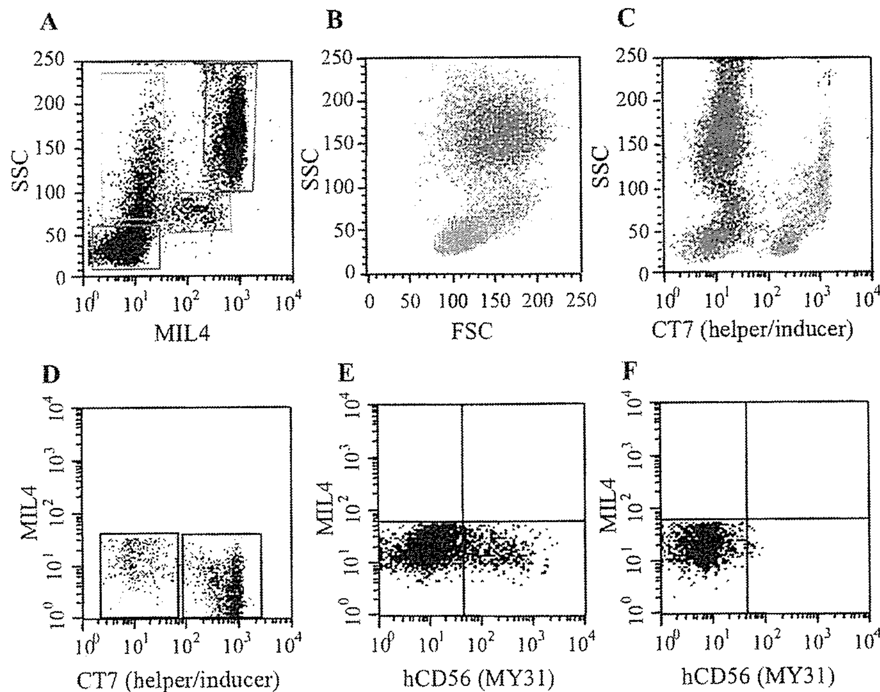

Fig. 2. Consecutive flow cytometry with MIL4/SSC and MIL4/CT7. Guinea pig leukocyte fractions were separated using consecutive flow cytometry with MIL4/SSC and MIL4/CT7. First, dead cells were excluded using propidium iodide and viable cells were gated using MIL4/SSC. Each of the gated colors in A is equivalent to the same color dots indicated by FSC/SSC (Fig. 2B) and CT7/SSC (Fig. 2C) and have been retrospectively confirmed by morphological analysis: lymphocytes (green), monocytes (red), granulocytes (blue) and MIL4$^-$SSC^{large} cells (orange). Separation of guinea pig PBL by MIL4/SSC 2-parameter flow cytometry (A), FSC/SSC (B), and CT7 (helper inducer)/SSC 2-parameter flow cytometry (C). Panel (D) shows further separation of the gated MIL4$^-$SSC^{large} population in panel A with MIL4/CT7 2-parameter flow cytometry into a CT7$^+$ fraction (right-gated fraction with inclusion body-positive cells) and a CT7$^-$ fraction (left-gated fraction with eosinophils plus basophils). (E) Cross-reactivity of human CD56 antibody with a CT7$^+$ cell fraction containing inclusion bodies (as described in Fig. 3B) was analyzed by gating with MIL4/hCD56. Approximately 6% of them were positive for hCD56. (F) In contrast, CT7$^-$ cells, consisting of eosinophils plus basophils, showed no cross-reactivity with MIL4/hCD56 cells. Only viable cells were analyzed, with dead cells excluded using propidium iodide.

(Fig. 3B). The MIL4$^-$SSC^{large}CT7^{high} cells had a mean diameter of approximately 12 μm, with nearly half of the cells containing a large cytoplasmic inclusion body. The inclusion body had an internal structure characterized by agglutinated or dispersed pachychromatic and reddish microparticles.

Thus, nearly 50% of the MIL4$^-$SSC^{large}CT7^{high} leukocytes were morphologically indistinguishable from previously reported Kurloff cells (Debout et al., 1984). The remaining half of the cell population had a smaller inclusion body with a mean diameter from 2 to 9 μm and an internal structure characterized by agglutinated, pachychromatic or reddish microparticles. Thus, this morphological characterization clearly confirmed five leukocyte fractions for the guinea pig: the lymphocyte, monocyte, neutrophil, the eosinophil plus basophil fraction in addition to the inclusion body-positive cell fraction that seemed unique to guinea pigs.

The unique MIL4$^-$SSC^{large}CT7^{high} leukocytes in the non-gated population of Fig. 2D were found to be identical with the gated SSC^{large}CT7^{high} population in Fig. 1A-b, as expected (data not shown). The distribution of the unique cells was also traced by two- parameter analysis with FSC/SSC. We found the cells distributed throughout the neutrophil (11.09 ±9.42% of neutrophils of the blue cell fraction in Fig. 2B), monocyte (30.45±11.22% of monocytes of the red cell fraction in Fig. 2B) and lymphocyte (1.27 ±0.75% of lymphocytes of the green cell fraction in Fig. 2B) cell populations.

Thus, our findings confirm previous observations that the PBL fractions of guinea pig leukocytes were very difficult to clearly identify using the traditional FSC/SSC flow cytometric assay. However, when guinea pig PBL were analyzed using a consecutive flow cytometric assay using MIL4/SSC followed by MIL4/CT7, we were able to accurately identify five

