Concanavalin A-mediated T cell proliferation is regulated by herpes virus entry mediator costimulatory molecule

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Concanavalin A-mediated T cell proliferation is regulated by Herpes Virus Entry Mediator costimulatory molecule

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Running title;
Role of HVEM on ConA-mediated T cell proliferation

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Abbreviations;
ConA, Concanavalin A; TCR, T cell receptor; Ag, antigen; APC, antigen presenting cell; DC, Dendritic cell; LN, Lymph node; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; 7-AAD, 7-amino-actinomycin D; TRAF, TNFR-associated factor; PI, propidium iodide

Abstract

T cell activation is regulated by two distinct signals, signal one and two. Concanavalin A (ConA) is an antigen-independent mitogen and functions as signal one inducer, leading T cells to polyclonal proliferation. CD28 is known to be one of major costimulatory receptors and to provide signal two in the ConA-induced T cell proliferation. Here, we have studied the implication of other costimulatory pathways in the ConA-mediated T cell proliferation by using soluble recombinant proteins consisting of an extracellular domain of costimulatory receptors and Fc portion of human IgG. We found that T cell proliferation induced by ConA, but not PMA plus ionomycin or anti-CD3 mAb, is significantly inhibited by HVEM-Ig, even in the presence of CD28 signaling. Moreover, the high concentration of HVEM-Ig molecules almost completely suppressed ConA-mediated T cell proliferation. These results suggest that HVEM might play more important roles than CD28 in ConA-mediated T cell proliferation.

Keywords

T Cell activation, Lectin, Ig-fusion molecule, costimulatory signal

Introduction

T cell activation is initiated by recognition of antigen (Ag) peptide in a context of MHC molecule and this recognition is done by a T cell receptor (TCR). T cell activation leads to proliferation, which is regulated by two distinct signals (Bretscher 1999). Signal one is generally derived from the TCR, thereby ensuring Ag-specific responses. Signal two is also called a costimulatory signal and is activated when a costimulatory receptor is engaged by its ligand. The signal two has no ability to induce T cell activation in the absence of the signal one, indicating its regulatory roles in the T cell response qualitatively as well as quantitatively. For the complex regulation of T cell fate following activation, multiple pairs of a receptor and ligand have been found to transmit various costimulatory signals.

Among costimulatory receptors, CD28 is known as a most potent costimulatory receptor. The CD28 is expressed on T cells and binds to CD80 and CD86 on antigen presenting cells (APCs) (Rudd et al. 2009). The role of CD28 has been investigated using CD28-deficient T cells or anti-CD28 mAb, and was found to enhance T cell proliferation through both IL-2 dependent (Khoruts et al. 1998) and independent (Appleman et al. 2000) mechanisms. CTLA-4, which shows sequence homology to CD28, shares ligands with CD28 and also binds to both CD80 and CD86 (Salomon and Bluestone 2001). Unlike CD28, CTLA-4 down-regulates T cell proliferation (Walunas et al. 1994) and is

rather called a coinhibitory receptor for T cell function (Sinclair 1999). HVEM, initially identified as a herpes virus receptor, regulates T cell proliferation positively or negatively depending on its ligands (Cai and Freeman 2009). HVEM binds to LIGHT, augmenting T cell proliferation and IFN-γ production (Harrop et al. 1998; Tamada et al. 2000), whereas its binding to BTLA and CD160 negatively regulates T cell proliferation (Cai et al. 2008; Sedy et al. 2005). ICOS also enhances T cell activation, although it seems to be more important for differentiation of activated T cells (McAdam et al. 2000). On the other hand, CD40 is expressed on APCs, while its ligand CD40L is expressed on activated T cells. The CD40-CD40L interaction up-regulates costimulatory ligands on Dendritic cells (DCs), one of potent APCs, which enhances T cell activation (Ma and Clark 2009). CD28 and ICOS belong to an Ig-super family, while HVEM and CD40 are categorized to a TNFR family. Many other molecules belonging to each family have been shown to regulate T cell activation (Rothstein and Sayegh 2003).

T cells can be also activated in an Ag-independent manner by a variety of mitogens such as anti-CD3 mAb, phorbol 12-myristate 13-acetate (PMA) plus ionomycin and concanavalin A (ConA). Anti-CD3 mAb directly stimulates a signal transducer associated with the TCR, while PMA and ionomycin stimulate protein kinase C (PKC) and Ca^{2+} influx, respectively, activating further downstream of TCR-initiated signaling. In contrast, an intracellular process of ConA-induced T cell activation has not been clearly addressed, although ConA is a widely used mitogen to study T cell activation.

ConA is a lectin isolated from Jack beans and is known to bind to a mannose moiety of cell surface glycoproteins including the TCR (Weiss et al. 1987). Based on the inability to activate T cells fully after depletion of APCs, ConA alone is not sufficient for T cell activation (Ahmann et al. 1978; Chatila et al. 1987), suggesting the involvement of signal two in this process as well. In fact, CD28 has been reported to provide the signal two in ConA-stimulated T cells (Perrin et al. 1997; Shahinian et al. 1993). However, it is not clear whether other costimulatory receptor(s) is involved in ConA-mediated T cell proliferation.

