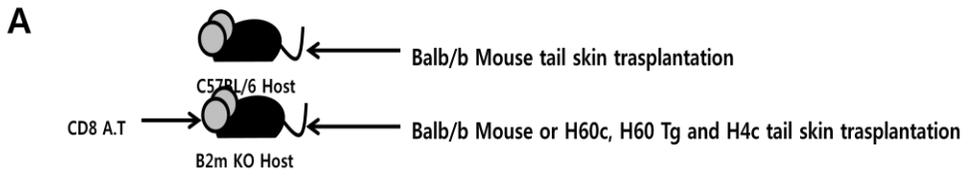
Fig 4. Hematopoietic cell origin mH-Ags mainly recognized via donor APCs, Skin cell origin mH-Ags recognized via Host APCs

A. BALB.B, H60c (hematopoietic cell expressed H60 mH-Ag), H60N (all of cell expressed H60 mH-Ag), H4c (all of cell expressed H4 mH-Ag) mouse tail skin transferred to C57BL/6 host. **B.** PBL, splenocyte and draining-LN cells were recovered on post transplant day 7, 10 and 14 from skin graft recipients and stained with CD11 mAb, CD8 mAb and PE-conjugated H4/H-2Kb, H60/H-2Kb tetramer. Splenocyte and LN data were not shown. **C.** Comparison of H60 mH-Ag-specific CD8 T-cell response and H4 mH-Ag-specific CD8 T-cell response in 4 groups host PBL, spleen and draining-LN at 7, 10, 14 days after skin grafts. **D.** Spleen cell and draining-LN cells derived skin transplantation host (Used BALB.B, H60c, H60N, H4c mice as a donor). Cells (1×10^7 per well) were activated 2h with H60, H4, VSV peptide ($10 \mu\text{g/ml}$) and BFA 4h for stop stimulation. Activated cell were fixing through 1% PFA and staining FITC-conjugated CD8 mAb, PE-conjugated H4/H-2K^b or H60/H-2K^b tetramer and APC-conjugated INF- γ mAb. Four mice were tested in each. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Student T test.

Direct presentations through Donor APCs were associated with higher H60 specific CD8 T-cell response than H4 specific CD8 T-cell response

To understand the mechanism for the competition out of H4-specific CD8 T cell response over H60-specific CD8 T cell response, I investigated the influence of antigen presentation pathway on the dominance and competition between the CD8 T cell responses with different specificity. First, to examine the role of direct presentation pathway in each antigen-specific CD8 T cell response, I transplanted skin from BALB.B and other congenic mouse strains onto $\beta 2m$ -deficient hosts to block the cross-presentation in the hosts, and checked the frequencies of CD8 T cells binding to each tetramer by flow cytometric analysis (Fig 5A). B6 normal hosts were included as controls. The results from the flow cytometric analysis showed that the frequencies of H60-tetramer binding CD8 T cells were highest in the $\beta 2m$ -deficient hosts transplanted with BALB.B skins, even though the H60-tetramer-binding CD8 T cells were also detected in the $\beta 2m$ -deficient hosts transplanted with skins from H60 congenic or Actin-H60 Tg mice. Regarding the H4-specific CD8 T cells, they were detected in the $\beta 2m$ -deficient hosts transplanted with H4-congenic skins, but the frequencies were lower compared to the levels detected in B6 hosts. Interestingly, the frequencies of H4-tetramer-binding CD8 T cells were very low when the $\beta 2m$ -deficient hosts were transplanted

with BALB.B (Fig. 5B, C). Taken together, these results demonstrated that direct presentation played significant role in H60-specific CD8 T cell response after skin-transplantation and the presence of competition enhanced the reactivity of H60-specific CD8 T cells.



C

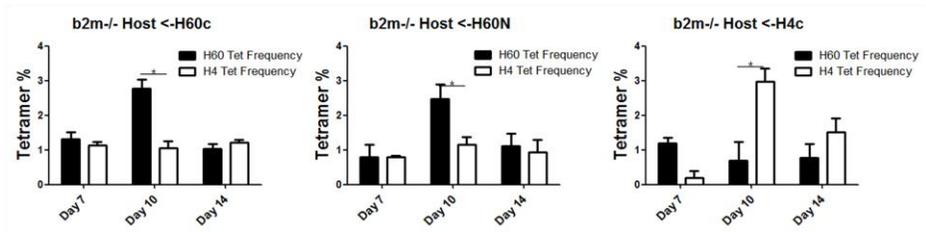
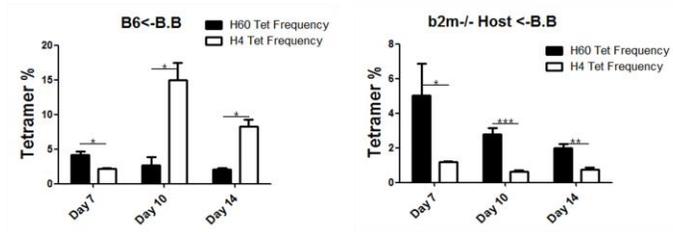


Fig 5. Donor APC affects H60 mH-Ag-specific CD8 T-cell is dominant than H4 in early phase graft rejection.