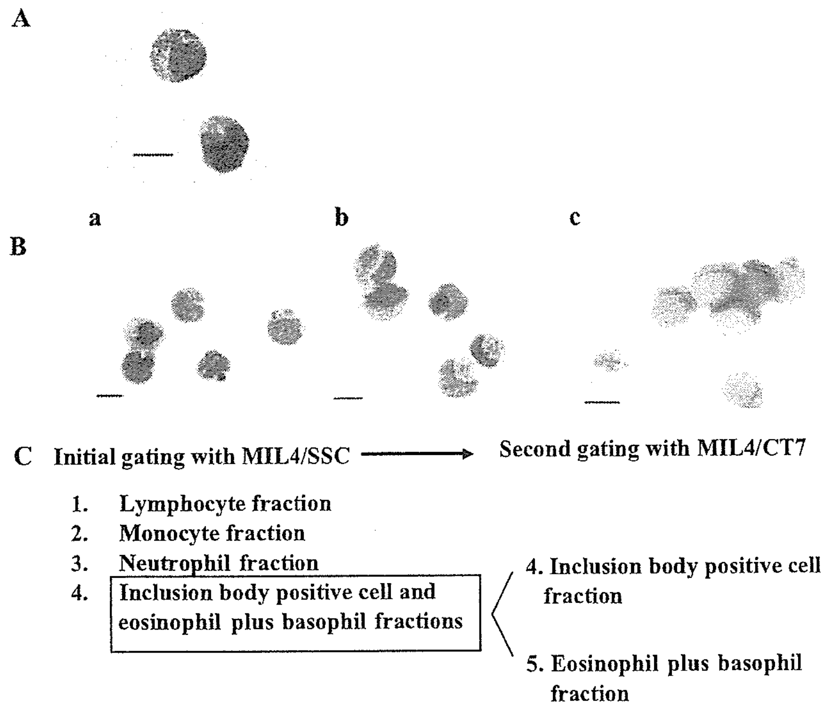

Fig. 3. Morphological character of $MIL4^{-}SSC^{large}CT7^{negative}$ cells and $MIL4^{-}SSC^{large}CT7^{high}$ cells. The $MIL4^{-}SSC^{large}CT7^{negative}$ and $MIL4^{-}SSC^{large}CT7^{high}$ cells shown in Fig. 2D were purified using flow cytometric sorting from normal guinea pig PBL. (A) May–Giemsa staining of $MIL4^{-}SSC^{large}CT7^{negative}$ cells revealed eosinophils (upper cell in panel A) and basophils (lower cell in panel A). (B) $MIL4^{-}SSC^{large}CT7^{high}$ cells stained by May–Giemsa (B-a and B-b) and PAS (B-c). Bar sizes in the panels are diameters of 10.0 μm. (C) Summary of fractionation procedure of guinea pig leukocytes. The guinea pig leukocytes were separated into five fractions by flow cytometric analysis. Using MIL4/SSC, we separated guinea pig leukocytes into five fractions: (1) lymphocytes, (2) monocytes, (3) neutrophils, (4) inclusion body-positive cells, and (5) eosinophils plus basophils. The $MIL4^{-}SSC^{large}$ fraction was further separated into $CT7^{+}$ (inclusion body-positive cell), $CT7^{-}$ (eosinophil plus basophil) populations by MIL4/CT7 2-parameter flow cytometry.

subtypes of leukocytes including the novel $MIL4^{-}SSC^{large}CT7^{high}$ cells (Fig. 3C).

3.4. The low cross-reactivity of human natural killer cell marker CD56 with $MIL4^{-}SSC^{large}CT7^{high}$ leukocytes

Since nearly 50% of the unique $MIL4^{-}SSC^{large}CT7^{high}$ leukocytes seemed to be morphologically indistinguishable from Kurloff cells in that they contained large cytoplasmic inclusion bodies in the cytoplasm, we investigated whether they would react with antibodies against human natural killer cells CD56 (Pouliot et al., 1996). Of the various human CD56 clones [leu19 (MY31, NCAM16.2 BD), human CD56 (B159 Pharmingen), human CD56 (NKI-nbi-1 Nichirei) and human CD56 (NKH-1 Coulter)] we used to screen for cross-reactivity with the $SSC^{high}MIL4^{-}CT7^{high}$ guinea pig cells (right-gated subpopulation of Fig. 2D), only leu19 (MY31, BD) proved to be cross-reactive, reacting with 6.3±2.4% of the unique leukocyte population (Fig. 2E). In contrast, $MIL4^{-}SSC^{large}CT7^{-}$ leukocytes (left gated subpopulation of Fig. 2D) did not react with any of the human CD56 clones (Fig. 2F). Thus, only a very small subpopulation of the $SSC^{high}MIL4^{-}CT7^{high}$ guinea pig cells cross-reacted with a human CD56 NK cell marker, a far lower rather of cross-reactivity with human CD56 antibodies than seen with Kurloff cells (Pouliot et al., 1996).

3.5. Cell surface analysis of the unique leukocyte fraction of guinea pigs

To characterize the unique cell fraction $MIL4^{-}SSC^{large}CT7^{high}$, leukocytes were further characterized by flow cytometry and were found to be reactive with helper/inducer antigens (CT7, H155), PanT (CT5, H159), CD45 (IH-1, H201), Thy-1 (H154), anti-asialo GM1 and FcR (20ED7) antibodies, but not with CD8 (CT6), B cell (Msgp9), IgM (31D2), MHC class II (R27E7) and MIL4 antibodies (Fig. 4). Moreover, these leukocytes proved to be more reactive with