In this study, we examined the role of other costimulatory receptors in T cell proliferation induced by mitogenic stimulation including ConA. We found that T cell proliferation induced by ConA, but not PMA plus ionomycin or anti-CD3 mAb, requires HVEM as signal two, which may predominate over major signal two CD28.

Materials and Methods

T Cell Culture and CFSE analysis

Lymph nodes (LNs: cervical, axillary, brachial, inguinal, mesenteric, periaortic and pancreatic) were isolated from ddy mice aged 8-10 weeks, which had been euthanized according to the guidelines of the Animal Care and Use Committee in Kanazawa University, and crushed to make single cell suspension. LN cells were labeled for 15 min at 37˚C with 2 μM CFSE (Molecular Probes) to track mitotic divisions of T cells following mitogenic stimulation. After 3 washes with PBS containing 5% heat-inactivated fetal bovine serum (FBS), CFSE-labeled LN cells were inoculated at 1.5 x 10^6 cells/ml along with the same density of non-labeled splenocytes prepared from the same mice. The cells were co-cultured in RPMI1640 containing 10% FBS supplemented with gentamycin (10 μg/ml) and stimulated by ConA (4 μg/ml, Sigma-Aldrich), PMA (2 ng/ml, Sigma-Aldrich) plus ionomycin (20 ng/ml, Sigma-Aldrich) or anti-CD3 mAb (1 μg/ml). Anti-CD3 mAb was obtained from hybridoma 145-2C11 culture (Leo et al. 1987). To provide costimulatory signal from CD28 exogenously, anti-CD28 mAb from hybridoma PV1 (Abe et al. 1995) was added to the culture at a concentration of 5 μg/ml. The cells were collected at various time points and treated with 7-amino-actinomycin D (7-AAD, BD Biosciences) or propidium iodide (PI, Sigma) to exclude dead cells from the subsequent analysis. In some experiments, $CD4^+$ or $CD8^+$ T cells were separately stained

with PE-labeled anti-CD4 mAb (GK1.5, Beckman Coulter) or anti-CD8 mAb (53-6.7, Beckman Coulter), respectively, before treatment with 7-AAD. The CFSE profiles of 7-AAD- or PI-negative live cells were examined by flow cytometry using FACScalibur and CELLQuest software (Becton Dickinson). The mean division numbers were determined from the CFSE profiles by calculating percent contribution of the initial cohort in each division peak. Statistical tests were performed using Student's independent *t* test. The results were considered to be significant at $p < 0.05$.

Preparation of soluble costimulatory molecules fused with IgG-Fc

In order to block costimulatory ligand-receptor interaction, Ig-fusion molecules were prepared as described previously (Kanaya et al. 2003; Matsui et al. 2003; Ono et al. 2004). In brief, COS-7 cells grown densely were infected with an excess amount of recombinant adenovirus carrying a CAG promoter and Ig-fusion gene, which expresses an extra-cellular region of each costimulatory receptor, HVEM, ICOS or CD40, connected in flame to a Fc region of human IgG1. Following the infection, cells were cultured in FBS-free DMEM for 72 h and the recombinant proteins were purified from the culture supernatant by protein-A column chromatography.

Results

Role of CD28 in LN cell proliferation stimulated by ConA

Firstly, we tried to examine the role of CD28 in ConA-stimulated LN cell proliferation. In our assay, LN cells isolated from mice were labeled with CFSE and mixed with non-labeled SPL cells before adding mitogens to the mixture. SPL cells contain APCs, which can provide an array of various costimulatory signals including CD28. At 60 h after mitogenic stimulation, several peaks with lower CFSE intensity were detected in the CFSE profiles, indicating multiple rounds of cell division during this time frame (Fig.1A). The addition of anti-CD28 mAb markedly enhanced proliferation of LN cells stimulated by PMA plus ionomycin or anti-CD3 mAb (Fig.1A), suggesting that CD28 stimulation from APCs in SPL cells is insufficient to fully activate LN cells under the conditions we used. In clear contrast, anti-CD28 mAb had no effects in the LN cell proliferation stimulated by ConA. To confirm this observation, we conducted time-course experiments and compared mean division numbers determined from the CFSE profiles (Fig.1B). Cell division was detected at 36 h or later and the mean division numbers were increased until 60 h. Consistent with the observation in Fig. 1A, the addition of anti-CD28 mAb significantly enhanced cell proliferation stimulated by PMA plus ionomycin or anti-CD3 mAb, but not ConA. These results suggest that the level of CD28 requirement as signal two might be different between ConA-initiated stimulation and other mitogenic

stimulation.

