이학석사학위논문

CD4 Help Enhances Expansion of Myeloids and DCs

CD4 T 세포 도움이 골수성 세포와 수지상 세포의 양적 증가에 미치는 영향 연구

2018 년 8 월

서울대학교 대학원

협동과정 유전공학 전공

오세화

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지도교수 최은영

이 논문을 이학석사학위논문으로 제출함

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오세화

오세화의 석사학위논문을 인준함
2018년 8 월

위 원 장 이동섭 (인)
부 위 원 장 최은영 (인)
위 원 윤홍덕 (인)

Abstract

CD4 Help Enhances Expansion of Myeloids and DCs

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CD4 T cell help has been known to induce DC "licensing" for an optimal CD8 T cell immune response during viral and pathogenic antigen immunization models. However, the exact role and mechanism for the DC licensing by CD4 help have not been fully identified. To define the role of CD4 T cell help, I tracked and compared the dynamics of myeloids and immature DCs during helped and helpless CD8 T cell responses to a cellular antigen, minor histocompatibility antigen H60. DC maturation and functionality was similar between helped and helpless responses. However, there was a quantitative increase in both myeloids and immature DC populations, in correlation with an increase in the

number of mature DCs. I identified a myeloid population, $MHCII^{+}$ and $MHCII^{-}CD11c^{-}CD11b^{+}Ly6C^{hi}Ly6G^{int}$ subsets, that were increased during the helped response. They had a phenotype similar to that of immature and mature DCs, suggesting a DC precursor population. The expansion of myeloids and DCs, and the DC precursor myeloid population during the helped response was also confirmed by comparison of the myeloid and DC cell kinetics during helped responses induced in B6 wild type and CD40-deficient mice. The same findings were observed in analysis of *in vitro* bone marrow cell culture kinetics. These data suggest that the role of CD4 help is not enhancement of DC maturation *bona fide*, but rather a quantitative increase of myeloid cells and DC precursors to subsequently increase mature DC population.

Key Words: CD4 help, CD40, DC, myeloid, expansion, H60

Student Number: 2015-22300

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List of Abbreviations and Symbols

APC: antigen-presenting cell

B6: C57BL/6J strain

DC: Dendritic Cell

H60c: B6C.CH60 strain

Helped: CD4 helper-sufficient

Helpless: CD4 helper-deficient

H60c: B6C.CH60 strain

Introduction

CD8 T cell immune responses have been classified according to the requirement of CD4 help: either CD4 help-independent or CD4 help-dependent. This classification has strong correlation with the type of antigen introduced into the host immune system. The primary CD8 T cell response is CD4 help-independent when a pathogenic antigen is introduced, and is CD4-dependent when a cellular antigen is introduced (1-3).

CD4 help-dependent responses require the concomitant activation of CD4 and CD8 T cells post-immunization (5-7). CD4 T cell help has been known to provide DC licensing to properly activate CD8 T cells. The "DC licensing," in which CD40-CD40L interaction and other help factors participate, induces DCs to reach a more mature state (8-10). The licensed DCs are then upregulated in costimulatory molecules and cytokines, which results in the qualification of DCs to activate a CD8 T cell immune response. On the other hand, pathogenic antigens have been considered to induce activation and maturation of DCs through inflammatory and TLR signaling, bypassing CD4 help, to initiate an optimal primary CD8 T cell immune response (2). However, this dichotomic classification turned out to be not valid; recent publications on CD8 T cell memory responses highlighted the necessity of CD4 help in antigen-specific memory CD8 T cell

generation. Regardless of a primary CD4 help-independent or CD4 help-dependent response, CD4 help was found to be needed to generate memory CD8 T cells (12-14). Thus, only the helped CD8 T cells during primary challenge were able to mount quick and efficient recall responses to antigen-re-exposure.

In the help process, many factors have been identified to be involved, each with a distinct function during the concomitant activation. The CD40-CD40L interaction between DCs and activated CD4+ T cells has been known as an essential signaling pathway for DC maturation and licensing (20-22). Other help factors include IL-15 and IL-12, which is secreted by licensed DCs to activate CD8 T cells (19, 30-31). CD70, which is upregulated on licensed DCs, is thought to be the mechanism of CD8 T cell activation during helped response (17-18). IL-2, secreted by activated CD4 T cells, induce activation and proliferation of interacting CD4 and CD8 T cells (15). Despite the accumulation of publications on the help factors, the molecular and cellular mechanism explaining exactly how CD4 help licenses DCs has remained to be established.

In order to understand the cellular mechanism regarding the DC licensing by CD4 help, I used a helped and helpless cellular antigen model, targeting an immunodominant minor histocompatibility antigen, H60. The H60-specific CD8 T cell response is induced against a single H-2K^{b}-presented peptide (LTFNYRNL) and is helper-dependent. B6 mice have null

expression of H60 and recognize the antigen as foreign. To induce solely a response to H60, B6.CH60 mice strain, a B6 mouse background with a congenic H60 region on chromosome 10, was used to immunize B6 mice. CD4 help was induced by the presence of the male Y chromosome, which contains a Hy-Dby locus that provides a CD4 epitope (NAGFNSNRANSSRSS) recognized by H2-A^b. Thus, immunization of female B6 hosts with male or female B6.CH60 splenocytes induces a helped or helpless model, respectively. In the presence of help, the primary H60-specific CD8 T cell immune response is efficient, and antigen is cleared. In contrast, helpless has a small, early burst of H60-specific CD8 T cells with persistence of antigen. In addition, the recall response is faster and larger when CD4 help was given during priming (28). Induction of helped H60-specific CD8 T cell response was dependent on intactness of CD40-CD40L interaction between DCs and activated CD4 T cells. When help was given to CD40-deficient or CD40L-deficient mice, the H60-specific CD8 T cell response was significantly decreased in comparison to wild type (WT) (29), typical of responses requiring CD4 help (22)..

Using the H60 model system, my study will show that CD4 help enhances the expansion of mature DCs and their precursors, myeloid cells and immature DCs. This refutes the previous consensus that CD4 help induces immature DCs to undergo a maturation process. I will show that CD4 help via

CD40 signaling increases myeloid cells and immature DCs along with an increase in mature DC numbers, pinpointing a distinct myeloid subset that shares a similar maturation phenotype as immature and mature DCs. The results from this study will provide insight into how CD4 help is involved in DC licensing and guarantees a proper CD8 T cell response.

Materials and Methods

Mouse. C57BL/6 (B6), B6.129P2-*Tnfrsf5*^{tm1Kik}/J (CD40-deficient mice) and B6.C-H60^{c}/DCR (B6.CH60) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained under specific pathogen free-conditions at the Center of Animal Resource Development of Seoul National University, College of Medicine, Korea and used for experiments at ages 8-12 weeks with the approval of the Institutional Animal Care and Use Committee of Seoul National University.

Immunization. A single-cell suspension (2 x 10^7 in 200 μL PBS) of splenocytes from B6.CH60 mice was injected I.p. to induce an H60-specific CD8+ T cell response.

Spleen and Lymph Node Cell Isolation. Spleens and lymph nodes were isolated from mice and stored in cell preparation solution. Spleen and lymph nodes were cut or teased apart into small sections in 2 mg/mL Collagenase D (Roche) and 20 ug/mL DNaseI (Roche)-containing media. Sections were incubated at 37 ◦C for 25 minutes. 0.5 M EDTA was then added and incubated at room temperature for 5 minutes. Media was then diluted with cell preparation solution and red blood cells were lysed. Remaining cells were counted and analyzed by flow cytometry.

Lineage Negative Bone Marrow Isolation and Culture. Bone marrow cells were flushed from femurs and tibiae of mice. BM Lin- cells were isolated by magnetic cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). 2 x 10^5 isolated Lin- cells were cultured in 1 mL of 10% fetal bovine serum containing DMEM culture media. 40 ng/mL GM-CSF were cultured with or without pre-coated 10 ug/mL FGK4.5 antibody 4 hours beforehand at 37◦C. Cells were then cultured at 37◦C for 4 days and then media was replaced with respective culture conditions. Cells were observed daily via microscopy and flow cytometry.

Antibodies and Flow Cytometry. Single-cell suspensions were stained with antibodies or H60 tetramers at 4◦C for 30 minutes in staining buffer (1x PBS containing 0.1% bovine serum and 0.1% sodium azide). Cells were analyzed using LSRII-Green (BD Pharmingen) or LSR Fortessa X-20 (BD Pharmingen) and data were analysed using the FlowJo Software (Tree Star, Ashland, OR, USA).

Statistical Analysis. Statistical significance was determined using Graphpad Prism software (version 7, GraphPad Software, San Diego, CA). Data are presented as the means ± s.e.m. *P* values were determined by Student's t-tests with $*P<0.05$,

$^{**}P<0.01$, $^{***}P<0.001$. A P-value <0.05 considered to indicate statistical significance.