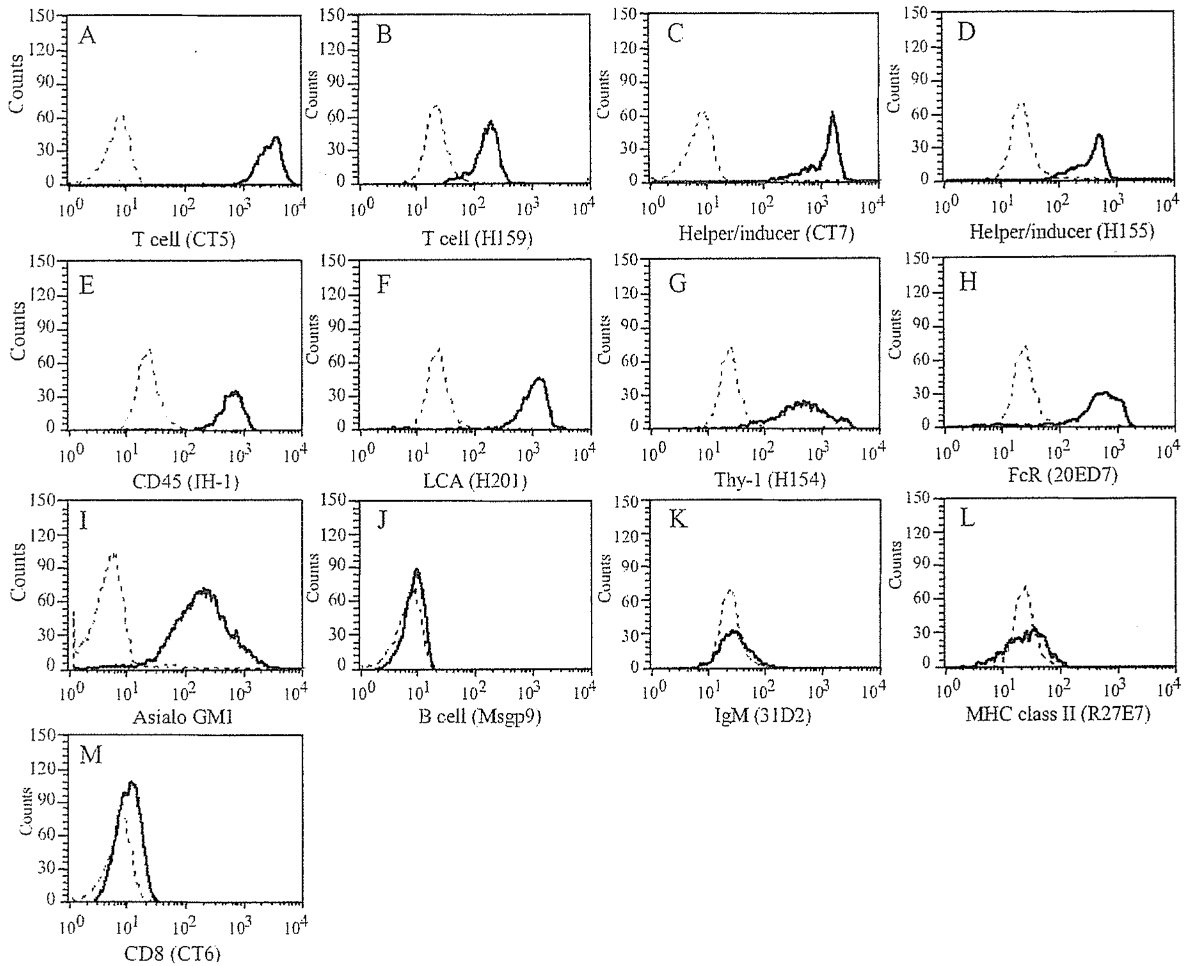

Fig. 4. Characteristics of the unique inclusion body-positive cells of guinea pig PBL as determined by flow cytometry. The MIL4$^-$SSC^{large}CT7^{high} fraction of PBL (orange fraction in Fig. 2) was further evaluated using the respective antibodies for the antigens expressed on their cell surface. Panels A, C, J, and M represent flow cytometric profiles of MIL4$^-$SSC^{large}CT7^{high} cells gated by APC-MIL4 and PE-CT7 followed by FITC-specific antibodies. Moreover, panels B, D, E, F, G, H, I, K and L represent the profiles of the unique cells gated by APC-MIL4 and FITC-CT7 followed by PE-specific antibodies.

helper/inducer antigens (CT7 or H155 mAbs) than lymphocytes in either peripheral blood (Fig. 2C) or spleen (data not shown). Guinea pig monocytes/macrophages were negative for reactivity with the CT7 antigen. Thus, because histograms of the various cell surface markers on the MIL4$^-$SSC^{large}CT7^{high} cells showed a haploid phase, we surmised that the unique cell population was a single leukocyte fraction of guinea pigs.

3.6. Normalization of the five leukocyte fractions in lymphoid tissues

The five leukocyte fractions of guinea pigs were distributed to the various lymphoid tissues by gating cells with MIL4/SSC followed by MIL4/CT7 (Fig. 5 and Table 2). As shown in Fig. 5A and Table 2, leukocyte fractions of spleen were gated as well as those of PBL. MIL4$^-$SSC^{large}CT7^{high} leukocytes were also similarly sorted to have a unique inclusion body in the cytoplasm (Fig. 5A-c). Lymph nodes (LN) and thymus were predominantly populated by lymphocytes and contained only 0.4% and 0.2% of MIL4$^-$SSC^{large}CT7^{high} leukocytes, respectively. BM cells were mainly comprised of myeloblasts and erythroblasts which were difficult to gate. In contrast, the MIL4$^-$SSC^{large}CT7^{high} leukocytes were clearly separated and they contained 1.6% of the total cells. Liver cells were also difficult to gate, but the unique cells in the liver could be separated using this consecutive flow cytometric procedure and were found in 4.3% of liver cells. The frequencies of these cells were 9.9% and 16.6% in peripheral blood and