ConA is known to have an affinity to surface glycoproteins and CD28 can be one of those glycoproteins. Therefore, it is possible that a high concentration of ConA also stimulates CD28 signaling. To examine this possibility, we treated LN cells with less concentrations of ConA and tested the effect of anti-CD28 mAb on their proliferation. As shown in Fig.2, in the absence of anti-CD28 mAb, ConA-induced proliferation was attenuated at less than 2 μg/ml in a concentration-dependent manner. Under the suboptimal conditions, anti-CD28 mAb significantly enhanced LN cell proliferation, suggesting that CD28 is also implicated in the ConA-stimulated LN cell proliferation.

Implication of HVEM in T cell proliferation induced by ConA, but not PMA plus ionomycin or anti-CD3 mAb

We next asked whether other costimulatory pathways are involved in ConA-induced proliferation. ICOS (Simpson et al. 2010) and HVEM (Harrop et al. 1998; Tamada et al. 2000) are receptors expressed on T cells to potentially transmit a costimulatory signal, while CD40 (Ma and Clark 2009) is expressed on APCs and changes expression pattern of costimulatory ligands. We prepared Ig-fusion molecules of ICOS, HVEM or CD40, which consist of their extra-cellular domain and a Fc portion of human IgG. Since these molecules are expected to inhibit the specific receptor-ligand interaction, we tested their impacts on cell proliferation induced by ConA, PMA plus ionomycin or anti-CD3 mAb in

the presence of anti-CD28 mAb (Fig.3). CD40-Ig and ICOS-Ig showed no effects on any mitogen-induced proliferation, whereas HVEM-Ig markedly inhibited the proliferation induced by the ConA, but not PMA plus ionomycin or anti-CD3 mAb, in a dose-dependent manner. Importantly, at the maximum concentration, HVEM-Ig almost completely inhibited the proliferation. The percentages of dead LN cells (CFSE and PI double-positive cells) were constantly 35-45% at 48 h in the presence or absence of HVEM-Ig (Fig.4), indicating that the HVEM-Ig-mediated inhibition of ConA-induced proliferation is not likely due to cell death of proliferating population. In order to further strengthen the contrasting action of HVEM-Ig on ConA versus anti-CD3 mAb mediated LN cell proliferation, we examined the inhibitory effect of HVEM-Ig on LN cells stimulated by an optimal concentration of ConA or a suboptimal concentration of anti-CD3 mAb in the presence or absence of anti-CD28 mAb (Fig.5). The inhibitory effect of HVEM-Ig on the ConA-mediated LN cell proliferation was again observed at the optimal concentration of ConA even in the absence of anti-CD28 mAb (Fig.5A). The LN cell prolifetaion induce by anti-CD3 mAb plus anti-CD28 mAb was resistant to the HVEM-Ig treatment for 48 h (Fig.3), therefore we next examined the effect of HVEM-Ig on LN cells stimulated under suboptimal condition at 60 h. Since the LN cell proliferation begun attenuated at less than 0.1 μg/ml (data not shown), we stimulated LN cells by anti-CD3 mAb at 0.01 μg/ml and found that the effect of HVEM-Ig was subtle even in the absence of anti-CD28 mAb (Fig.5B). Thus, the LN cell proliferation induced by ConA is

fundamentally different from that induced by anti-CD3 mAb.

The cell population isolated from LN contains mainly T cells but also other types of cells such as B cells. Although ConA has been used as a T cell mitogen, it is possible to stimulate B cell proliferation by binding to their surface glycoproteins. T cell population contains two major subsets expressing either CD4 or CD8 molecules, which have different roles in adaptive immunity. To examine the effect of HVEM-Ig on T cell proliferation more precisely, LN cells were stained with a PE-labeled anti-CD4 mAb or anti-CD8 mAb and separately analyzed by FACS (Fig.6). The inhibitory effect of HVEM-Ig was comparable to that observed with whole LN cell population (Fig.3) and showed no clear difference between $CD4^+$ and $CD8^+$ T cell subsets. These results strongly suggest that HVEM is implicated in ConA-stimulated proliferation in both $CD4^+$ and $CD8^+$ T cells.

Discussion

In this study, we found that ConA-mediated T cell proliferation is greatly inhibited by HVEM-Ig. This inhibitory effect was not observed in T cell proliferation induced by PMA plus ionomycin or anti-CD3 mAb, indicating the specific involvement of HVEM-related signaling in ConA-mediated T cell activation.

LIGHT has been identified as a ligand for HVEM (Mauri et al. 1998) and might be one of the target candidates for soluble HVEM-Ig inhibiting HVEM-mediated costimulatory signal. HVEM is also expressed on immature DCs as well as T cells and LIGHT enhances DC maturation through the interaction with HVEM (Morel et al. 2001). Thus, HVEM-Ig might inhibit T cell proliferation through an indirect effect against APCs in addition to the direct effect against T cells. On the other hand, HVEM is also known to bind to BTLA and CD160 expressed on T cells (del Rio et al. 2010) and to induce coinhibitory signal (Cai et al. 2008; Sedy et al. 2005), raising the possibility that HVEM-Ig might stimulate this coinhibitory signaling. However, BTLA was reported to induce cell death in T cells in some cases (Deppong et al. 2008), but we did not detect any additional T cell death by HVEM-Ig treatment (Fig.4). Furthermore, HVEM-Ig had no impacts on LN cell proliferation stimulated by PMA plus ionomycin or anti-CD3 mAb (Fig.3). Thus, HVEM-Ig most likely inhibits the HVEM-mediated costimulatory signal, however, further study is needed to clarify how HVEM regulates the ConA-mediated T cell

activation.