A. C57BL/6 CD8 T-cell adoptive transferred to $\beta 2m^{-/-}$ host before skin graft.

After 4weeks later A.T, BALB.B, H60c, H60N, H4c mouse tail skin transfer to CD8 adoptive transferred $\beta 2m^{-/-}$ host. **B.** PBLs were recovered on post

transplant day 7, 10, 14 from skin graft recipients and stained with CD11 mAb, CD8 mAb and PE-conjugated H4/H-2Kb, H60/H-2Kb tetramer. **C.**

Comparison of H60 mH-Ag-specific CD8 T-cell response and H4 mH-Ag-specific CD8 T-cell response at Day 7, 10, 14 after skin graft. Four mice were tested in each. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Student T test.

Indirect presentations through Host APCs were associated with changing immune hierarchy of H60 to H4 mH-Ag

Then, to investigate the effect of cross-presentation on CD8 T cell response specific for hematopoietic cell-restricted H60 and for ubiquitously expressed H60, B6 hosts were transplanted with the skins from H60congenic or from Actin-H60-Tg mice on $\beta 2m$ - background and the PBLs were analyzed via flow cytometry after staining with H60-tetramer (Fig 6A). The results from the flow cytometric analysis showed that the frequencies of H60-specific CD8 T cell response were higher in the hosts transplanted with skins from Actin-H60-Tg mice on $\beta 2m$ - background (Fig. 6B, C). These results demonstrated that the cross-presentation was important for the induction of CD8 T cell response specific for ubiquitously expressed H60.

Therefore, I could conclude that the presentation pathways, indirect or direct pathway, played role in induction of CD8 T cell responses specific for hematopoietic cell restricted antigens and ubiquitously expressed antigens. And the competition between the pathways determines the dominancy after skin transplantation.

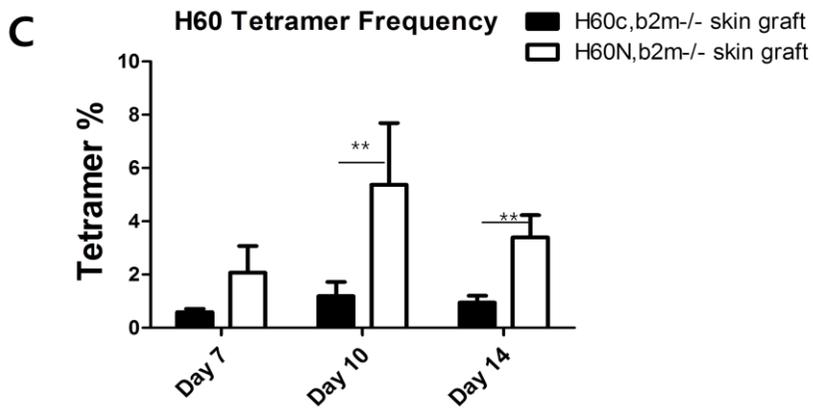
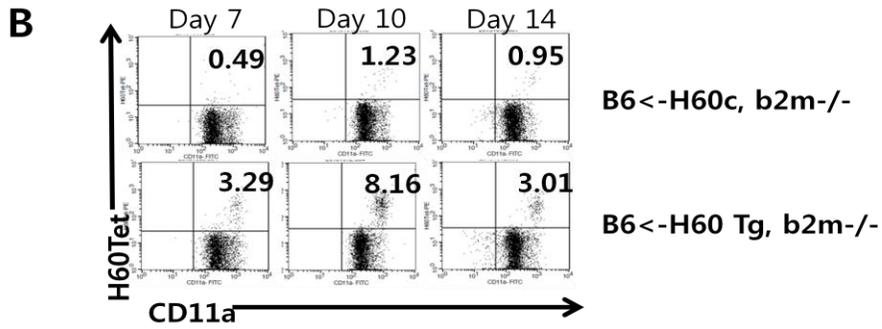
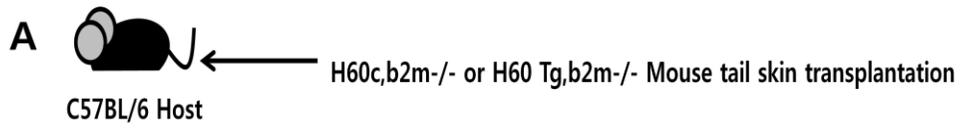


Fig 6. Host APC affects H4 mH-Ag-specific CD8 T-cell is dominant than H60 in later phase graft rejection.