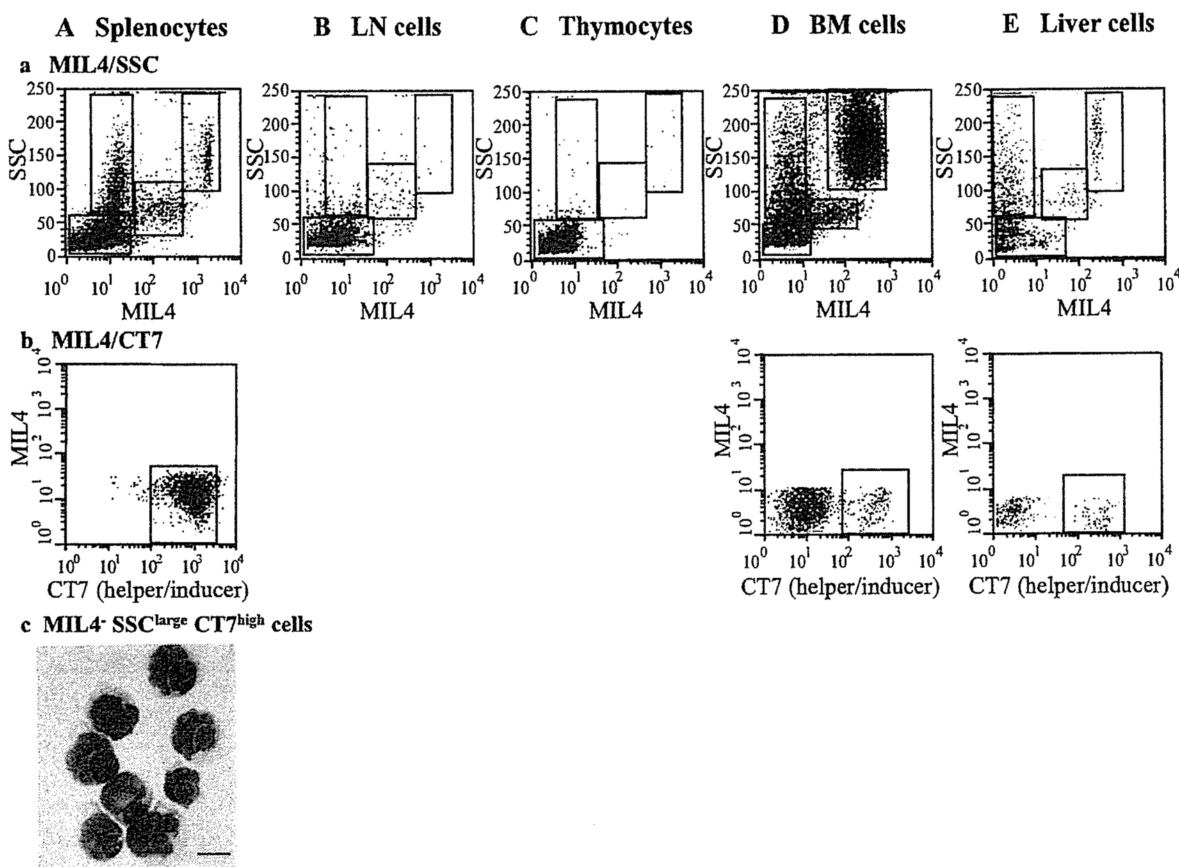

Fig. 5. Distribution pattern of leukocyte fractions in various guinea pig tissues. Cells isolated form various tissues of normal guinea pigs were analyzed by the novel two-parameter cytometric procedure: the MIL4/SSC parameter (a), followed by the MIL4/CT7 parameter (b). MIL4^{-}SSC^{large}CT7^{high} cells (c) from spleen were sorted and their morphological character confirmed using May–Giemsa staining. Tissue distribution of guinea pig leukocyte fractions are shown for spleen (A), lymph node (B), thymus (C), bone marrow (D) and liver (E). Dead cells were excluded using propidium iodide and viable cells were analyzed.

spleen, respectively (Table 2 and Fig. 5A). Thus, the MIL4^{-}SSC^{large}CT7^{high} leukocytes are abundant in the spleen and PBL, but are comparatively rare in the LN, BM and thymus of healthy guinea pigs. Fewer eosinophils and basophils are found in spleen cells (non-gated population in Fig. 5A-b) than in PBL (gated population in Fig. 2D). In summary, in our study we have been able to identify and separate the five fractions of guinea pig leukocytes, not only in PBL but also in the other lymphoid tissues. The novel flow cytometric technique presented here promises to dramatically improve the immunological analysis of guinea pig animal models.

4. Discussion

In this study, we found that MIL4, which is an antibody against porcine peripheral blood neutrophils, eosinophils and basophils (Haverson et al., 1994), cross-reacted with guinea pig neutrophils and moderately reacted with guinea pig monocytes/macrophages, but did not recognize lymphocytes, eosinophils, basophils and the MIL4^{-}SSC^{large}CT7^{high} cells. Based on this observation, we distinguished guinea pig leukocyte fractions using consecutive flow cytometry of MIL4/SSC followed by MIL4/CT7 gating. The CT7 mAbs used for leukocyte typing in this study were screened and selected based on reports that CT7 recognizes a surface helper/inducer T cell (Steerenberg et al., 1991; Tan et al., 1985).

Light-scatter gating, commonly used to differentiate leukocyte cell types in humans and other species, proved ineffective for guinea pig cell differentiation because the MIL4^{-}SSC^{large}CT7^{high} cells were widely dispersed throughout the light-scatter profiles of guinea pig granulocytes, lymphocytes and monocytes/macrophages. Using a traditional FSC/SSC gate, with the MIL4^{-}SSC^{large}CT7^{high} cells located between the

Table 2
Mean percentage of leukocyte fractions in various tissues of normal guinea pigs obtained by using MIL4/SSC and MIL4/CT7 parameters[a]

Tissue	Leukocyte fraction	Mean	±SD
Blood	Lymphocytes	48.7	14.1
	Monocytes	4.7	2.6
	Neutrophils	33.6	14.2
	Inclusion body-positive cells	9.9	6.4
	Eosinophils and basophils	1.8	0.6
Spleen	Lymphocytes	68.2	7.2
	Monocytes	4.4	1.6
	Neutrophils	3.1	1.4
	Inclusion body-positive cells	16.6	6.2
	Eosinophils and basophils	0.7	0.4
Thymus	Lymphocytes	96.5	0.6
	Monocytes	0.6	0.2
	Neutrophils	0.1	0.0
	Inclusion body-positive cells	0.2	0.0
	Eosinophils and basophils	0.0	0.0
LN	Lymphocytes	94.8	1.4
	Monocytes	1.1	0.4
	Neutrophils	0.1	0.1
	Inclusion body-positive cells	0.4	0.0
	Eosinophils and basophils	0.0	0.0

[a] Leukocytes of guinea pigs were gated into 5 fractions by flow cytometry using MIL4/SSC followed by MIL4/CT7. The MIL4$^-$SSC^{large} fraction was separated into CT7$^+$ and CT7$^-$ cells. Data are expressed as the mean±SD.

monocyte and granulocyte populations on the SSC, clean leukocyte fractions could not be obtained, as MIL4$^-$SSC^{large}CT7^{high} cells contaminated approximately 30% of the monocyte and 10% of the granulocyte fractions, as well as a minor fraction of lymphocytes. Since non-lymphocytes are usually gated based on light scatter, and lymphocytes are gated based on CD45 fluorescence (Nicholson et al., 1996), we tried to gate guinea pig lymphocytes using CD45/SSC. This alternative gating approach also failed to effectively separate guinea pig leukocyte fractions, this time because each fraction's cells were stained with the same intensity of CD45 (data not shown).

The MIL4$^-$SSC^{large}CT7^{high} population contained inclusion bodies of varying sizes, with nearly half having the large inclusion body. This finding suggests that the MIL4$^-$SSC^{large}CT7^{high} cells containing the large inclusion bodies are similar to KC cells containing a large PAS-positive inclusion body. KC cells are also reported to be circulating mononuclear cells with NK-like function in vitro (Berendsen and Telford, 1966; Debout et al., 1984, 1999). The surface phenotype of MIL4$^-$SSC^{large}CT7^{high} was highly positive for helper/inducer antigens (CT7 or H155), T cell antigens (CT5, H159), Thy-1 (H154), CD45 (IH-1, H201), asialoGM1 and FcR (20ED7). However, it was negative for CD8 (CT6), B cell antigens (Msgp9), IgM (31D2), MHC Class II (R27E7) and MIL4. Thus, the surface of MIL4$^-$SSC^{large}CT7^{high} cells exhibited some NK and some T cell markers, but exhibited neither B cell markers nor definite CD3 antigen.