Another important point we should stress is that HVEM-Ig blocked ConA-induced T cell proliferation even in the presence not just absence of anti-CD28 mAb. CD28 has been previously reported to enhance T cell proliferation induced by Ag-independent mitogenic stimuli including ConA (Perrin et al. 1997; Shahinian et al. 1993). Consistently, in our experiments, anti-CD28 mAb indeed enhanced LN cell proliferation induced by suboptimal concentration (0.25 - 1.0 μg/ml) of ConA (Fig.2), although anti-CD28 mAb showed no significant effects at higher ConA concentrations (2.0 and 4.0 μg/ml). These results suggest that at least two distinct costimulatory pathways, CD28 and HVEM, are implicated in the ConA-stimulated T cell proliferation. CD28 belongs to Ig-super family and has been shown to bind PI3-kinase and Grb2 via its cytoplasmic region (Rudd et al. 2009), whereas HVEM is one of TNFR family members and binds to TNFR-associated factors (TRAFs) (Wallach et al. 1999), suggesting their independent function on T cell regulation. HVEM might activate an NF-κB pathway by binding to TRAF (Wallach et al. 1999) and function as not only signal two but also signal one in part. More importantly, the highest concentration of HVEM-Ig almost completely abolished cell division of T cells stimulated by ConA (Figs.3 and 4), leading us to speculate that HVEM signaling may predominate over CD28 signaling. CD28 expression was up-regulated on the CD3-positive T cells after stimulation by ConA (data not shown). This result is consistent with the previous report in which Yokoyama et al. showed up-regulation of CD28 after

stimulation by ConA as well as anti-CD3 mAb or PMA (Yokoyama et al. 1988). In our pilot experiment, CD28 up-regulation induced by ConA was inhibited by HVEM-Ig. Thus, CD28 expression is tightly regulated, however, it is difficult to speculate a direct link between HVEM and CD28 function.

ConA has been extensively used in the model of autoimmune hepatitis and $CD4^+$ T cells as well as NKT cells were suggested to play a role in this model (Kaneko et al. 2000; Tiegs et al. 1992; Toyabe et al. 1997; Yang et al. 2010). In addition, ConA has been reported to show anti-tumor effects, which are mediated by the intrahepatic activation of both NK cells (Miyagi et al. 2004) and $CD8^+$ T cells (Chang et al. 2007; Lei and Chang 2009). ConA is also known to induce anti-fungal response (Felipe et al. 1995). This effect seems to be mediated by IFN-γ production through development of Th1 cells, leading to the activation of phagocytes (Kaposzta et al. 1998). Based on our finding, HVEM might be also implicated in these immune responses and is a potential target for their modulation. Further studies on the precise roles of HVEM in ConA-stimulated T cell proliferation would be helpful for not only understanding the basic mechanism of T cell activation but also developing the therapeutic approach for modulating T cell responses.

Conclusions

The T cell proliferation induced by ConA, but not PMA plus ionomycin or anti-CD3 mAb, is regulated by HVEM, which may predominate over major costimulatory molecule CD28. This finding would not only show unique requirement of the ConA-mediated T cell proliferation, but also provide valuable information to exploit ConA for various applications.

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Figure legends

Fig. 1. Anti-CD28 mAb enhances LN cell proliferation induced by PMA plus ionomycin or anti-CD3 mAb, but not ConA. A. LN cells were labeled with CFSE and combined with SPL cells. The cells were stimulated by ConA (4 μg/ml), PMA (2 ng/ml) plus ionomycin (20 ng/ml) or anti-CD3 mAb (1 μg/ml) in the presence or absence of anti-CD28 mAb (5 μg/ml). After 60 h stimulation, CFSE profiles of LN cells were analyzed by FACS. B. The mixture of CFSE-labeled LN cells and non-labeled SPL cells was stimulated as described in A and harvested at the indicated time points before FACS analysis. The mean division number was determined from each CFSE profile as described in the *Materials and Methods*. The data represents the average ± SEM from three independent experiments. * denotes a significant difference with $p < 0.05$.

Fig. 2. ConA-induced LN cell proliferation is enhanced by anti-CD28 mAb only under suboptimal conditions. The mixture of CFSE-labeled LN cells and non-labeled SPL cells was stimulated by various concentrations of ConA (0.125, 0.25, 0.5, 1, 2 or 4 μg/ml) in the presence (■) or absence (○) of anti-CD28 mAb (5 μg/ml). Cells were harvested at 48 h and analyzed by FACS. The mean division number was determined from each CFSE profile as described in the *Materials and Methods*. The data represents the average ± SEM from three independent experiments. * denotes a significant difference with $p < 0.05$.

