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Expansion of PD-1-Positive Effector CD4 T Cells in an Experimental Model of SLE: Contribution to the Self-Organized Criticality Theory

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We have developed a systems biology concept to explain the origin of systemic autoimmunity. From our studies of systemic lupus erythematosus (SLE) we have concluded that this disease is the inevitable consequence of over-stimulating the host's immune system by repeated exposure to antigen to levels that surpass a critical threshold, which we term the system's "self-organized criticality". We observed that overstimulation of CD4 T cells in mice led to the development of autoantibody-inducing CD4 T cells (*ai*CD4 T) capable of generating various autoantibodies and pathological lesions identical to those observed in SLE. We show here that this is accompanied by the significant expansion of a novel population of effector T cells characterized by expression of programmed death-1 (PD-1)-positive, CD27^{low}, CD127^{low}, CCR7^{low} and CD44^{high}CD62L^{low} markers, as well as increased production of IL-2 and IL-6. In addition, repeated immunization caused the expansion of CD8 T cells into fully-matured cytotoxic T lymphocytes (CTL) that express Ly6C^{high}CD122^{high} effector and memory markers. Thus, overstimulation with antigen leads to the expansion of a novel effector CD4 T cell population that expresses an unusual memory marker, PD-1, and that may contribute to the pathogenesis of SLE.

The cause of systemic lupus erythematosus (SLE) remains unknown (5, 17) and attempts to experimentally induce SLE have so far been not fruitful. However, we have succeeded in inducing experimental SLE in mice by repeated antigen stimulation (21). From the stand point of systems biology, our results suggest that SLE is the inevitable consequence of over-stimulating one's immune system to levels that exceed critical threshold, or what we term the systems' self-organized criticality (21). The key observation is that overstimulation with any antigen, including keyhole limpet hemocyanin (KLH), ovalbumin (OVA) or staphylococcal enterotoxin B (SEB), leads to the development of autoantibody-inducing CD4 T (*ai*CD4 T) cells. We observed that these cells had undergone T cell receptor (TCR) revision, were capable of inducing a variety of autoantibodies and could induce differentiation of CD8 T cell into cytotoxic T lymphocytes (CTL) *via* antigen cross-presentation, and that this ultimately leads to the development of an autoimmune condition in mice indistinguishable from SLE.

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In the present study, we further characterized this *ai*CD4 T cell population with respect to surface marker expression and cytokine production in mice immunized 12x with KLH, OVA or SEB. These *ai*CD4 T cells exhibited *de novo* TCR revision, and express the novel programmed death-1 marker PD-1. We discuss the role of these effector CD4 T cells in the pathogenesis of SLE.

MATERIALS AND METHODS

Animal studies

Animal studies using BALB/c female mice (Japan SLE, Inc., Hamamatsu, Japan) were approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations. Mice (8 weeks-old) were repeatedly immunized with 100μg Keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO), 500μg ovalbumin (OVA) (grade V; Sigma), 25μg staphylococcal enterotoxin B (SEB) (Toxin Technologies, Sarasota, FL) or PBS by means of i.p. injection every 5 days.

Detection of cell-surface molecules by flow cytometry

Surface staining was done in the dark on ice for 30 min in PBS. APC (allophycocyanin)-conjugated antibody against CCR7 (4B12), FITC-conjugated antibodies against CD45RB (C363-16A) and CD27 (LG.3A10), PerCP Cy5.5 (Peridinin-chlorophyll proteins cyanin 5.5)-conjugated antibodies against CD4 (RM4-5) and CD8α (53-6.7), PE-conjugated antibodies against CD122 (5H4) and CD62L (MEL-14), and purified antibodies against CD3ε (145-2C11) and CD28 (37.51) were purchased from BioLegend (San Diego, CA). FITC-conjugated antibodies against CD44 (IM7) and Ly6C (AL-21), and PE-conjugated antibodies against PD1 (CD279; J43) were purchased from BD PharMingen (San Diego, CA). PE-conjugated antibody against CD127 (A7R34) was purchased from eBioscience (San Diego, CA). Samples were analyzed on a BD PharMingen FACSCalibur, and raw data was analyzed using CellQuest software (BD PharMingen).