Results

DC maturation status is similar between helped and helpless regardless of differences in cellularity

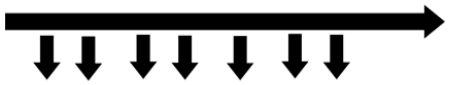
I wanted to define the role of CD4 help in the context of the cellular antigen model by using the minor histocompatibility antigen H60. I compared the DC maturation status between helped and helpless to confirm that of the previous publication (29). Unlike the previous data, I did not transfer H60-specific J15 CD8 T cells to create a more realistic immunization model. I injected female B6 mice with male or female double congenic CD45.1 and H60 splenocytes, resulting in a helped or helpless model, respectively (Figure 1A). The mice were sacrificed and analyzed daily for 7 days post-immunization. The peritoneum (the antigen injection site), the draining mesenteric and inguinal lymph nodes, and the spleen were observed for total cellularity and analyzed by flow cytometry.

I observed that mice were properly immunized, based on the increasing amount of H60-specific CD8 T cells in the helped condition (Figure 1B). The total cellularity kinetics differed between helped and helpless, specifically with increased numbers on day 5 in the spleen and peritoneum (Figure 1C). Additionally, There was a total expansion of cellularity on days 1 and 2, which coincides with the observed timepoint of CD4 help and DC

licensing.

I gated on pDCs and mature XCR1+ CD8a+ cDC1s and CD11b+ cDC2 subsets, adherent to current mature DC gating strategies (Figure 1D). I analyzed the DC maturation status of all 3 subsets using phenotypic markers such as CD40, CD80, CD86 and MHCII, which are known CD4 help factors and DC maturation indicators. CD70, a help-associated marker, is upregulated after CD4 T cell help to induce an optimal CD8 T cell immune response. Despite some differences in pDC CD86 MFI expression level, the total mature DC phenotype was similar between helped and helpless (Figure 1E). DC maturation status between helped and helpless was similar regardless of a difference in total cellularity.

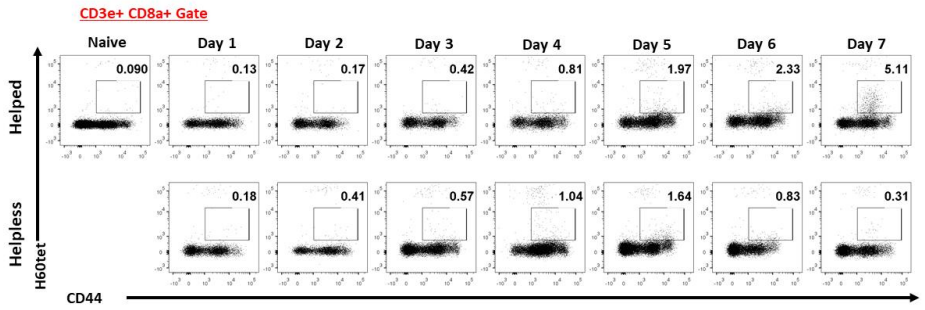
A



D 1-7: sacrificed and analyzed by flow cytometry

Helped: 2 x 10^7 CD45.1c.H60c.♂ splenocytes
Helpless: 2 x 10^7 CD45.1c.H60c.♀ splenocytes

B



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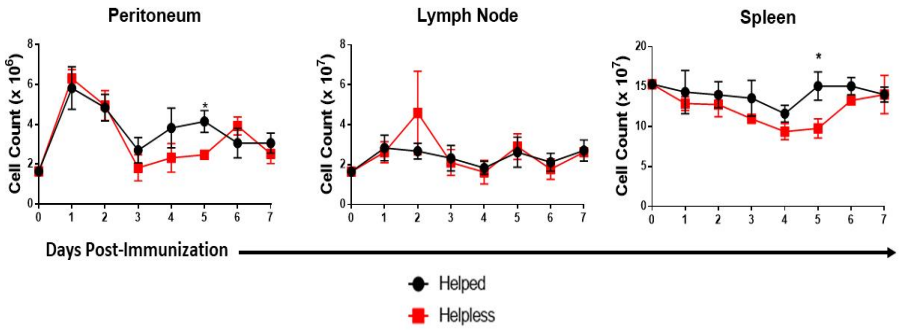
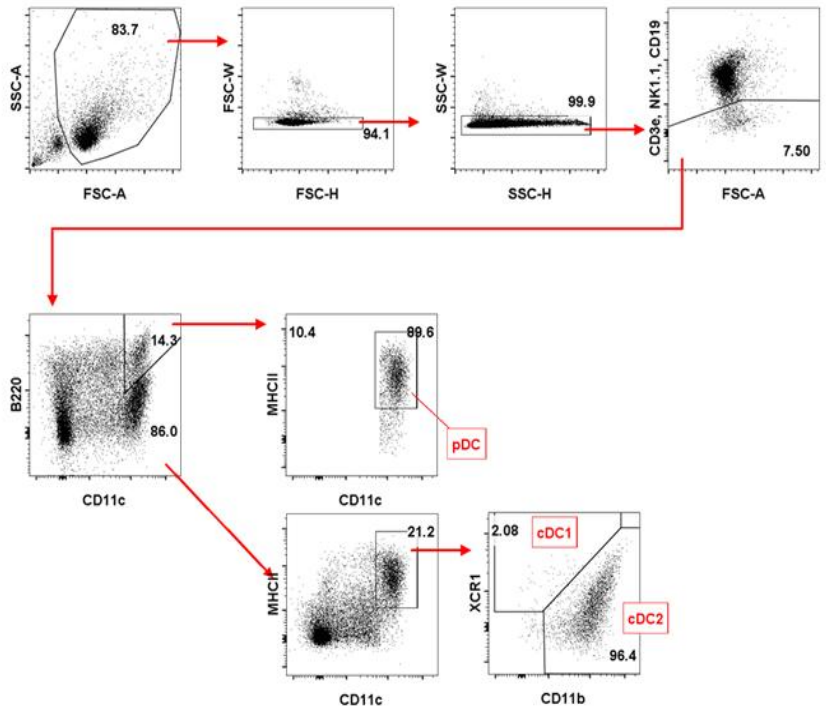


D


83.7
SSC-A
FSC-A
FSC-W
94.1
FSC-H
SSC-W
99.9
SSC-H
CD3e, NK1.1, CD19
7.50
FSC-A
B220
14.3
86.0
CD11c
MHCII
10.4
89.6
pDC
CD11c
MHCII
21.2
CD11c
XCR1
2.08
cDC1
cDC2
96.4
CD11b

E

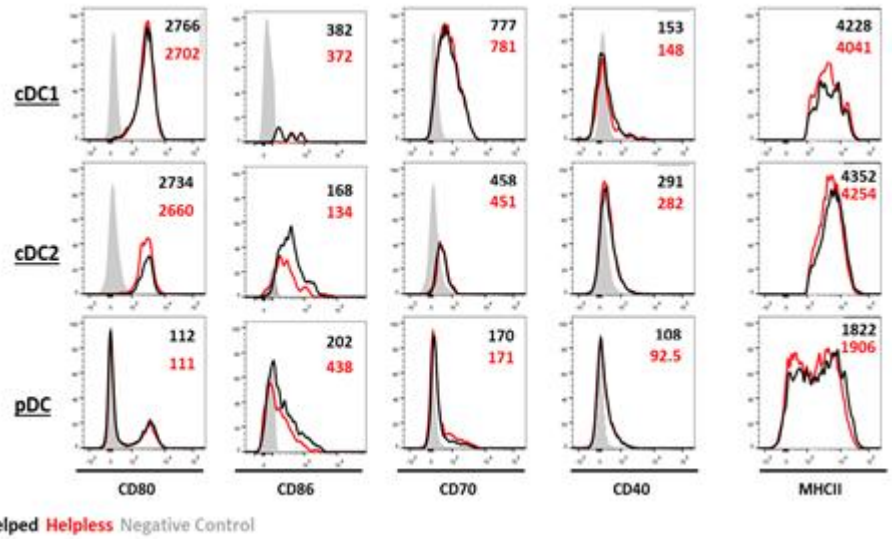

cDC1
2766
2702
382
372
777
781
153
148
4228
4041
cDC2
2734
2660
168
134
458
451
291
282
4352
4254
pDC
112
111
202
438
170
171
108
92.5
1822
1906
CD80
CD86
CD70
CD40
MHCII
Helped Helpless Negative Control

Figure 1. Total Cellularity and mature DC maturation status

(A) Experimental scheme of immunization of female B6 mice with double congenic CD45.1 x H60 splenocytes and daily sacrifice analysis. (B) Flow cytometric data from spleen of sacrificed mice daily. Parent frequency is total CD8+ T cells. (C) Total Cellularity kinetics of organs from mice daily. (D) Gating strategy of pDCs and mature cDC1 and cDC2 (E) Phenotype of pDCs and mature cDCs in the spleen on day 2. All data (b-e) are representative of 4 mice (n=4 per experiment). Data are presented as means ± SEM. *P<0.05, **P<0.01 by student's *t*-test.