Previously, KC cells, noted for their large cytoplasmic inclusion bodies, were purified from the spleen of estradiol-inoculated guinea pigs by Percoll-gradient centrifugation (Debout et al., 1991; Pouliot et al., 1996). Most of the cells were shown to be cross-reactive with human NK cell marker CD56, suggesting that the cells might be homologues of human NK cells (Pouliot et al., 1996). Furthermore, KC cells were reported to be positive for CT7, but negative for 8EB7, CT6 and B-lymphocyte lineage antigens (CT10, IgM) (Debout et al., 1991). In contrast, only clone MY31 (B.D.) of the CD56 antigen reacted with the MIL4$^-$SSC^{large}CT7^{high} population of spleen cells from normal guinea pigs, and that only at a rate of 6%, as described above. This low cross-reactivity with human CD56 for the MIL4$^-$SSC^{large}CT7^{high} cells compared to that for purified KC cells is not surprising, since KC cells make up only a part of one subpopulation of MIL4$^-$SSC^{large}CT7^{high} cells, that with large inclusion bodies. We have here introduced methods for identifying and isolating the MIL4$^-$SSC^{large}CT7^{high} cells contained in approximately 10% of PBL and 17% spleen cells.

Although FSC/SSC gating puts both the eosinophil and basophil as well as the monocyte subsets in the same fraction (data not shown), we made it possible to separate the two guinea pig leukocyte fractions. An identification of eosinophils and basophils of guinea pigs will be beneficial to the study of immediate-type hypersensitivity reactions in guinea pig models. Therefore, the identification of five distinct subtypes of guinea pig leukocytes, including one unique MIL4$^-$SSC^{large}CT7^{high} cell type, paves the way for examining the role of each of these cell types in the infections and inflammatory conditions that can be uniquely studied in guinea pigs.

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Requirements for the functional expression of OX40 ligand on human activated $CD4^+$ and $CD8^+$ T cells

Running title: Functional OX40L on human T cells

Kayo Kondo[a], Kazu Okuma[a], Reiko Tanaka[a], Li Feng Zhang[a], Akira Kodama[a], Yoshiaki Takahashi[b], Naoki Yamamoto[b], Aftab A. Ansari[c], and Yuetsu Tanaka[a]

[a)] Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan

[b)] AIDS research center, National Institute of Infectious Diseases, Tokyo, Japan

[c)] Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA.

Correspondence to: Prof. Y. Tanaka, Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan.

Tel: +81-98-895-1202; Fax: +81-98-895-1437

e-mail: yuetsu@s4.dion.ne.jp

Abbreviations used: OX40L, OX40 ligand; PBMCs, peripheral blood mononuclear cells; APCs, antigen-presenting cells; mAb, monoclonal antibody; FCM, flow cytometry; TCR, T cell receptor; TGF, transforming growth factor

KEY WORDS: human OX40L, OX40, T cells, TCR stimulation, TGF-β1, IL-12, IL-4

ABSTRACT

Interaction between OX40 expressed on activated T cells and its ligand (OX40L) on antigen presenting cells (APC) provides a co-stimulatory signal for T cells to promote acquired immunity. In the present study, we have examined various culture conditions for optimum OX40L expression on T cells stimulated with immobilized anti-CD3/CD28 monoclonal antibodies (mAbs). While the day 3 primed T cells expressed minimal OX40L, after repeated stimulations both the $CD4^+$ and $CD8^+$ T cells became OX40L-positive as determined by flow cytometry. IL-12 interfered with the OX40L expression. Among activated T cells, a higher frequency of $CD8^+$ T cells expressed OX40L than $CD4^+$ T cells. By blocking OX40L-OX40 interaction by an anti-OX40 mAb, the number of $OX40L^+$ T cells significantly increased. Screening of various cytokines showed that TGF-β1 was capable of induction of OX40L on the activated T cells within 3 days. The OX40L expressed on T cells was functional, as they bound soluble OX40 and stimulated HIV-1 production from HIV-1-chronically infected cell lines expressing OX40. Altogether, the present study indicates that functional OX40L is inducible on human activated $CD4^+$ and $CD8^+$ T cells, and that the expression is enhanced by TGF-β1.

(191words)

INTRODUCTION

OX40/OX40L (CD134/CD252) represent a growing number of pairs of co-stimulatory molecules and ligands which have been reported to be critical for T cell proliferation, survival, cytokine production and the generation of memory T cells [1-5]. The OX40/OX40L are members of the tumor necrosis factor receptor (TNFR)–TNF super-family with OX40 being transiently expressed primarily by activated T cells following ligation of the T cell receptor (TCR). Its cognate ligand OX40L (also termed gp34) [6, 7] has a broad tissue distribution which includes dendritic cells, Langerhans cells, B cells, natural killer cells, vascular endothelial cells and mast cells, and is not normally detectable on resting cells, but can be up-regulated following activation [8-23]. The patho-physiological relevance of such broad tissue distribution of OX40L requires further study. The interaction between OX40 and OX40L has been described as being highly plastic, a characteristic of the TNFR–TNF super-family which was recently confirmed by the delineation of the crystal structure of the OX40/OX40L complex [24].

While initially the expression of OX40L was thought to be restricted to non-T cells, recent studies appear to question this view. Thus, it has been shown that OX40L is in fact inducible on long-term cultured human $CD4^+$ and $CD8^+$ cytotoxic T cell lines in addition to T cell lines transformed by human T cell leukemia virus type-I (HTLV-I) [16, 25, 26]. In the murine system, it has been reported that functional OX40L can be induced on TCR-transgenic T cells when they are stimulated in vitro [27, 28]. More recently the data from Soroosh et al [29] suggested that indeed OX40L expressed by murine T cells participates in an unique T-T cell signaling system by interaction with OX40 and that such interactions are critical for long-term survival of such T cells. While these studies have been performed using murine T cells, a role for such T-T cell interaction and the optimum conditions and requirements for OX40L expression by human T cells have yet to be defined. Thus, the present studies were conducted to first define the culture conditions which promote OX40L expression by human T cells *in vitro*. We show herein that activated human $CD4^+$ and $CD8^+$ T cells both can express functional OX40L *in vitro* specially following repeated stimulation, and that TGF-β1 is a strong inducer of OX40L. The role of OX40L expressed by T cells is discussed.