Cytokine assays

CD4 T cells were isolated from immunized mice using MACS beads (Miltenyi Biotec), and stimulated *in vitro* with plate-bound anti-CD3 (2μg/ml) and anti-CD28 (5μg/ml) antibodies at 37 °C for 2 days. Cytokines, IL-4, IFNγ, IL-2 and IL-6, in culture supernatants were measured by using ELISA (Invitrogen/BioSource International; Camarillo, CA).

RESULTS

Expansion of PD-1-expressing effector CD4 T cells after repeated immunization with antigen

BALB/c mice were immunized 12x with KLH, OVA or SEB to generate *ai*CD4 T cells (21). Analysis of total CD4 T cells revealed the significant expansion of a population expressing $CD27^{low}$, $CD45RB^{low}$, $CD122^{high}$ and $PD\text{-}1^{high}$ markers in the repeat-immunized mice but not in the control non-immunized mice (Figure 1).

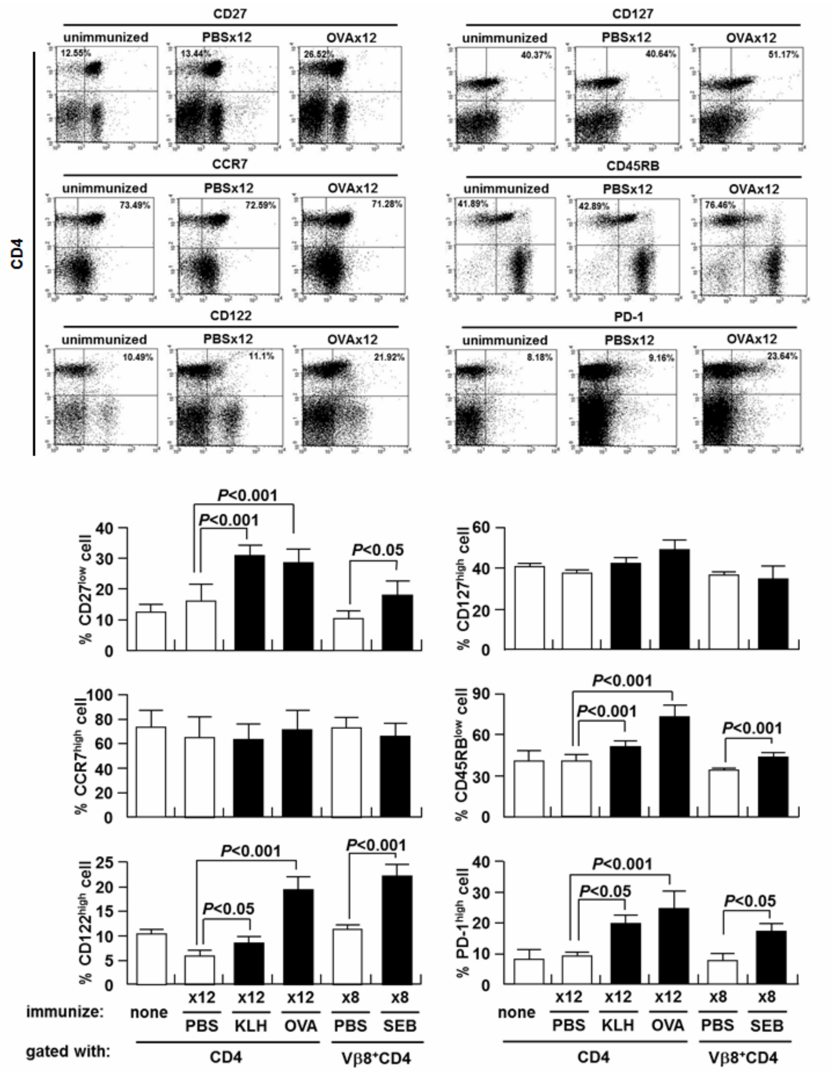

Figure 1.
CD4 T cell surface markers. BALB/c mice were repeatedly injected i.p. with 100µg of keyhole limpet hemocyanin (KLH), 500µg of ovalbumin (OVA), 25µg of staphylococcal enterotoxin B (SEB), or PBS every 5 days. The CD4 T cells from immunized mice were stained with the respective antibodies and analyzed by flow cytometry (upper). Bar graphs represent the mean ± SD of marker expression (n = 5) (down).