Expansion of Immature Myeloids and DCs are Enhanced in the Presence of CD4 Help

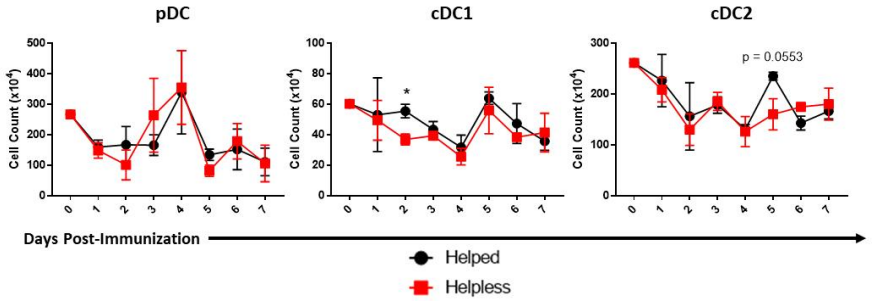
CD4 help plays a necessary role in mature DC enhancement, in which myeloid cells and immature DCs differentiate into mature DCs. This can be detected by a negative correlation in the ratio between myeloids and immature DCs, and mature DCs. I compared the mature DC number kinetics between helped and helpless daily for 7 days post-immunization. Despite a similar DC maturation phenotype (Figure 1E), the mature DC number was higher in helped than in helpless at certain timepoints, though they differed depending on the DC subset. $XCR1^+$ cDC1 numbers were increased on day 2, $CD11b^+$ cDC2s on day 5; however, pDCs were similar between helped and helpless mice (Figure 2A).

I next sought to view the population kinetics of myeloids and immature DCs and compare them to that of mature DCs. I subdivided myeloids and immature DCs based on CD11c and MHCII expression (Figure 2B). Myeloids were categorized as $CD11c^-$ populations, whereas immature DCs contain $CD11c^{int}$ and $MHCII^-$ $CD11c^{hi}$ subsets. Myeloids and DCs were analyzed by flow cytometry, (Figure 2C) and then numbers were calculated and compared between helped and helpless. Myeloid population kinetics differed not only between helped and helpless but also between the $MHCII^+$ and $MHCII^-$ subsets (Figure 2D). The $MHCII^-$ $CD11c^-$ subsets in the myeloid cells were increased in

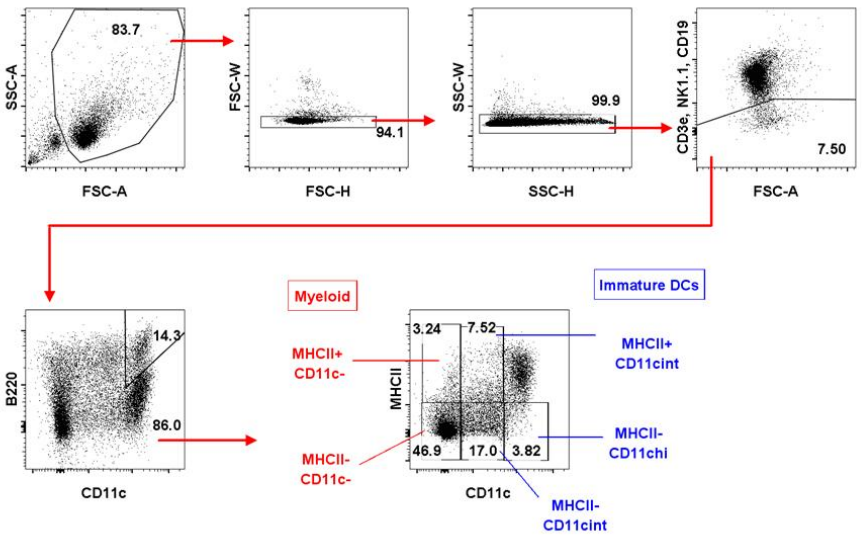
helped mice on day 5 in comparison to that of helpless mice. Immature DCs were similarly subdivided into 3 subsets. Similarly to the myeloid subsets, their kinetics differed between helped and helpless, and amongst the 3 subgroups (Figure 2E). $MHCII^{-}$ $CD11c^{int}$ were increased on day 4 whereas $MHCII^{+}$ $CD11c^{int}$ immature DCs were increased on day 3 in helped in comparison to helpless.

To clarify the role of CD4 help in mature DC enhancement, I compared the myeloid, immature DC, and mature DC populations on days 2 and 5, when the mature cDC numbers were increased in helped. Despite an increase in subsets in helped on day 2 and 5, the ratios between the 3 groups remained similar between helped and helpless (Figure 2F). Based on these data, CD4 help appears to enhance the expansion of all myeloid and DC subsets.

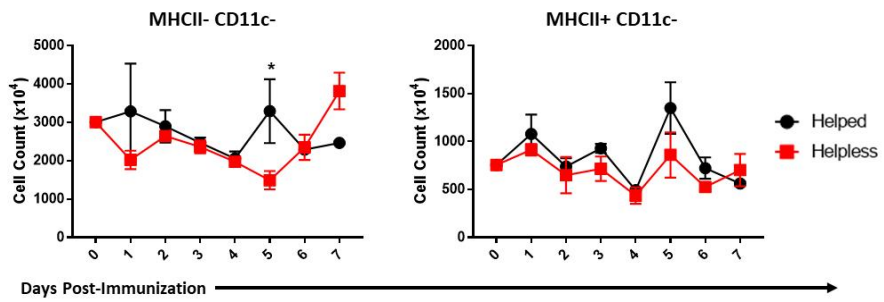
A


pDC
cDC1
cDC2
Cell Count (x10⁴)
p = 0.0553
*
Days Post-Immunization
Helped
Helpless

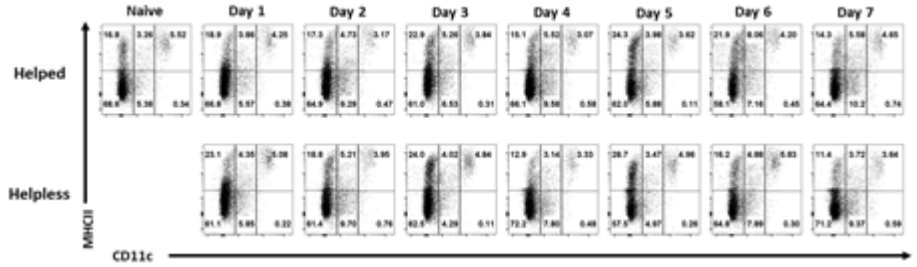
B


83.7
SSC-A
FSC-A
FSC-W
94.1
FSC-H
SSC-W
99.9
SSC-H
CD3e, NK1.1, CD19
7.50
FSC-A
14.3
86.0
B220
CD11c
Myeloid
Immature DCs
MHCII+
CD11c-
MHCII-
CD11c-
3.24
7.52
46.9
17.0
3.82
MHCII
CD11c
MHCII+
CD11cint
MHCII-
CD11chi
MHCII-
CD11cint

C


MHCII- CD11c-
MHCII+ CD11c-
Cell Count (x10⁴)
*
Days Post-Immunization
Helped
Helpless

D



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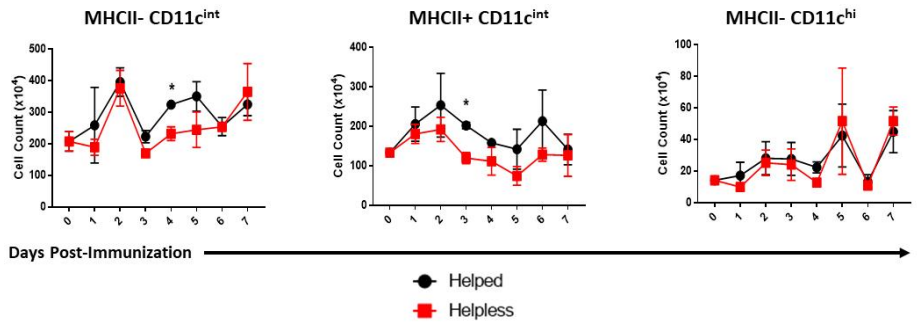