MATERIALS AND METHODS

Reagents

Medium used was RPMI-1640 medium (Sigma, St. Louis, MO.) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Sigma) (referred to as RPMI medium). No antibiotics were added. Recombinant human IL-2 (rIL-2) was provided courtesy of the US NIH AIDS Research and Reference Reagent Program. The recombinant cytokines rIL-12, rIL-4, rIL-10, rIL-17, rIL-18 rIFN-γ and rTGF-β1 were purchased from Peprotec (London, UK). The fluorescent mAbs specific for human CD4 and CD8 (PE or PC5-labeled) and Streptavidin-PE were purchased from Beckman-Coulter (CA, USA). The mAbs produced in our laboratory included mouse IgG1 anti-OX40L (clone 5A8) [30], rat IgG2b anti-HCV (clone Mo-8) [31], mouse IgG1 anti-human OX40 (clone B-7B5) [32], rat IgG2b anti-OX40 (clone W4-54 which was generated from a WKA rat and capable of blocking OX40/OX40L interaction), control mouse IgG1 mAb TAXY-8 anti-HTLV-I tax antigen [33]. The mAbs from clones B-7B5 and W4-54 recognized different epitopes of OX40 and did not interfere with each other in terms of binding to the same molecule (unpublished). These in-house mAbs were purified from SCID mouse ascites fluids by gel filtration using Superdex G-200 (Amersham Bioscience, Uppsala, Sweden). These mAbs were labeled using FITC- or Cy5-labeling kits (Dojin, Tokyo, Japan or Amersham) according to the manufacture's instructions. Human T cell negative isolation kit, magnetic beads conjugated with anti-human CD3 and anti-human CD28 mAbs, and magnetic beads conjugated with anti-human CD4 or CD8 were all purchased form Dynal Corp (NY, USA). Biotinylated recombinant soluble human OX40 (sOX40, CD134-muIgG2a/biotin fusion protein) and OX40L (sOX40L, CD134L-muCD8/biotin) were purchased from Ancell (MN, USA). A control for mouse IgG2a was purchased from Ancell. Neutralizing mAbs specific for human TNF-α and TNF-β were purchased from R&D (MN, USA). Anti-CD28 mAb with stimulating activity was purchased from R&D (USA).

Cell Lines

A HIV-1-chronically infected ACH-2 cell line expressing OX40 was derived by OX40-gene transfection as described previously [32]. Other OX40-, OX40L- and control-gene transfected cell lines used in the present study were generated from the human T cell line Molt-4 expressing human CCR5 (kindly provided by Dr. M. Baba of Kagoshima University, Japan), and the human promonocytic cell line U1 which was derived from the HIV-1 chronically-infected U937 cell line. These cell lines were transfected by electroporation using 10-15 μg of the individual plasmid, as described previously [32]. For the selection of transfectants, 1 μg/ml puromycin was added to the culture media. The expression of the OX40L or OX40 protein on the cell surface of the transfected cells was confirmed by an

immuofluorescence followed by flow cytometric analysis.

Stimulation of T Cells *in vitro*

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (5 U/ml) blood of normal healthy donors using standard density gradient centrifugation with the use of the human lymphocyte separation medium (Sigma). The cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% BSA (BSA-PBS). PBMCs were re-suspended at 1 x 10^6 cells/ml in RPMI medium supplemented with 20 U/ml IL-2. Then, 1 ml of the cell suspension was dispensed into individual wells of 12-well plates (B-D, NJ, USA) and mixed with the anti-CD3/CD28 magnetic beads (anti-CD3/28 immunobeads) at a cell to bead ratio of 1:1, and cultured either in the presence or absence of exogenous IL-12, IL-4 or other recombinant cytokines at 20 ng/ml for 3 days at 37°C in a 5% CO2 humidified incubator. In some experiments, PBMCs were stimulated by plate bound OKT-3 (0.05, 0.5 or 5μg/ml) and soluble anti-CD28 mAb (1 μg/ml). In addition, for some experiments, T cells were purified using a human $CD3^+$ T cell negative isolation kit. Viable cell numbers were assessed on an aliquot of such cells utilizing staining with 0.1% eosin-Y in PBS. After 3 days, activated cells were harvested, adjusted to 2 x 10^5 cells/ml and further stimulated using the same conditions every 3 days. The anti-OX40 blocking mAb (W4-54) or control mAb (Mo-8) was added to T cell-stimulation cultures at 5 μg/ml.

Flow Cytometry (FCM)

Cells to be analyzed were incubated in a FACS buffer (PBS containing 0.1% NaN3 and 2% FCS) containing 2 mg/ml human IgG on ice for 15 mimutes for the blocking of Fc receptors. Without washing, the cells were then subjected to staining with a set of dye-conjugated mAbs, 5A8-FITC and B-7B5-Cy5, with either anti-CD4-PE or anti-CD8-PE on ice for 30 mimutes. Then after washing with FACS buffer, cells were fixed in 1% PFA-containing FACS buffer and analyzed using FACSCalibur, and data obtained were analyzed using Cell Quest software (BD). In order to determine whether activated T cells can bind OX40L and OX40, Fc-blocked cells were incubated with biotinylated sOX40 or sOX40L at 2.5 μg/ml together with either anti-CD4-PC5 or CD8-PC5 for 30 mimutes on ice followed by staining with PE-labeled streptavidin (Beckman Coulter) for 30 mimutes on ice. After washing with FACS buffer, cells were fixed in 1% PFA-containing FACS buffer and analyzed. In the experiments that were conducted to demonstrate the ability of W4-54 mAb to block the transfer of OX40 to OX40L and vice versa, aliquots of the Molt4/OX40L cells were mixed with Molt4/OX40 cells in the presence of 5 μg/ml W4-54 or control rat IgG for 30 mimutes on ice. These cell aliquots were then incubated with pre-determined

optimum concentrations of the 5A8-FITC and B-7B5-Cy5 mAb and subjected to flow cytometric analysis.