Fig. 3. Soluble HVEM-Ig molecules strongly suppress LN cell proliferation induced by ConA, but not PMA plus ionomycin or anti-CD3 mAb. The mixture of CFSE-labeled LN cells and non-labeled SPL cells was stimulated by ConA (4 μg/ml), PMA (2 ng/ml) plus ionomycin (20 ng/ml) or anti-CD3 mAb (1 μg/ml), along with anti-CD28 mAb (5 μg/ml) in the presence or absence of various concentrations of CD40-Ig (▲), ICOS-Ig (■) or HVEM-Ig (○) (0, 2, 6, 18 or 54 μg/ml) for 48 h. The mean division number was determined from each CFSE profile and the relative proliferation of LN cells to the control (no Ig-fusion molecule) is shown as % mean division number (B). The data represents the average ± SEM from three independent experiments. * denotes a significant difference with $p < 0.05$. Panel A shows typical CFSE profiles of the LN cells following mitogenic stimulation for 48 h in the presence or absence of HVEM-Ig, CD40-Ig or ICOS-Ig (54 μg/ml).

Fig. 4. HVEM-Ig induces no additional cell death in ConA-activated LN cells. The mixture of CFSE-labeled LN cells and non-labeled SPL cells was stimulated by ConA (4 μg/ml) and anti-CD28 mAb (5 μg/ml) in the presence or absence of HVEM-Ig (0, 10 or 50 μg/ml) for 48 h. Cells were stained with PI (2 μg/ml) and analyzed by FACS. Cell death was expressed as the percentage of PI-positive cells in CFSE-positive LN cell population (B). The data represents the average ± SEM from three independent

experiments. N.S.; not significant. Panel A shows typical FACS plots of the LN and SPL cell mixture stimulated for 48 h in the presence of various concentrations of HVEM-Ig.

Fig. 5. HVEM-Ig suppresses LN cell proliferation induced by an optimal concentration of ConA, but not a suboptimal concentration of anti-CD3 mAb, in the presence or absence of anti-CD28 mAb. LN cells were labeled with CFSE and combined with SPL cells. A. The cells were stimulated by ConA (4 μg/ml) alone (lower panel) or along with anti-CD28 mAb (5 μg/ml) (upper panel) in the presence or absence of HVEM-Ig (50 μg/ml) for 48 h. B. The cells were stimulated by anti-CD3 mAb (0.01 μg/ml) alone (lower panel) or along with anti-CD28 mAb (5 μg/ml) (upper panel) in the presence or absence of HVEM-Ig (50 μg/ml) for 60 h. CFSE profiles of LN cells were analyzed by FACS and one of similar profiles from two independent experiments is shown.

Fig. 6. ConA-induced proliferation of $CD4^+$ or $CD8^+$ T cells is strongly suppressed by the addition of HVEM-Ig. The mixture of CFSE-labeled LN cells and non-labeled SPL cells was stimulated by ConA (4 μg/ml) and anti-CD28 mAb (5 μg/ml) in the presence or absence of various concentrations of CD40-Ig (▲), ICOS-Ig (■) or HVEM-Ig (ᑎ) (0, 2, 6, 18 or 54 μg/ml) for 48 h. Cells were stained with a PE-labeled anti-CD4 mAb or anti-CD8 mAb and separately analyzed by FACS. The mean division number was determined from each CFSE profile and the relative proliferation of $CD4^+$ or $CD8^+$ T cells to the control

(no Ig-fusion molecule) was shown as % mean division number (B). The data represents the average ± SEM from three independent experiments. * denotes a significant difference with $p < 0.05$. Panel A shows typical CFSE profiles of $CD4^+$ or $CD8^+$ T cells stimulated by ConA and anti-CD28 mAb in the presence or absence of CD40-Ig, ICOS-Ig or HVEM-Ig (54 μg/ml) for 48 h.