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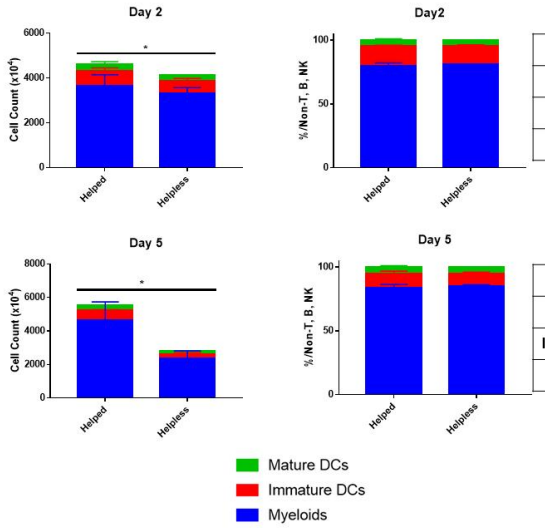


Day 2	Helped	Helpless
Mature DC	4.5 ± 1.33	4.365 ±.415
Immature DC	15.235 ± .745	14.715 ± .955
Myeloid	80.15 ± 2.05	80.8 ± 0.6

Day 2	Helped	Helpless
Mature DC	4.795 ± 1.175	4.95 ±.01
Immature DC	11.675 ± 1.705	9.9 ± 1.2
Myeloid	83.45±2.85	85.05±1.15

Figure 2. Number and Total Frequency of Myeloids, and Immature and Mature DCs

(A) Mature DC number kinetics in the spleen post-immunization. (B) Gating of myeloid and immature DC subsets (C) Flow cytometric data of myeloid and DC subsets from the spleen post-immunization (D) Myeloid number and (E) immature DC number kinetics in the spleen post-immunization (F) Ratio of immature myeloids, immature DCs, and Mature DCs in the spleen on day 2 and 5 post-immunization. All data (a-f) are representative of 4 mice (n=4 per experiment). Data are presented as means ± SEM. *P<0.05, **P<0.01 by student's *t*-test.

MHCII$^-$ and MHCII$^+$ Ly6C^{hi} Ly6G^{int} DC Myeloids are Increased in Helped and have a DC Precursor Phenotype

All myeloids, immature DCs and mature DCs were increased in cellularity at distinct timepoints in helped over helpless. In the hematopoietic cell lineage, the differentiation pathway is known to start from CD11c$^-$ myeloid subsets. These CD11c$^-$ cells can differentiate to become immature and mature DCs as well as other hematopoietic lineage cells. I hypothesized that CD4 help may increase a distinct myeloid subset that subsequently results in the expansion of immature and mature DC populations. MHCII$^-$ and MHCII$^+$ CD11c$^-$ myeloids were shown to be increased throughout days 1 to 5 (Figure 2D), which suggest that they may both contribute to the increased DC subsets or they share the same lineage, one subset could be the precursor of the other.

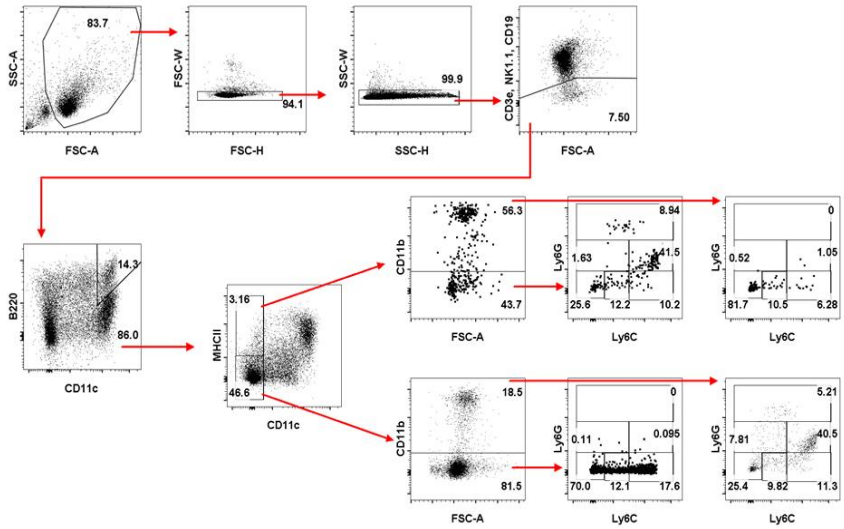
I defined the myeloid subsets into finer, clearly defined subsets using CD11b, Ly6C and Ly6G expression (Figure 3A). Within the myeloid subsets, the MHCII$^-$ and MHCII$^+$ CD11b$^+$ Ly6C^{hi} Ly6C^{int} myeloids increased on days 2 and 5, similar to that of the mature DC subset (Figure 3).

I next sought to define the MHCII$^-$ and MHCII$^+$ CD11c$^-$ CD11b$^+$ Ly6C^{hi} Ly6C^{int} myeloids based on known DC, monocyte, neutrophil, and hematopoietic cell surface markers. I hypothesized that these subsets may be DC precursors that contribute to the immature and mature DC subset numbers. I compared the

phenotype of MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloids to that of immature DCs, and mature cDC1 and cDC2 subsets in both helped and helpless (Figure 3C). I observed no differences in costimulatory and help factor expression levels between helped and helpless. The expression levels of CD40 and CD86 in MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloids were similar to that of immature DCs and mature cDC2s. CD80 expression in MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloids were similar to that of mature DCs and they had unexpected higher CD70 expression levels in comparison to mature DCs. MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloids shared similar CD210 or IL-10r expression levels to mature cDC2s, which suggest a role in suppression.

Overall, the MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloid population in helped was expanded in comparison to helpless. These cells were phenotypically similar to immature and mature DCs. The high CD70 expression may suggest that they are able to interact with CD4 T cells and potentially activate CD8 T cells.

A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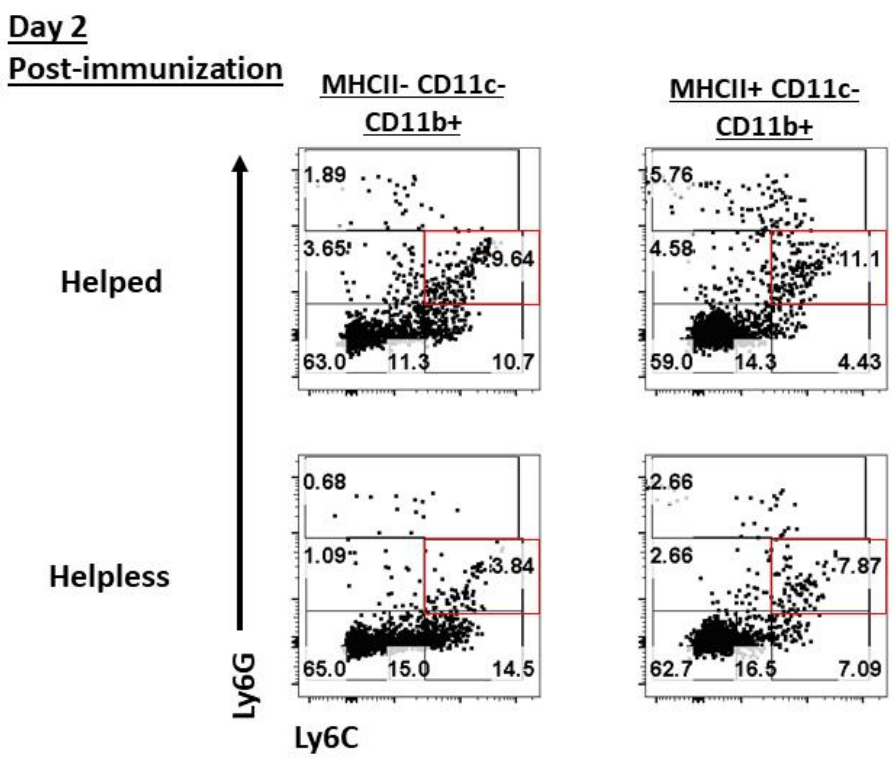
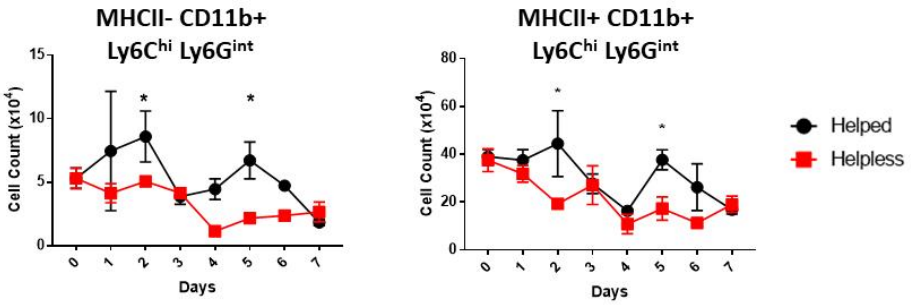
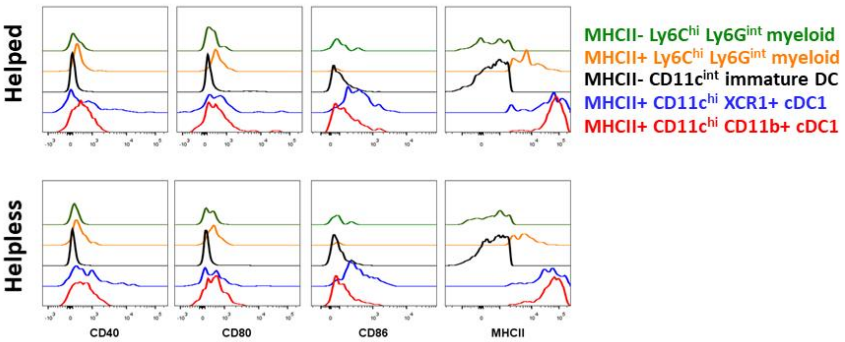
SSC-A
FSC-A
FSC-W
FSC-H
94.1
SSC-W
SSC-H
99.9
CD3e, NK1.1, CD19
7.50
FSC-A
B220
14.3
86.0
CD11c
MHCII
3.16
46.6
CD11c
CD11b
56.3
43.7
FSC-A
Ly6G
8.94
1.63
41.5
25.6
12.2
10.2
Ly6C
Ly6G
0
0.52
1.05
81.7
10.5
6.28
Ly6C
CD11b
18.5
81.5
FSC-A
Ly6G
0
0.11
0.095
70.0
12.1
17.6
Ly6C
Ly6G
5.21
7.81
40.5
25.4
9.82
11.3
Ly6C