OX40 Stimulation Assay

Two HIV-1-chronically infected cell lines (ACH-2 and U1) that had been transfected with human OX40 gene, (ACH-2/OX40 and U1/OX40) ([32], and Takahashi et al., unpublished) were suspended at 2 $\times 10^5$ cells/ml and co-cultured with equal number of previously activated T cells in a volume of 0.5 ml in a 48-well plate (BD) in RPMI medium for 24-48 hours at 37°C in a 5% CO_2 humidified incubator. To neutralize endogenous TNF, anti-human TNF-α and TNF-β mAbs (at 5 μg/ml each) were included to block the potential effects of these cytokines synthesized endogenously. HIV-1 replication was determined by quantification of HIV-1 core p24 antigen in the supernatant fluid from such cultures utilizing commercial ELISA kits (Retro-Tec, NY, USA).

RESULTS

Induction of OX40L on Human T cells by Repeated Stimulation.

Results from a series of preliminary studies carried out confirmed previous observations that the expression of both OX40L and OX40 were undetectable on resting T cells isolated from fresh human PBMCs from a number of normal donors as determined by standard FCM (data not shown). In efforts to examine the expression of OX40L and OX40 on activated human T cells, PBMCs were incubated *in vitro* with anti-CD3/28 immunobeads. Pre-determined optimum concentrations of IL-2 and either IL-12 or IL-4 were added to the cultures to generate prototype Th1 or Th2-like populations of human $CD4^+$ and $CD8^+$ T cells in efforts to examine them for the expression of OX40L and OX40. After 3 days in such culture conditions, the cultures were harvested and the expression of OX40L and OX40 by the $CD4^+$ and $CD8^+$ T cells was examined by a triple-color staining method. As seen in Fig.1, while >75% of the $CD4^+$ T cells and 16-30% of the $CD8^+$ T cells expressed cell surface OX40, < 1.5 % of these cells expressed OX40L irrespective of the Th1 or Th2-inducing culture conditions. In efforts to determine if repeated stimulation would facilitate OX40L expression, similar cultures were set up except fresh anti-CD3/28 immunobeads and the same cytokine mixtures were added on days 3 and 6, and the cultures harvested on day 9. Such repeated stimulation had interesting results. Thus, while IL-12 had a modest effect on OX40L expression by $CD4^+$ T cells (an increase from 1.4 to 17.3%) most of which co-expressed OX40, similar cultures incubated in IL-4 led to a dramatic increase (from 1.3 to 58.0%) in the frequency of $CD4^+$ T cells that co-expressed OX40 and OX40L. On the other hand, while similar repeated stimulation in the presence of IL-12 gave similar effects on the co-expression of OX40L by $CD8^+OX40^+$ T cells (an increase from 1 % to 14.6%) as $CD4^+$ T cells, stimulation in the presence of IL-4 not only led to an increase in $OX40^+OX40L^+$ (double positive (DP)) cells (from 0.6% to 36.2%) but also a discrete population of $CD8^+$ T cells that expressed only OX40L (0.1% to 45.7%). Repeated studies performed on different days using similar culture conditions showed some degree of variability in the levels of OX40L expression depending upon the donor of the PBMCs (data not shown). However, the basic trend was the same in that whereas the inclusion of IL-12 always led to a modest increase in the frequency of $OX40^+OX40L^+$ (DP) cells within both the $CD4^+$ and the $CD8^+$ T cell populations upon repeated stimulation, the inclusion of IL-4 consistently led to an increase exclusively in $OX40^+OX40L^+$ (DP) cells within the $CD4^+$ T cell population and both $OX40^+OX40L^+$ (DP) and single $OX40L^+$ (SP) cells in the $CD8^+$ T cell population (Fig.1 and data not shown). Since previous reports utilizing T cells from TCR transgenic mice suggested that weak TCR stimulation is more critical for OX40L expression [34], microtiter plates were coated with the OKT-3 mAb at 5, 0.5 and 0.05 μg/ml for 1 hours at 37°C, and then triplicate wells were cultured with human PBMCs for 3 days. Analyses of activated T cells from these cultures, however, failed to show any

detectable changes in OX40L expression by T cells (data not shown), indicating that differences exist between murine TCR transgenic cells and human bulk T cells in terms of signals required for OX40L expression.

sOX40 Binding by T cells.

In order to examine whether the OX40L molecules expressed by these activated human T cells were functional, we examined the capacity of the repeatedly activated T cells to bind sOX40 by FCM. Fig.2 shows that while sOX40 binds preferentially to the re-stimulated $CD8^+$ T cells but not $CD4^+$ T cells, sOX40L binds preferentially to the re-stimulated $CD4^+$ T cells but not $CD8^+$ T cells. In addition, while a markedly higher frequency of the IL-4-treated $CD8^+$ T cells bound sOX40 than the IL-12-treated $CD8^+$ T cells, sOX40L bound preferentially to the re-stimulated $CD4^+$ T cells cultured in IL-12 as compared with IL-4. These data suggest that the induced OX40L molecules were functional in terms of their ability to bind OX40, and that the patterns of the expression of functional OX40L and OX40 by activated T cells differed depending on activation conditions and whether the T cells were of the $CD4^+$ or $CD8^+$ phenotypes.

Effect of Anti-OX40 Blocking mAb on OX40L Expression

It has previously been shown in the murine system that OX40L expression on activated T cells is down-regulated by cell to cell contact when co-cultured with OX40-expressing cells [29]. In addition, we have shown that human OX40L as well as OX40 molecules can be inter-cellularly transferred, indicating that co-expression of OX40L and OX40 by a single cell or co-cultures of cells individually expressing OX40L and OX40 in the same culture may interfere with OX40L expression and/or detection. In efforts to study OX40L expression without the involvement of interactions between these 2 molecules, a series of mAb specific for OX40 were first screened for their individual ability to block such interactions between OX40 and OX40L. A mAb clone W4-54 was thus identified that was capable of inhibiting the binding of sOX40L to membrane bound OX40. In addition, the effect of the addition of W4-54 on the binding of sOX40 to its cognate ligand OX40L was also studied. We initially utilized the OX40- and OX40L-transfected Molt4 cell line for these studies in efforts to restrict the analysis to a study between these 2 molecules and avoid the potential role of other cell surface molecules expressed by mixed cell populations in primary cultures. Fig.3 demonstrates the data obtained. Thus, as seen in Fig 3a the addition of the W4-54 mAb completely blocked the ability of sOX40L to bind to the Molt4 cell membrane-expressed OX40 (top panel). Similarly, the addition of the same W4-54 mAb markedly decreased the binding of sOX40 to the Molt4 membrane bound OX40L (bottom panel). The partial