In particular, we observed expansion of CD4 T cells exhibiting an effector phenotype, i.e. $CD27^{low}$ and $CD44^{high}CD62L^{low}$ (Figure 2A). Furthermore, these $CD44^{high}CD62L^{low}$ CD4 T cells uniquely expressed the PD-1 marker upon repeated immunization with OVA (Figure 2C). In contrast, T cell memory markers such as $CD127^{high}$, $CCR7^{high}$ and $CD44^{high}CD62L^{high}$ were comparable between the repeat-immunized and control non-immunized mice. Further, CD4 T cells isolated from both groups produced comparable amounts of IFNγ and IL-4 (Figure 3), whereas CD4 T cells from mice immunized 12x with KLH produced significantly higher amounts of IL-2 and IL-6 compared to the controls.

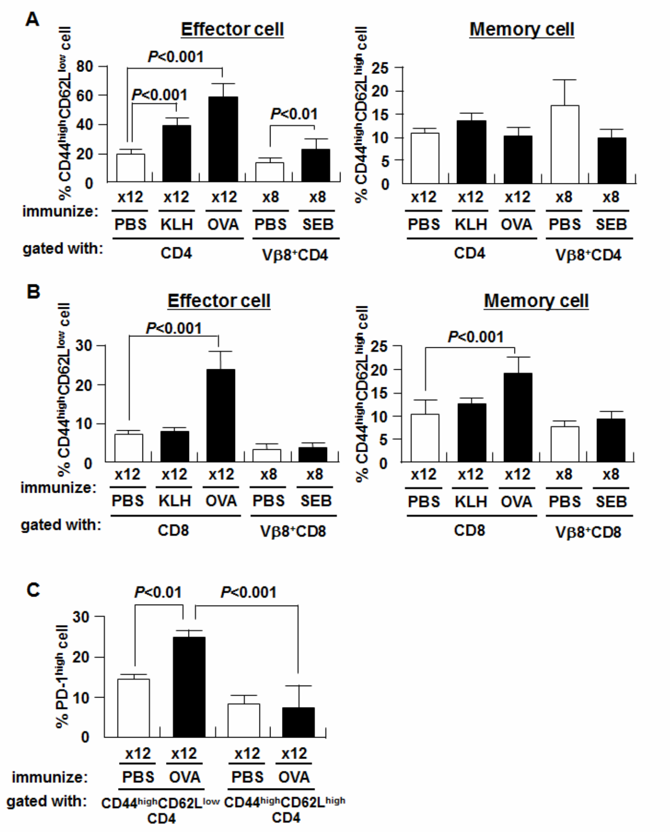

Figure 2. Effector and memory cell markers on CD4 T cells (A) and CD8 T cells (B) as determined by CD44^{high}CD62L^{low} and CD44^{high}CD62L^{high} expression. (C) Expression of PD-1 marker on effector and memory CD4 T cells.