Fig.1

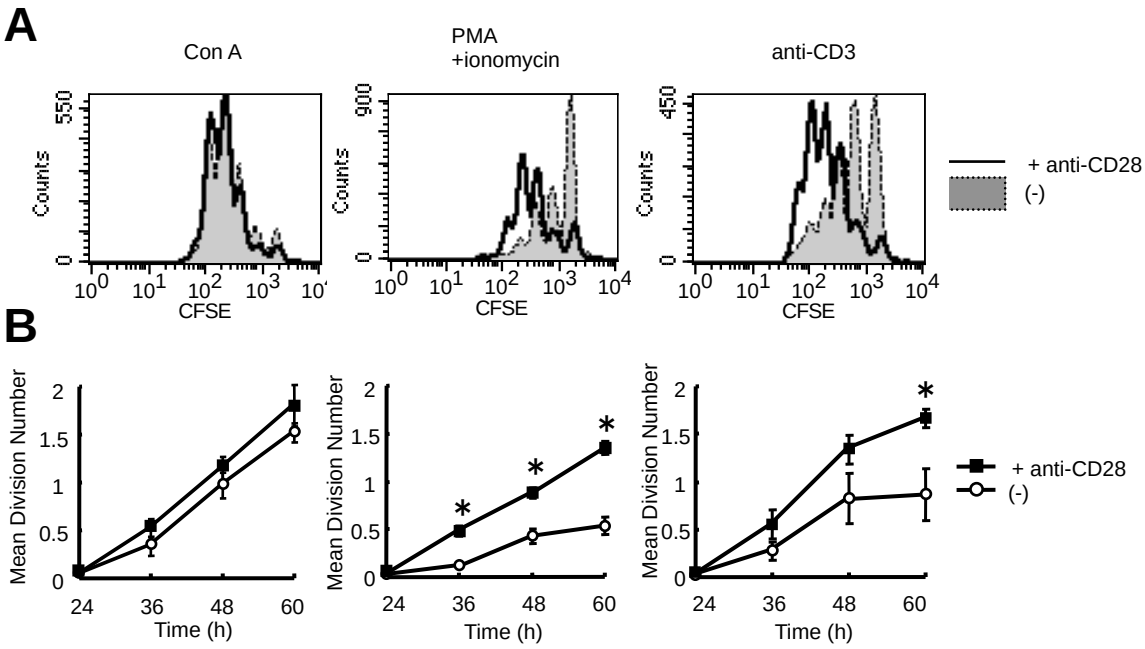

A
Con A
PMA
+ionomycin
anti-CD3
Counts
CFSE
+ anti-CD28
(-)
B
Mean Division Number
Time (h)
+ anti-CD28
(-)

Fig.2

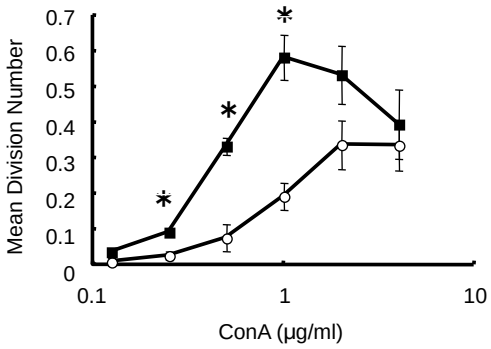
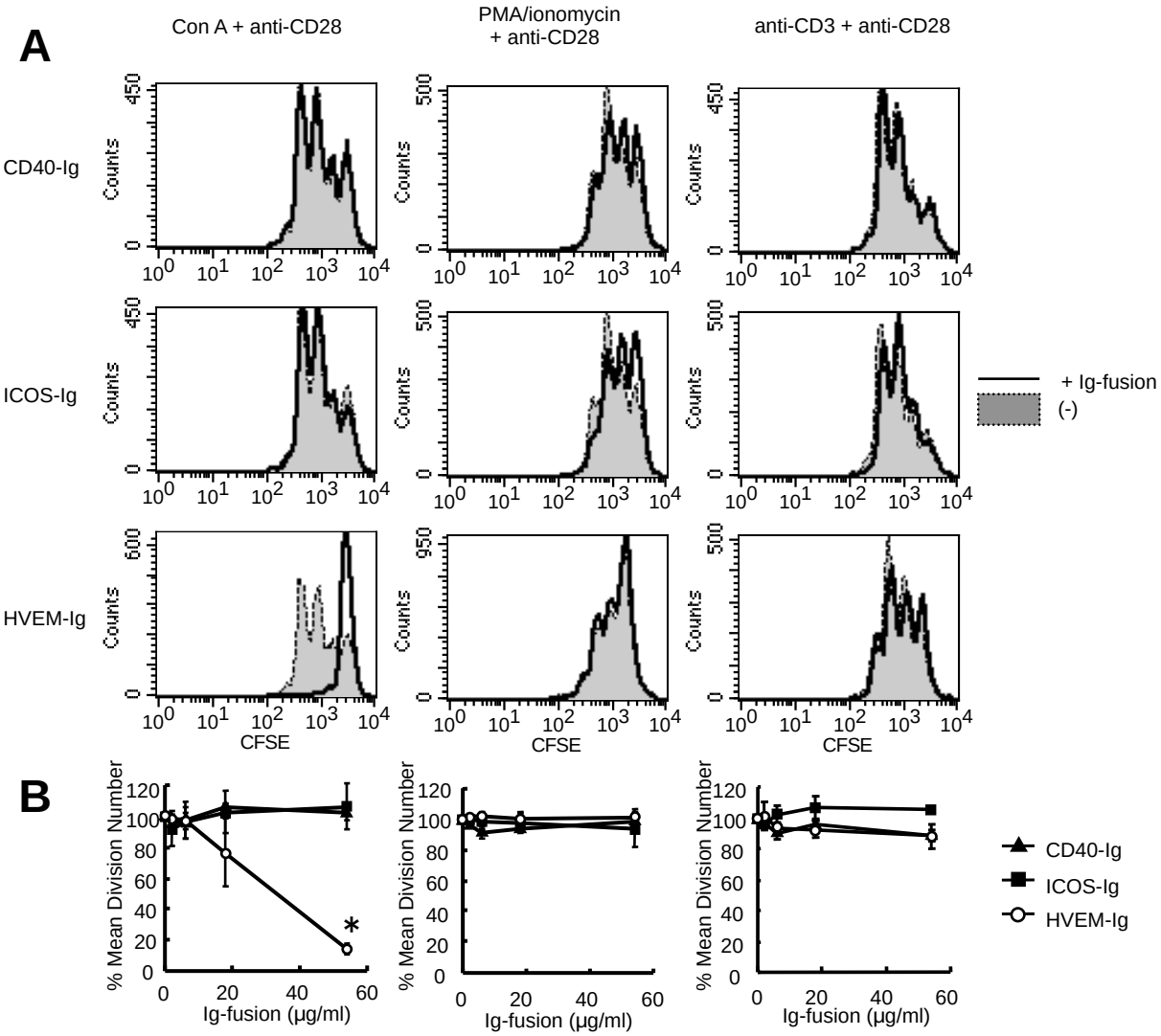