B


Day 2
Post-immunization
MHCII- CD11c- CD11b+
MHCII+ CD11c- CD11b+
Helped
Helpless
1.89
3.65
9.64
63.0
11.3
10.7
5.76
4.58
11.1
59.0
14.3
4.43
0.68
1.09
3.84
65.0
15.0
14.5
2.66
2.66
7.87
62.7
16.5
7.09
Ly6G
Ly6C


MHCII- CD11b+
Ly6C^hi Ly6G^int
Cell Count (x10^4)
Days
MHCII+ CD11b+
Ly6C^hi Ly6G^int
Helped
Helpless

C


Helped
Helpless
MHCII- Ly6C^hi Ly6G^int myeloid
MHCII+ Ly6C^hi Ly6G^int myeloid
MHCII- CD11c^int immature DC
MHCII+ CD11c^hi XCR1+ cDC1
MHCII+ CD11c^hi CD11b+ cDC1
CD40
CD80
CD86
MHCII

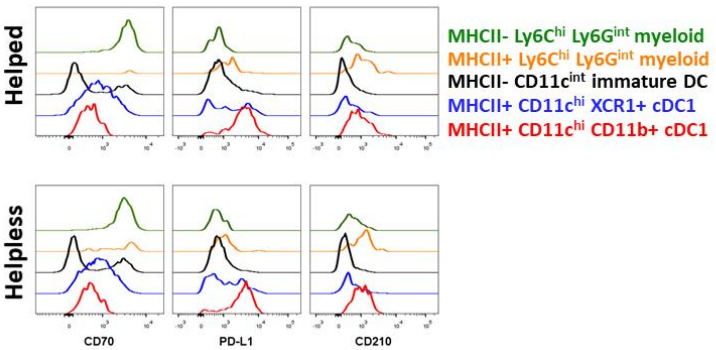

Helped
Helpless
MHCII- Ly6C^hi Ly6G^int myeloid
MHCII+ Ly6C^hi Ly6G^int myeloid
MHCII- CD11c^int immature DC
MHCII+ CD11c^hi XCR1+ cDC1
MHCII+ CD11c^hi CD11b+ cDC1
CD70
PD-L1
CD210

Figure 3. MHCII+ and MHCII- Ly6C^{hi} Ly6G^{int} DC myeloids, DC precursors, are expanded during CD4 Help

(A) Gating strategy of Ly6C and Ly6G in Myeloid Subsets. (B) Flow Cytometric data and cellularity kinetics of increased MHCII^{-} and MHCII^{+} CD11c^{-} CD11b^{+} Ly6C^{hi} Ly6C^{int} Myeloid Subsets in the Spleen (C) Phenotype of MHCII^{-} and MHCII^{+} CD11c^{-} CD11b^{+} Ly6C^{hi} Ly6C^{int} Myeloids on Day 2 in the spleen. All data (b, c) are representative of 4 mice (n=4 per experiment). Data are presented as means ± SEM. *P<0.05, **P<0.01 by student's *t*-test.

CD40 is Needed to Enhance Expansion of Myeloids and DCs, and MHCII^{+} and MHCII^{-} Ly6C^{hi} Ly6G^{int} Myeloids

The effect of CD4 help on myeloid and DC expansion was seen in helped but the mechanism was still undefined. CD40 signaling during CD4 help is known to induce DC maturation and licensing; however, I hypothesized that the CD40 may actually be the key factor behind myeloid and DC expansion. Myeloids and immature DCs were shown to express CD40 (Figure 3C), which suggested that they are able to receive CD40 signaling.

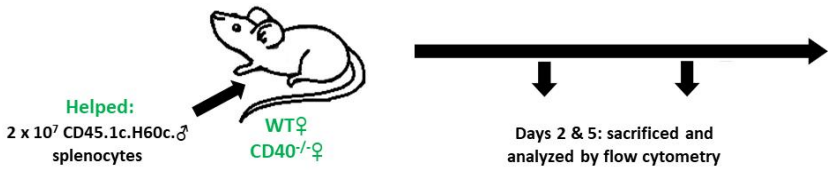
To better define the role of CD40 during CD4 help, I immunized WT and CD40-deficient mice (CD40 KO) with male H60 congenic splenocytes to induce a helped condition without the CD40-CD40L interaction. I sacrificed and analyzed the spleen on days 2 and 5 (Figure 4A). Despite the absence of CD40, mature DCs were formed and had similar MHCII levels (Figure 4B). This suggested that CD40 was not needed for DC maturation. However, the number of mature DCs was higher in WT than in CD40-deficient helped mice. Mature cDC1 and cDC2 populations were increased in WT helped mice on day 2 post-immunization (Figure 4C).

To rule out the role of CD40 signaling in mature DC enhancement, I compared the ratio of immature populations to mature DCs in both frequency and cellularity. I found that WT had a larger immature DC proportion in comparison to CD40 KO helped mice (Figure 4D). However, both WT and CD40 KO

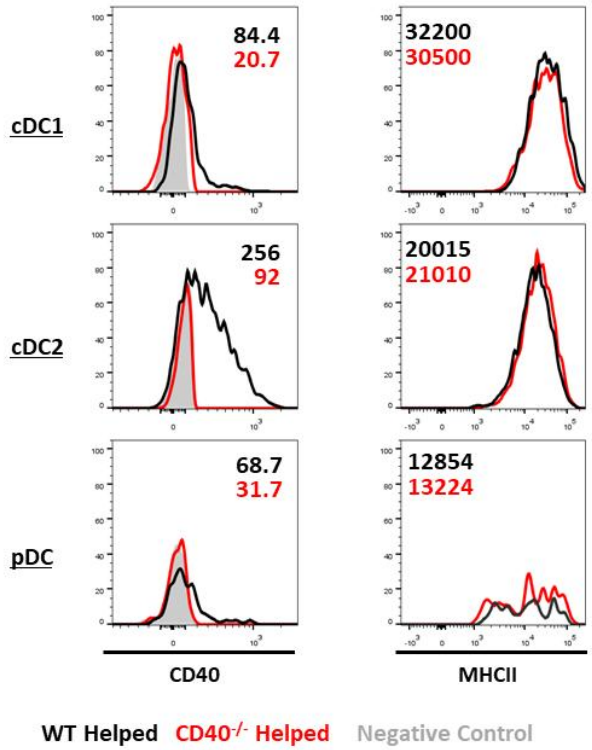
helped maintained similar proportions between day 2 and 5 despite DC number differences between both timepoints. CD40 signaling did not seem to be necessary for DC maturation or enhancement. WT helped had an enhanced increase in all subsets whereas CD40 KO helped condition failed to do so.

I confirmed if CD40 signaling was required for the increase of MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloid subsets as well. WT helped were composed of more MHCII+ and MHCII^{-} Ly6C^{hi} Ly6C^{int} myeloid cells in both frequency and numbers on day 2 after induction. CD40 signaling correlated with enhanced expansion of total myeloids and DCs as well as the increase of the MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloid numbers.