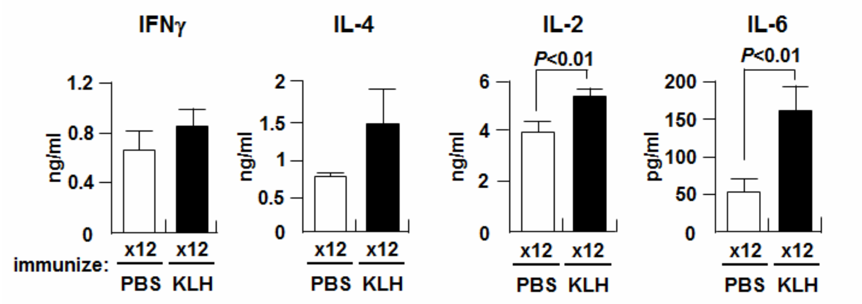

Figure 3. Cytokine production from CD4 T cell subsets. Mice were immunized 12x with 100µg of KLH and CD4 T cells obtained 9 days after the final immunization were sorted and stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 antibodies for 2 days. Culture supernatants were assayed for IL-4, IFNγ, IL-2 and IL-6.

Expansion of conventional effector and memory CD8 T cells after repeated immunization with antigen

Examination of the CD8 T cell populations revealed significant expansion of cells expressing CD122^{high}, Ly6C^{high} and CD44^{high}CD62L^{low} effector (14, 24) and CD44^{high}CD62L^{high} memory (24) markers, in repeat-immunized mice versus non-immunized controls (Figures 2B, 4). These cells also expressed higher levels of the PD-1 memory marker, indicating that these CD8 T cells represent a fully-stimulated, authentic effector-memory CD8 T cell type, which is ubiquitously distributed in the spleen and blood but not in lymph nodes.