Fig.3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Con A + anti-CD28
PMA/ionomycin
+ anti-CD28
anti-CD3 + anti-CD28
CD40-Ig
ICOS-Ig
HVEM-Ig
Counts
CFSE
+ Ig-fusion
(-)
B
% Mean Division Number
Ig-fusion (μg/ml)
CD40-Ig
ICOS-Ig
HVEM-Ig

Fig.4

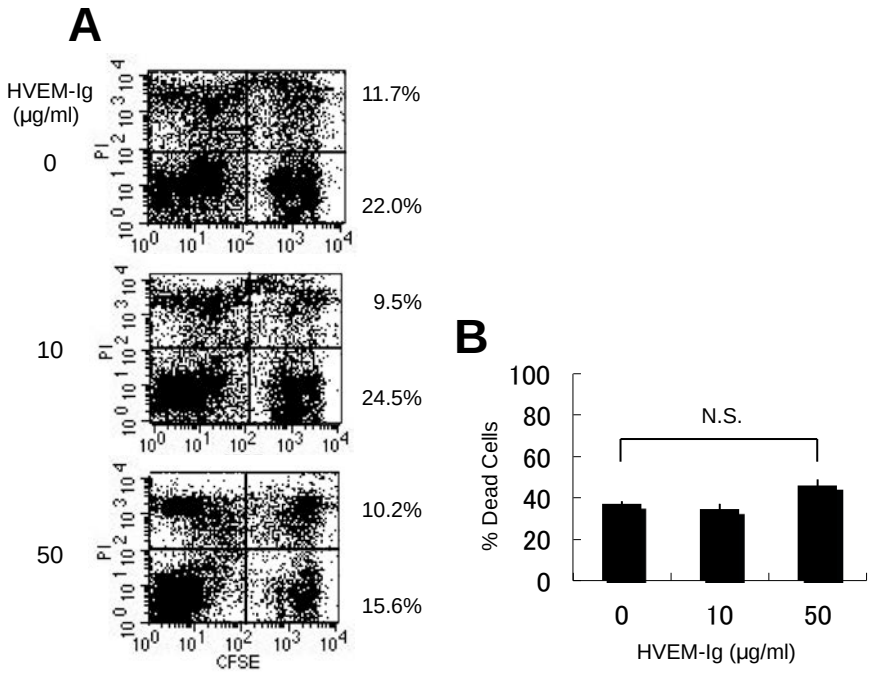
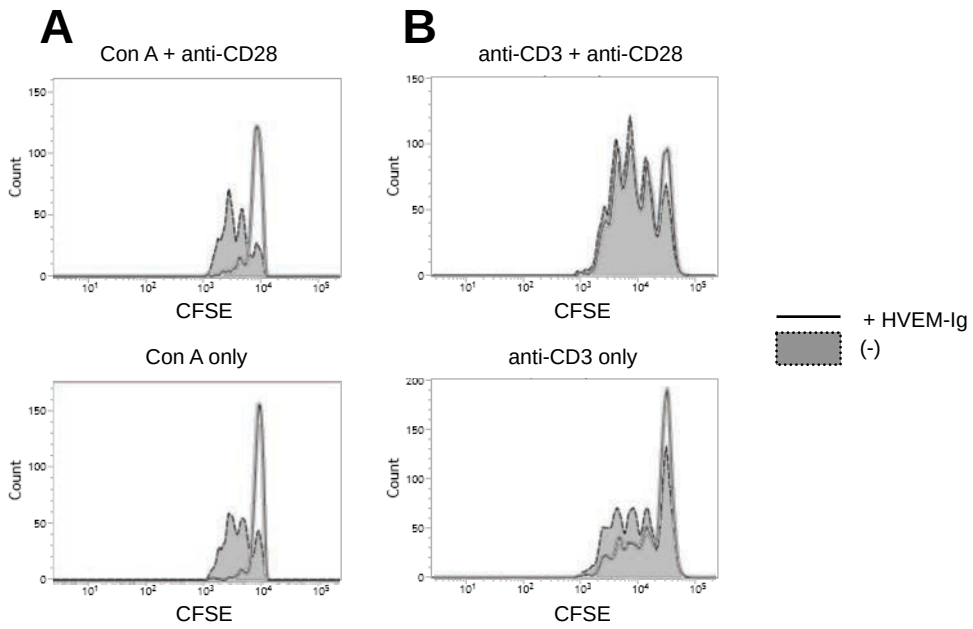