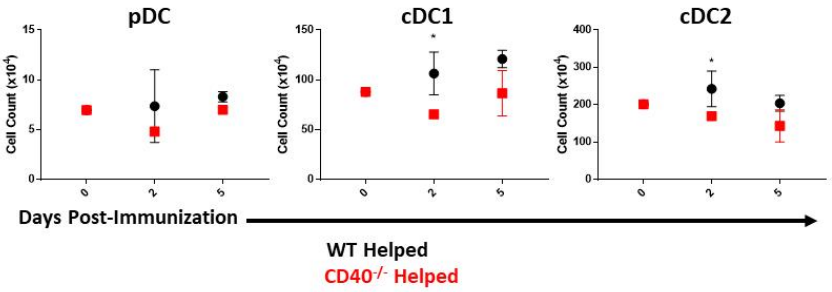
A


Helped:
2 x 10⁷ CD45.1c.H60c.♂
splenocytes
WT♀
CD40-/-♀
Days 2 & 5: sacrificed and
analyzed by flow cytometry

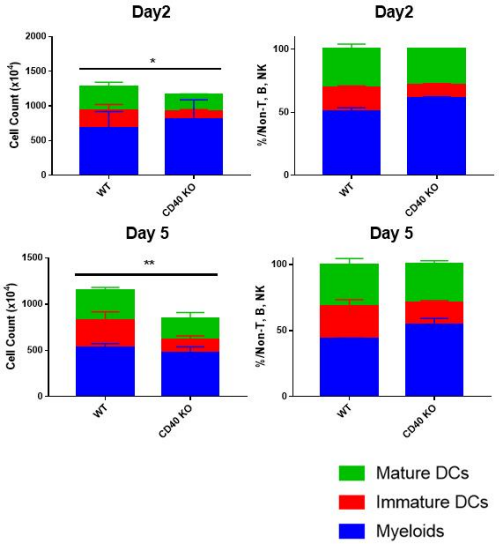
B


cDC1
cDC2
pDC
84.4
20.7
32200
30500
256
92
20015
21010
68.7
31.7
12854
13224
CD40
MHCII
WT Helped
CD40-/- Helped
Negative Control

C


pDC
cDC1
cDC2
Cell Count (x10⁴)
Days Post-Immunization
WT Helped
CD40-/- Helped

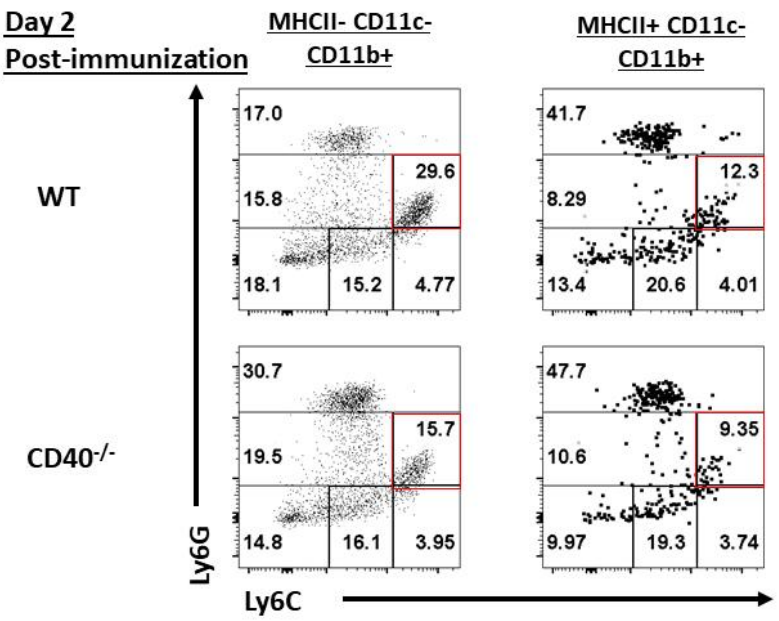
D



Day 2	WT Helped	CD40 KO Helped
Mature DC	30.75±3.95	28.4±1.4
Immature DC	15.39±2.4	13.81±1.09
Myeloid	54.005±2.495	57.875±1.475

Day 5	WT Helped	CD40 KO Helped
Mature DC	31.8±4.5	29.3±2.6
Immature DC	20.48±5.06	16.23±1.73
Myeloid	47.745±.575	54.655±4.375

E



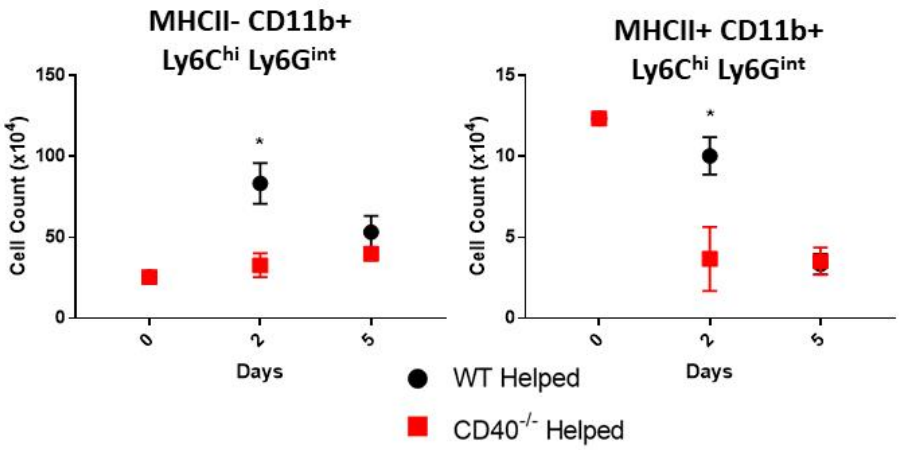

MHCII- CD11b+
Ly6C^hi Ly6G^int
Cell Count (x10^4)
150
100
50
0
*
0
2
5
Days
MHCII+ CD11b+
Ly6C^hi Ly6G^int
Cell Count (x10^4)
15
10
5
0
*
0
2
5
Days
WT Helped
CD40^-/- Helped

Figure 4. CD40 Deficient Mice Have Limited Expansion of Myeloids and DCs, and MHCII^{+} and MHCII^{-} Ly6C^{hi} Ly6G^{int} Myeloids

(A) Experimental Scheme of WT and CD40 KO Helped. (B) DC maturation phenotype on day 2 in the spleen of WT and CD40 KO Helped. (C) Mature DC numbers on days 2 and 5 in the spleen post-immunization (D) Ratio of Immature myeloids, DCs, and mature DCs in the spleen on day 2 and 5. (E) Flow cytometric data and numbers of MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloid analyzed days 2 and 5 post-immunization of WT and CD40 KO Helped. All data (b-e) are representative of 4 mice (n=4 per experiment). Data are presented as means ± SEM. *P<0.05, **P<0.01 by student's *t*-test.

CD40 stimulation Enhances Expansion of Myeloids and DCs, including MHCII^{+} and MHCII^{-} Ly6C^{hi} Ly6G^{int} Myeloids, *in vitro*

Myeloid and DC numbers are affected *in vivo* from killing by cytotoxic CD8 T cells and other phagocytes, and by migration induced during the immune response. I sought to see if the enhanced expansion of myeloids and DCs, and the distinct MHCII^{-} and MHCII^{+} Ly6C^{hi} Ly6C^{int} myeloid subsets could be recapitulated using an *in vitro* hematopoietic cell culture model. This would create a closed system model in which differentiation and expansion potential could be observed without the effects of migration or killing induced by cytolytic activity.

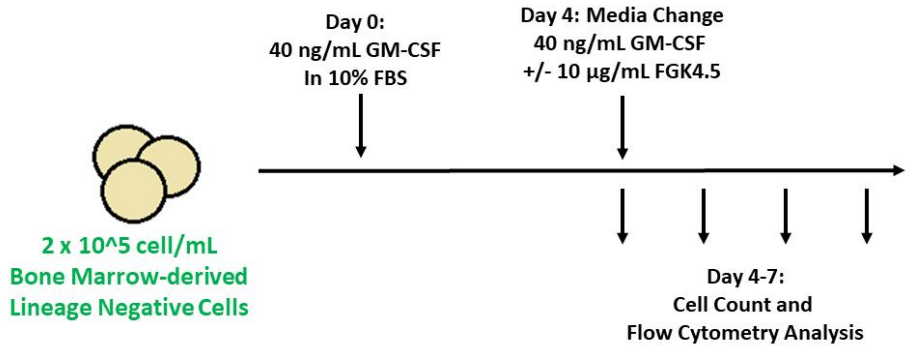
I isolated lineage negative cells from the bone marrow, the source of hematopoietic cells. These lineage negative cells consist of pluripotent hematopoietic stem cells and multipotent progenitors and precursors. All lineage markers (CD3e, CD19, B220, CD11b, CD11c, Ly6G, and Ly6C) were sorted out. I cultured lineage negative cells in GM-CSF-containing media and supplemented with or without α-CD40 stimulating FGK4.5 antibody during the media change on day 4, which consists of mostly of myeloid and immature DCs (Figure 5A). I counted and analyzed cells daily by flow cytometry.