A. H60c, $\beta 2m^{-/-}$ mice (hematopoietic cell only expression H60 and APC is not expression b2m molecule) or H60N, $\beta 2m^{-/-}$ mice (all cell expression H60 and APC is not expression b2m molecule) skin graft to C57BL/6 mice.

B. PBLs were recovered on post transplant day 7, 10, 14 from skin graft recipients and stained with CD11 mAb, CD8 mAb and PE-conjugated H4/H-2Kb, H60/H-2Kb tetramer.

C. Comparison of H60 mH-Ag-specific CD8 T-cell response and H4 mH-Ag-specific CD8 T-cell response at Day 7, 10, 14 after skin graft in host received H60c, $\beta 2m^{-/-}$ skin or H60N, $\beta 2m^{-/-}$ skin.

Last panel of Fig 6C is comparison of H60 specific CD8 T-cell response between hosts received H60c, $\beta 2m^{-/-}$ skin grafts and H60N $\beta 2m^{-/-}$ skin grafts. Four mice were tested in each group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, Student T test.

Discussion

Minor Ag mismatch organ transplantation is a useful model to study immunodominance. In particular, the H60 and H4 mH-Ags that are known to be the most immunodominant peptides in this mouse model. Among the mH-Ags, H60 is of hematopoietic origin and was found to dominate the B6 anti-BALB.B immune response during both the primary and secondary challenges. (17. 18) H4 mH-Ag is also of hematopoietic origin, yet is widely expressed in epithelial cells and other cell types. (19) H4 was also found to be a dominant mH-Ag in skin transplantation.

Wettstein and Colombo's (6) study reported that H4^b mH-Ag showed the highest binding affinity (K^b) to MHC molecule. Furthermore, the CTL assay revealed that H4-specific T cells are the most efficient after skin transplantation. In contrast, another group showed H60 mH-Ag response was immunodominant in BALB.B to C57BL/6 heart transplantation. (20) Several previous studies have showed the immunodominance of H4 and H60 mH-Ag in various transplantation models. Unfortunately, previous studies have focused on which mH-Ag was dominant rather than the roles of other mH-Ag in various transplantation setting.

In our skin transplantation setting, the H4 mH-Ag response was more

dominant than the H60 mH-Ag response. Kwun. et al. (5) also showed that the H60 mH-Ag specific response had second immunodominance in skin transplantation. Although we found that The CD8 T-cell response for H4 was both bigger and more dominant over than H60 overall, the CD8 T-cell response for H60 was found to be briefly dominant over that for H4. H60 mH-Ag response was dominant at the early phase, and later hierarchy was changed to a H4 mH-Ag response. We therefore questioned what mechanism was involved in this phenomenon. Among the various possible factors, we thought differential APC distributions may affect this hierarchical change phenomenon.

Basically, minor H antigens were recognized by CD8 T-cells and induced a mH-Ag-specific immune response through APC. The antidonor immune response is initiated by host T-cells activated in the host's second lymphoid organ through two APC present pathways: The direct and the indirect pathway. In the skin transplantation model, inducing an Ag-specific T-cell response via the direct and indirect pathways was important for the graft rejection process (21). Direct allo-response is sufficient to induce the acute graft rejection of skin transplantation (22). CD8 T-cell activated through the direct presentation pathway is thought to cause on acute phase immune response, whereas T-cells activated through the indirect pathway is involved

in chronic rejection (23). In the transplantation model of antigen presentation via the two pathways for activation of T-cells, there are time differences. Host APC takes five days to move to a graft site and if host APC is transplanted to the site, the host APC still needs at least five days (16). We thought that T-cell activation via donor APC therefore mainly happened within five day time limit after a skin graft and, activation via host APC mainly happens five days later. We questioned the relevance of mH-Ag-specific CD8 T-cell responses and the two pathways of APC presentation. To find the location of the main site of T-cell activation via APC, we checked the main activation site of mH-Ag-specific CD8 T-cell in our transplantation model. Spleen, lumbar, inguinal LN (we referred to it as the draining-LN) were the main sites where CD8 T-cell numbers had increased after skin transplantation (Fig. 1). We therefore investigated mH-Ag-specific CD8 T-cell response at PBL, spleen and draining-LN. The H60 mH-Ag-specific CD8 T-cell response was dominant over the H4 mH-Ag-specific response in PBL, spleen and draining-LN at seven days after the skin graft (Fig. 2). mH-Ag-specific CD8 T-cell response were reversed at ten days after skin graft. Hierarchy of immunodominance was H60 mH-Ag to H4 mH-Ag. So, we have questions about role of H60 mH-Ag in mH-Ag mismatch skin transplantation. H60 mH-Ag-specific T-cell responses were also important