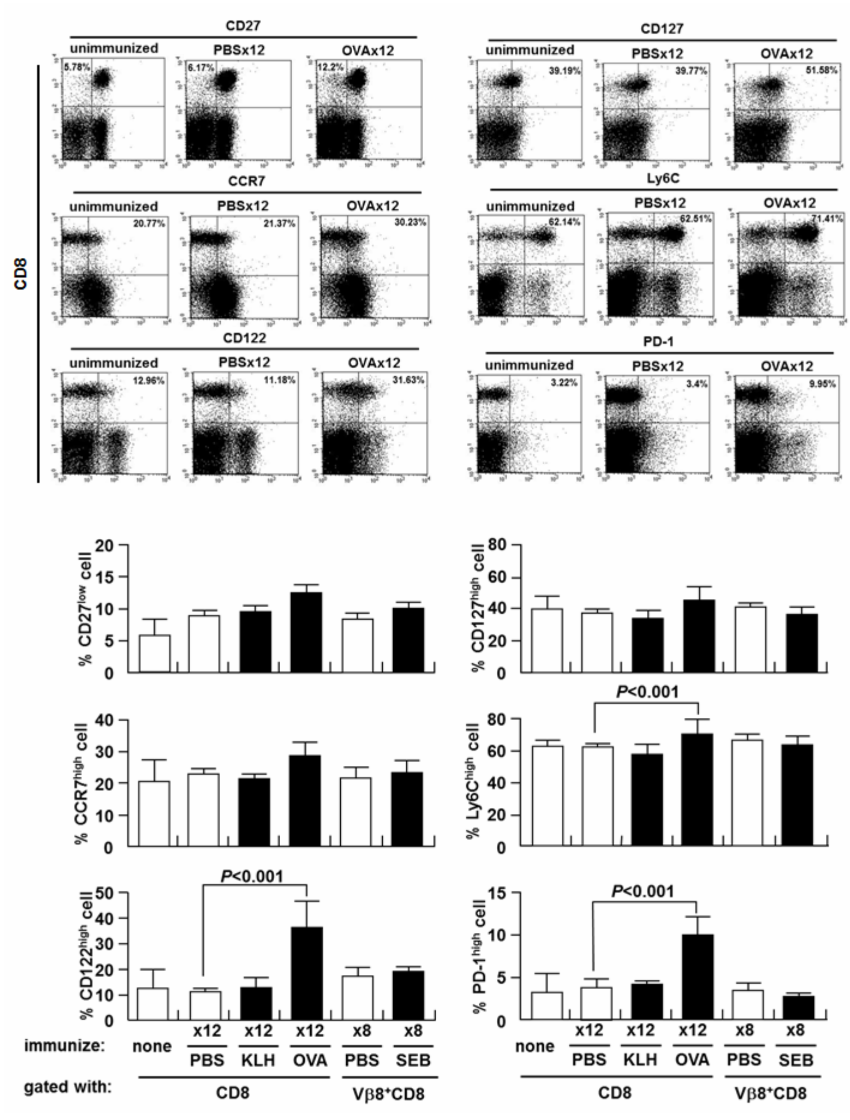

Figure 4. CD8 T cell surface markers. CD8 T cells from immunized mice were stained with the respective antibodies and analyzed by flow cytometry (upper). Bar graphs represent the mean ± SD of marker expression (n = 5) (down).

DISCUSSION

Upon encounter with antigen, naïve CD4 T cells normally mature into $CD27^{low}$, $CD127^{low}$, $CCR7^{low}$, $CD44^{high}CD62L^{low}$ effector cells (13, 22). These effector cells subsequently differentiate into memory cells associated with increased expression of CD27, CD62L, CD127 and CCR7, and finally PD-1 (3). PD-1 belongs to the CD28 superfamily and is expressed on regulatory T cells (Treg) (4), T follicular helper cells (Tfh) (6), memory T cells, and exhausted CD8 T cells (3, 7). PD-1 down-modulates T cell production of cytokines such as IFNγ, TNFα and IL-2 (18), and delivers negative signals resulting in the induction of T cell tolerance (7, 18).

Here we show that repeated immunization of BALB/c mice resulted in a significant increase in CD4 T cells expressing $CD27^{low}$, $CD45RB^{low}$ and $CD122^{high}$ markers. $CD45RB^{low}$ and $CD122^{high}$ are markers of effector and memory cells (13). Further, effector markers such as $CD27^{low}$ (23) and $CD44^{high}CD62L^{low}$ (22) were also increased. However, the memory marker CCR7, that induces migration of lymphocytes to the T cell area of lymph node or mucosal lymphoid organ (16), was similar to the control group, suggesting that the CD4 T cells that expand in response to repeated immunization exert their effects locally. Expression of CD127, a receptor for IL-7 that is important for the survival of memory T cell (11), was also similar between the repeat immunization and the control groups. Furthermore, $CD44^{high}CD62L^{high}$ memory markers were also comparable between the two groups. CD44 is a lymphocyte activation marker (10) and CD62L is a homing molecule for central lymphoid organs (16).

Taken together, these findings indicate that effector, but not memory CD4 T cells are expanded upon repeated immunization with antigen. However, this effector CD4 T cell population is unique in that it also expresses the PD-1 marker and shows increased production of IL-2. Although production of IL-2 is normally suppressed when PD-1 is expressed (9, 18), it has also been reported that stronger signaling through CD28 and/ or IL-2 receptor can overcome PD-1 inhibitory signaling (2, 15). Thus, it is possible that these PD-1-expressing effector CD4 T cells are activated.

Indeed, previous studies have shown that this PD-1-expressing CD4 T cell population is significantly increased in the spleen and kidney of NZB/W F1 mice (8) as well as in the peripheral blood of patients with SLE (12). Furthermore, treatment of NZB/W F1 mice with anti-PD-L1 antibody results in hyperactivation of T cells, which exacerbates lupus nephritis (8). We have previously shown that transfer of CD4 T cells from repeatedly immunized mice to naïve recipients results in the generation of lesions identical to SLE, and anti-CD4 T antibody treatment almost completely blocked the induction of autoantibodies and CTL and the generation of tissue injury in mice (21). Thus, this novel PD-1-expressing effector CD4 T cell seems important in the pathogenesis of lupus, and may be important in defining the lupus-inducing *ai*CD4 T cell.

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