Total cellularity in the CD40 stimulated culture was expanded by almost 1.5 to 2 times that of solely GM-CSF culture (Figure 5B). pDCs and mature cDCs numbers were

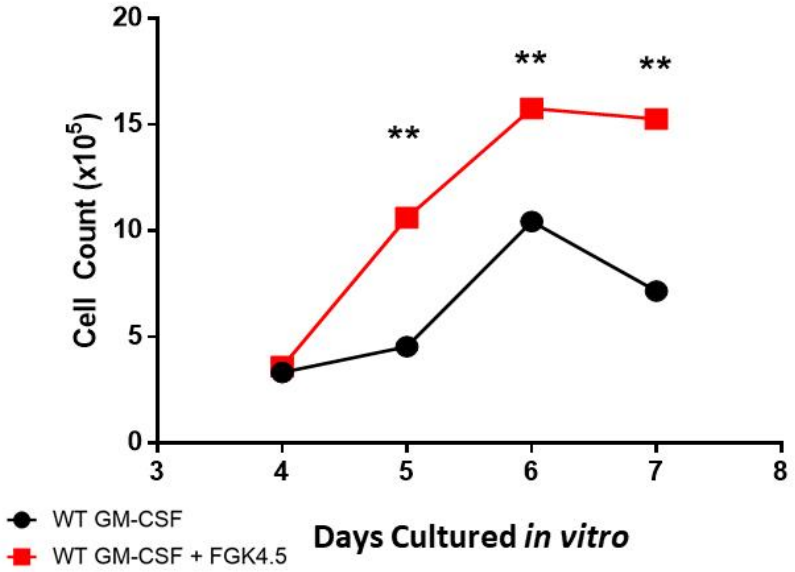
expanded in the presence of CD40 stimulation. Mature cDC1 numbers were increased almost 5 times in the presence of FGK4.5, whereas cDC2 numbers were doubled (Figure 5C). Despite the difference in total cellularity and mature DC numbers, the ratio of myeloids and DCs were maintained throughout day 6 (Figure 5D). The decrease in mature DC frequency in the GM-CSF condition on day 7 was due to apoptosis (data not shown). The similar ratios maintained between FGK4.5 supplemented and non-supplemented media suggests a total expansion of myeloid and DC subsets via CD40 signaling.

Since the DC and myeloid expansion was enhanced in the presence of FGK4.5, I confirmed that the $MHCII^{-}$ and $MHCII^{+}$ $CD11c^{-}$ $CD11b^{+}$ $Ly6C^{hi}$ $Ly6C^{int}$ myeloid subsets were also expanded in the presence of FGK4.5 (Figure 5E). Similar to the results seen with the *ex vivo* cells, $MHCII^{-}$ and $MHCII^{+}$ $Ly6C^{hi}$ $Ly6C^{int}$ myeloid numbers were almost doubled when CD40 stimulation was given. This data suggest that CD40 signaling enhances the expansion of myeloids and DCs.

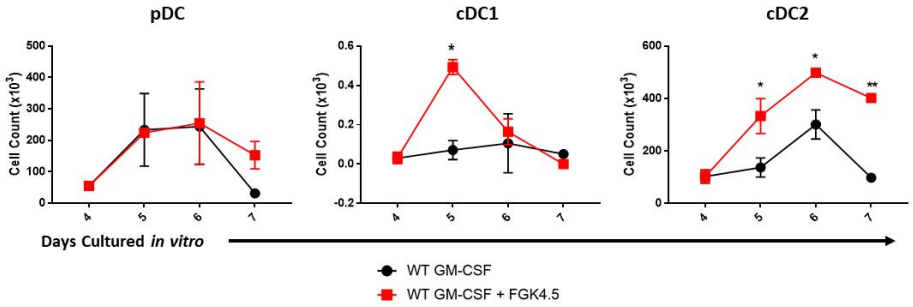
A


Day 0:
40 ng/mL GM-CSF
In 10% FBS
Day 4: Media Change
40 ng/mL GM-CSF
+/- 10 µg/mL FGK4.5
2 x 10^5 cell/mL
Bone Marrow-derived
Lineage Negative Cells
Day 4-7:
Cell Count and
Flow Cytometry Analysis

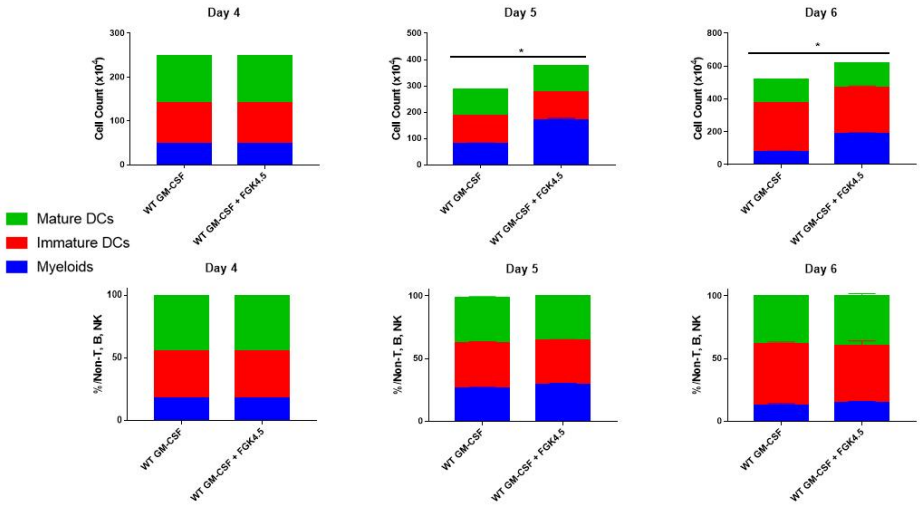
B


Cell Count (x10^5)
20
15
10
5
0
3
4
5
6
7
8
**
**
**
WT GM-CSF
WT GM-CSF + FGK4.5
Days Cultured in vitro

C



D



Day 5	GM-CSF	GM-CSF + FGK4.5
Mature DC	36.37±.53	35.325±.425
Immature DC	36.06±.84	35.325±.425
Myeloid	26.295±.455	29.205±.705

Day 6	GM-CSF	GM-CSF + FGK4.5
Mature DC	37.975±.175	39.4±1.5
Immature DC	49.255±.955	45.9±2.65
Myeloid	12.665±.885	14.51±.96