for graft rejection and graft rejection time was increased when the host had H60 mH-Ag-specific memory CD8 T-cell (Fig. 3). We can then question how the H60 mH-Ag-specific T-cell response was dominant over H4 in the early phase and how the hierarchy of immunodominance was changed. We found the H60 mH-Ag was more dominant over H4 mH-Ag when Ag was mainly presenting via donor APC (at the early phase of the rejection) (Fig. 5). H4 mH-Ag-specific CD8 T-cell response were increased when a host APC was participating in presentation (Fig. 6).

According our data, the time difference in the APC's presentation pathway influenced the formation of the hierarchy of immunodominant mH-Ag-specific CD8 T-cells.

Reference

1. Teh, H. S., J. Bennink, and H. Von Boehmer. 1982. Selection of the T cell repertoire during ontogeny: limiting dilution analysis. *Eur. J. Immunol.* 12: 887–892.
2. Theobald, M., and D. Bunjes. 1993. Pretransplant detection of human minor histocompatibility antigen-specific naive and memory interleukin-2-secreting T cells within class I major histocompatibility complex (MHC)-restricted CD8⁺ and class II MHC-restricted CD4⁺ T-cell subsets. *Blood* 82: 298–306.
3. Perreault, C., D. C. Roy, and C. Fortin. 1998. Immunodominant minor histocompatibility antigens: the major ones. *Immunol. Today* 19: 69–74.
4. Anderson, C. C., and P. Matzinger. 2001. Immunity or tolerance: opposite outcomes of microchimerism from skin grafts. *Nat. Med.* 7: 80–87.
5. Jean Kwun, and Stuart J. Knechtle 2011. Primary Vascularization of the Graft Determines the Immunodominance of Murine Minor H Antigens

during Organ Transplantation. *J Immunol* 2011; 187:3997-4006.

6. Wettstein, P. J., and M. P. Colombo. 1987. Immunodominance in the T cell response to multiple non-H-2 histocompatibility antigens. IV. Partial tissue distribution and mapping of immunodominant antigens. *J. Immunol.* 139: 2166–2171.

7. Lechler, R. I., G. Lombardi, J. R. Batchelor, N. Reinsmoen, and F. H. Bach. 1990. The molecular basis of alloreactivity. *Immunol. Today* 11: 83–88.

8. Benichou, G., P. A. Takizawa, C. A. Olson, M. McMillan, and E. E. Sercarz. 1992. Donor major histocompatibility complex MHC peptides are presented by recipient MHC molecules during graft rejection. *J. Exp. Med.* 175:305.

9. Fangmann, J., R. Dalchau, and J. W. Fabre. 1992. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. *J. Exp. Med.* 175:1521.

10. Liu, Z., N. S. Braunstein, and N. Suciú-Foca. 1992. T cell recognition of allopeptides in context of syngeneic MHC. *J. Immunol.* 148:35.
11. Benichou, G., P. A. Takizawa, C. A. Olson, M. McMillan, and E. E. Sercarz. 1992. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. *J. Exp. Med.* 175: 305–308.
12. Illigens, B. M., A. Yamada, N. Anosova, V. M. Dong, M. H. Sayegh, and G. Benichou. 2009. Dual effects of the alloresponse by Th1 and Th2 cells on acute and chronic rejection of allotransplants. *Eur. J. Immunol.* 39: 3000–3009.
13. Lee, R. S., M. J. Grusby, L. H. Glimcher, H. J. Winn, and H. Auchincloss, Jr. 1994. Indirect recognition by helper cells can induce donor-specific cytotoxic T lymphocytes in vivo. *J. Exp. Med.* 179:865.
14. Popov, I. A., E. V. Fedoseyeva, P. L. Orr, M. R. Garovoy, and G. Benichou. 1995. Direct evidence for in vivo induction of CD81 cytotoxic T

cells directed to donor MHC class I peptides following mouse allotransplantation. *Transplantation* 60:1621.

15. Larsen, C. P., and T. C. Pearson. 1997. The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr. Opin. Immunol.* 9: 641–647.