A
HVEM-Ig (μg/ml)
0
10
50
PI
CFSE
11.7%
22.0%
9.5%
24.5%
10.2%
15.6%
B
% Dead Cells
N.S.
HVEM-Ig (μg/ml)

Fig.5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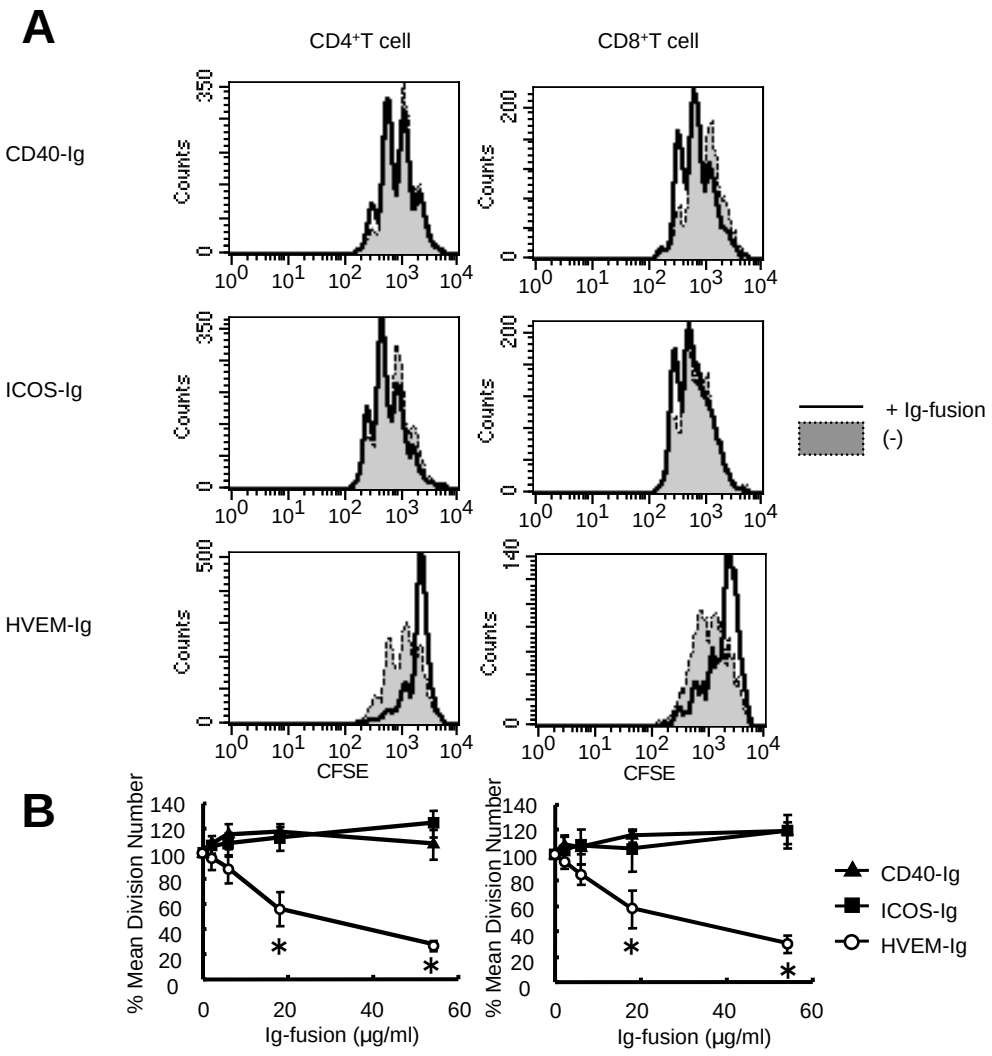
Con A + anti-CD28
Count
CFSE
Con A only
Count
CFSE
B
anti-CD3 + anti-CD28
Count
CFSE
anti-CD3 only
Count
CFSE
+ HVEM-Ig
(-)

Fig.6


A
CD4+T cell
CD8+T cell
CD40-Ig
ICOS-Ig
HVEM-Ig
Counts
CFSE
+ Ig-fusion
(-)
B
% Mean Division Number
Ig-fusion (μg/ml)
CD40-Ig
ICOS-Ig
HVEM-Ig