E

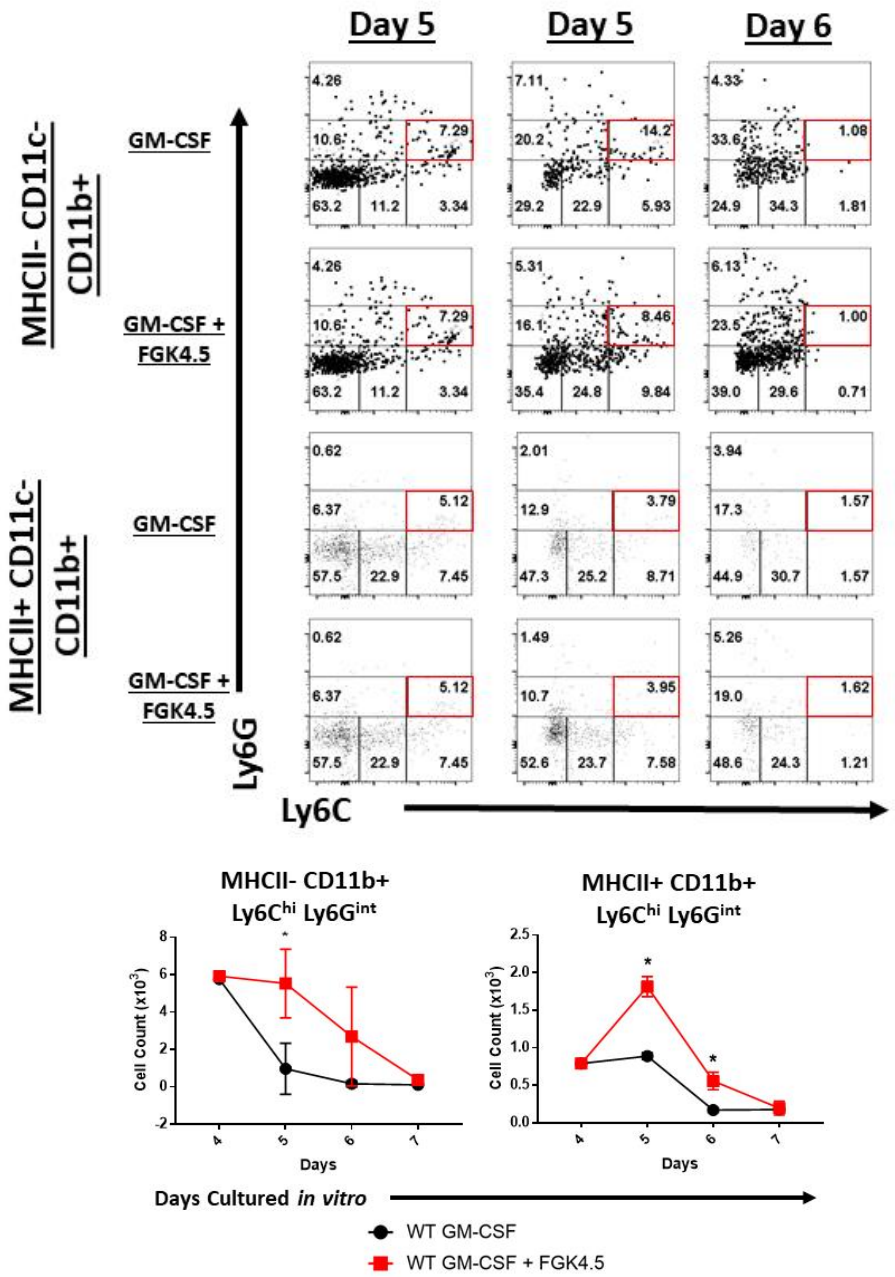

Day 5
Day 5
Day 6
MHCII- CD11c- CD11b+
MHCII+ CD11c- CD11b+
GM-CSF
GM-CSF + FGK4.5
GM-CSF
GM-CSF + FGK4.5
Ly6G
Ly6C
4.26
10.6
7.29
63.2
11.2
3.34
7.11
20.2
14.2
29.2
22.9
5.93
4.33
33.6
1.08
24.9
34.3
1.81
4.26
10.6
7.29
63.2
11.2
3.34
5.31
16.1
8.46
35.4
24.8
9.84
6.13
23.5
1.00
39.0
29.6
0.71
0.62
6.37
5.12
57.5
22.9
7.45
2.01
12.9
3.79
47.3
25.2
8.71
3.94
17.3
1.57
44.9
30.7
1.57
0.62
6.37
5.12
57.5
22.9
7.45
1.49
10.7
3.95
52.6
23.7
7.58
5.26
19.0
1.62
48.6
24.3
1.21
MHCII- CD11b+
Ly6Chi Ly6Gint
MHCII+ CD11b+
Ly6Chi Ly6Gint
Cell Count (x10³)
Days
Days Cultured in vitro
WT GM-CSF
WT GM-CSF + FGK4.5

Figure 5. CD40 stimulation Enhances Expansion of Myeloids and DCs, including MHCII^{+} and MHCII^{-} Ly6C^{hi} Ly6G^{int} Myeloids, *in vitro*

(A) Experimental Scheme of culturing lineage negative BM cells and media change on day 4. (B) Total Cellularity of *in vitro* Cultured WT BM-derived Lin^{-} Cells in GM-CSF or with FGK4.5 supplementation. (C) Mature DC Cellularity Kinetics and (D) ratio of myeloids and DCs *in vitro* (E) Flow Cytometric Data and Numbers of MHCII^{-} and MHCII^{+} CD11c^{-} CD11b^{+} Ly6C^{hi} Ly6C^{int} Myeloid cells *in vitro.* All data (b-e) are representative of 3 independent cultures (n=3 per experiment). Data are presented as means ± SEM. *P<0.05, **P<0.01 by student's *t*-test.

Discussion

In this study, I demonstrated that the role CD4 help is not associated with DC maturation or enhancement, that is, implementing a qualitative difference, which is the general consensus. Instead, it was demonstrated that CD4 help enhances the increase of the number of myeloids and DCs. In particular, it increases the number of a $MHCII^-$ and $MHCII^+$ $CD11c^-$ $CD11b^+$ $Ly6C^{hi}$ $Ly6C^{int}$ myeloid subset, that has a phenotype similar to that of immature and mature DCs. This suggests that this subset could be DC precursors whose expansion leads to the increase of the number of immature and mature DCs during the helped response.

Regarding the help factor that drives this enhancement of expansion is possibly CD40. The CD40 KO helped model and an *in vitro* culture supplemented with a-CD40 stimulating FGK4.5 antibody confirmed that the population numbers and kinetics seen in helped versus helpless phenomenon was observe *in vivo* inetics of WT mice. The CD40 deficient helped model had a limited expansion of total subsets, including the $MHCII^-$ and $MHCII^+$ $CD11c^-$ $CD11b^+$ $Ly6C^{hi}$ $Ly6C^{int}$ myeloid subset. In addition, the addition of CD40 stimulation *in vitro*, in which cells grow within a closed system, had total expansion of all subsets and cellularity as well as the DC precursor subset.

Despite a general consensus of the necessity of CD40 signaling during CD4 help for DC maturation, it has been known to have other roles during hematopoiesis. In addition to maturation, CD40 signaling can induce survival by suppressing apoptosis (24, 25). Previous studies with B cells and myeloids have shown CD40 to induce or inhibit differentiation (26, 27). I hypothesized that CD40 signaling during CD4 help would have an alternative role for DC licensing, or qualitative enhancement of DC populations. This role could be the enhancement of the DC maturation process *bona fide*. This indicates that the DC maturation status would not be influenced by the presence of CD4 help. The data pertaining to the DC maturation phenotype of mature DCs were similar in helped and helpless are in line with this suggestion. This is also consistent with the previous publication showing the maturation status of DCs in conjugation with helped and helpless monoclonal CD8 T cells specific for H60 (28).

Rather than the need for DC maturation during helped, my data suggest that this cellular antigen model may require a quantitative increase of myeloid cells and DCs to induce an expansion of antigen-specific CD8 T cells and antigen clearance. This increased number of myeloid cells and DCs would lead to the quantitative increase of mature DC cell numbers, resulting in the quantitative increase of activated effector CD8 T cells seen in the previous publication (28). Alternatively, this may suggest that

myeloids and immature DCs may also have the ability to present antigen, contributing to enhanced antigen-specific CD8 T cell expansion.

Despite information provided by this data, the $MHCII^-$ and $MHCII^+$ $CD11c^-$ $CD11b^+$ $Ly6C^{hi}$ $Ly6C^{int}$ myeloid populations are still unknown in their lineage. I require further lineage study and transcriptome analysis of this population to provide insight into how they might contribute to the increasing immature and mature DC population.

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요약 (국문 초록)

CD4 T 세포 도움은 바이러스 및 세포성 항원에 대한 최적의 CD8 T 세포 면역 반응이 일어날 수 있도록 수지상 세포의 “라이센싱”을 유도하는 것이라고 알려져 있다. 그러나 CD4 T 세포 도움에 의한 수지상 세포 라이센싱의 정확한 기작과 그 역할은 아직 완전히 밝혀지지 않았다. 따라서 본 연구에서 CD4 T 세포 도움의 역할을 구체적으로 규명하기 위하여, CD4 T 세포의 도움 유무에 따른 CD8 T 세포 반응이 일어나는 동안 수지상 세포 및 골수성 세포의 변화를 추적하였다. 놀랍게도 기존의 많은 보고들과는 달리 CD4 T 세포 도움 유무에 따른 수지상 세포의 성숙과 기능에는 큰 차이가 없음을 확인하였다. 하지만 CD4 T 세포 도움이 존재할 때에 성숙된 수지상 세포의 수가 증가하였고 동시에 미성숙한 수지상 세포와 골수성 세포의 수 또한 증가된 것을 발견하였다. 특히, CD4 T 세포 도움 시 양적으로 증가한 골수성 세포는 $MHCII^{+}$ 및 $MHCII^{-}$ 이면서 $CD11c^{-}CD11b^{+}Ly6C^{hi}Ly6G^{int}$ 인 세포로 구성되어 있었으며 구체적인 표현형이 미성숙 및 성숙된 수지상 세포의 표현형과 유사하였기 때문에 수지상 세포 전구체 집단일 것이라고 생각된다. CD4 T 세포 도움 시 증가하였던 골수성 세포, 수지상 세포 및 수지상 세포 전구체가 CD40 결핍 마우스를 면역하였을 때에는 감소하였으며, 시험관 내에서 골수 세포 배양 시 CD40 자극에 의해서는 다시 증가함을 확인하였다. 이러한 결과들을 바탕으로, 나는 CD4 T 세포 도움의 역할은 수지상 세포의 성숙을 촉진하기보다는 골수성 세포와 수지상 세포 전구체의 수적 증가에 기여함을 통해 성숙된 수지상 세포 집

단을 늘리는 것이라고 제안한다.

주용어: CD4 도움, CD40, 수지상 세포, 골수 세포, H60
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