16. Susanna C, Matthew L, Philippe B. 2011. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nature Medicine* 17: 744–749

17. Choi, E. Y., Y. Yoshimura, G. J. Christianson, T. J. Sproule, S. Malarkannan, N. Shastri, S. Joyce, and D. C. Roopenian. 2001. Quantitative analysis of the immune response to mouse non-MHC transplantation antigens in vivo: the H60 histocompatibility antigen dominates over all others. *J. Immunol.* 166: 4370–4379.

18. Choi, E. Y., G. J. Christianson, Y. Yoshimura, T. J. Sproule, N. Jung, S. Joyce, and D. C. Roopenian. 2002. Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. *Immunity* 17: 593–603.

19. Yadav, R., Y. Yoshimura, A. Boesteanu, G. J. Christianson, W. U. Ajayi, R. Shashidharamurthy, A. K. Stanic, D. C. Roopenian, and S. Joyce. 2003. The H4b minor histocompatibility antigen is caused by a combination of genetically determined and posttranslational modifications. *J. Immunol.* 170: 5133–5142.

20. Kwun, J., H. Hu, E. Schadde, D. Roenneburg, K. A. Sullivan, J. DeMartino, W. J. Burlingham, and S. J. Knechtle. 2007. Altered distribution of H60 minor H antigen-specific CD8 T cells and attenuated chronic vasculopathy in minor histocompatibility antigen mismatched heart transplantation in *Cxcr3*^{-/-} mouse recipients. *J. Immunol.* 179: 8016–8025.

21. Benichou, G., A. Valujskikh, and P. S. Heeger. 1999. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. *J. Immunol.* 162:352–358.

22. Auchincloss, H., Jr., R. Lee, S. Shea, J. S. Markowitz, M. J. Grusby, and L. H. Glimcher. 1993. The role of “indirect” recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. *Proc. Natl. Acad. Sci. USA* 90: 3373–3377.

23. Illigens, B. M., A. Yamada, N. Anosova, V. M. Dong, M. H. Sayegh, and G. Benichou. 2009. Dual effects of the alloresponse by Th1 and Th2 cells on acute and chronic rejection of allotransplants. *Eur. J. Immunol.* 39: 3000–3009.

국문 초록

부조직적합항원의 면역원성은 주조직적합성복합체 제시 펩타이드의 다양성에 의해 결정된다. 주조직적합항원이 일치하는 공여자와 수혜자의 이식에서 주조직적합성복합체 제시 펩타이드의 차이는 T세포의 활성을 유도하여 이식실패의 요인이라고 생각되고 있다. 다양한 B6 anti-BALB.B와 같은 부조직적합항원 불일치 조건에서 T세포의 반응은 면역우성 현상에 의해 몇 종류의 항원에 집중되어 발생한다. H60와 H4는 B6 anti BALB.B 이식 조건에서 우성을 가지는 부조직적합항원 이라고 알려져 왔다. 본 연구에서는, 주조직적합항원 일치 피부이식 실험에서, 다양한 세포에서 발현되는 항원 혹은 조혈세포에서만 발현되는 항원 특이적인 CD8 T세포 반응을 이해 하고자 하였다. H60 (조혈세포 제한적 발현) 와 H4 (다양한 세포에서 발현)에 대한 CD8 T세포의 반응을 유세포분석을 통해 장기적으로 관찰하였고, 이를 통하여 H60 특이적인 CD8 T세포의 빈도가 H4 특이적인 CD8 T세포보다 먼저 증가 하는 것을 확인했다. H4 특이적인 CD8 T세포 반응은 H60 특이적인 CD8 T세포의 반응보다 우성을 보이지만, H60 특이적인 memory CD8 T세포를 형성 하게 되면

H4 특이적인 CD8 T세포의 증가를 감소시키고, 이식 조직의 거부 반응이 빠르게 진행되었다. H60 특이적인 CD8 T세포가 공여자의 항원제시세포를 통하여 직접적인 항원 제시 방법으로 면역반응이 발생하면 H4 특이적인 CD8 T세포반응에 비하여 면역우성을 가지며, 수혜자의 항원제시세포를 통한 간접적인 제시 방법으로 면역 반응이 발생할 때에는 H4 특이적인 반응이 면역 우성을 가진다. 종합적으로, 이전 연구들의 개념과는 반대로, H4 특이적인 CD8 T세포 반응 뿐 아니라 H60 특이적인 반응도 조직거부 반응에 중요하다. 또한, 면역원성의 변화는 T세포에 어떤 항원제시세포가 항원을 제시 하는가에 달려 있다고 할 수 있다.

주요어: 피부 이식, 부조직접합항원, 면역우성체계, 항원 제시

학번: 2011-23804