blocking in this latter case may be due to differences in the affinity of the mAb to bind to the sOX40 and the affinity of sOX40 to bind to the Molt4 membrane expressed OX40L. The ability of the W4-54 mAb to inhibit interaction between OX40 and OX40L is further exemplified by the data shown in Fig. 3b. Thus, when the OX40 transfected Molt4 cells were co-cultured with the OX40L-transfected Molt4 cells and the mixed cells analyzed for OX40 and OX40L expression in the presence of the control antibody, there was significant interaction between these transfected cells (bottom left panel). However, the addition of the W4-54 mAb to the mixture of the transfected cells completely inhibited such interaction as seen with the staining profile (bottom right panel). The top left and right panels depict the control profiles in which the OX40- and OX40L-transfected Molt4 cells were mixed with mock-transfected Molt4 cells. In efforts to determine the physiological relevance of these findings, cultures of primary T cells were similarly examined for the expression of OX40L and OX40 following *in vitro* culture in media containing either IL-12 or IL-4 in the presence of either the same blocking W4-54 antibody and control Mo-8 mAb.

Addition of this blocking anti-OX40 mAb to the T cell cultures stimulated by anti-CD3/28 (as described under Fig.1) did not show any significant increase in the OX40L expression after the first stimulation (Fig.4, top panel), indicating that the numbers of OX40L molecules on the T cells were few at early stage of stimulation. But after third stimulation, the antibody treatment showed a marked increase in the frequency of $OX40L^+$ cells in both $CD4^+$ and $CD8^+$ T cell subpopulations treated with IL-12 (Fig.4, bottom panel), indicating that the OX40L molecules detected on the activated human T cells were endogenously synthesized but not transferred from other cells. In addition, these data highlight the problems associated with the analysis of OX40 and OX40L expression on such cultured cells if performed without preventing the interactions between these 2 molecules either by direct cell to cell interaction or by the transfer of soluble forms of such molecules.

The Effect of TGF-β1

We screened a number of recombinant human cytokines in combination with IL-2 that might influence the expression of OX40L by activated T cells. As shown in Fig.5, among the cytokines tested, only TGF-β1 showed a low (by $CD4^+$ T cells) to modest (by $CD8^+$ T cells) enhancement in the induction of OX40L on the activated T cells following as little as 3 days in culture *in vitro*. The cytokines that failed to induce OX40L included IL-10, IL-13, IL-17, IL-18, IFN-γ and IFN-β (Fig.5, and unpublished data), which was not secondary to the dose of the cytokine utilized. Of interest is also to note that IL-12 and IL-13 also increased the frequency of $CD8^+$ T cells that express OX40. Studies carried out on primary cultures of PBMCs from another donor incubated *in vitro* in media containing TGF-β1 for 3 days showed that TGF-β1 influenced the expression of OX40L by a higher frequency of the $CD8^+$ T cell population

than the $CD4^+$ T cell population (see Fig. 6a). In addition, there was a distinct population of $CD8^+$ T cells that only expressed OX40L but not OX40. In efforts to determine if the TGF-β1-induced OX40L was functional, aliquots of the TGF-β1-induced PBMCs were incubated with biotinylated sOX40 or biotinylated sOX40L followed with streptavidin-PE. As seen in Fig. 6b, OX40L expressed by the TGF-β1-treated $CD4^+$ and $CD8^+$ T cells was quite functional (giving values of 24.3%- and 61.7%-positive, respectively). However, TGF-β1 treatment in fact rendered the OX40 molecules non-functional as shown by a reduction of percentages of $CD4^+$ T cells bound by sOX40L from 33.2 to 3.4%. In order to exclude the possibility that the OX40L was passively acquired and synthesized by non-T cells in PBMCs, we stimulated purified $CD3^+$ T cells in the presence of both TGF-β1 and anti-OX40 blocking mAb (W4-54), and confirmed that TGF-β1 indeed induced the endogenous expression of OX40L on both the $CD4^+$ and $CD8^+$ T cells as early as 12 hours (data not shown).

Cell Stimulating Function of OX40L Expressed on T cells.

Finally, in order to confirm that the OX40L induced on the activated human T cells are biologically functional (in addition to their potential to bind their cognate ligand), we tested whether these $OX40L^+$ T cells could stimulate HIV-1 production from HIV-1-chronically infected T cell (ACH-2) and the monocytic cell lines (U1) expressing OX40. Activation by the intracellular NF-κB pathway has been shown to be the pathway by which OX40 stimulation induces HIV-1 replication in such cells [32]. Fig.7 shows that human bulk T cells repeatedly stimulated in the presence of anti-CD3/CD28 immunobeads and IL-4 as well as those stimulated once in the presence of the immunobeads and TGF-β1 could stimulate HIV-1 replication in the OX40-expressing T and monocytic cell lines (Fig.7 a and b, respectively). It is apparent that the cell activation which resulted in HIV-1 replication was mediated by OX40 stimulation by OX40L expressed on the T cells since this induction of HIV-1 was significantly inhibited by the addition of anti-OX40L blocking mAb (clone 5A8).

Altogether, the present data indicate that functional OX40L is inducible on both $CD4^+$ and $CD8^+$ *in vitro* activated human T cells which is dependent on not only the degree of cell activation but also on the culture environment.

DISCUSSION

The data shown herein support the previous findings made by Takasawa et al. [25], who showed that long-term cultured cytotoxic CD4^{+} and CD8^{+} T cell clones express OX40L after stimulation *in vitro*. The data reported herein extend these previous findings and describe new culture conditions that lead to the induction of OX40L on human CD4^{+} and CD8^{+} T cells. Importantly, we demonstrated for the first time that the OX40L molecules induced on the T cells were functional, as they were capable of not only binding soluble recombinant OX40 but also stimulating OX40-expressing cells (Figs.2 and 7). Importantly, the OX40L molecules detected on the activated T cells were endogenously synthesized, but not those transferred from other non-T cells [35] as demonstrated by using the anti-OX40 blocking mAb (W4-54) during *in vitro* activation in the cultures. Interestingly, the findings herein also show for the first time that the immune-suppressive cytokine TGF-β1 accelerated OX40L expression by primary cultures of activated human CD4^{+} and CD8^{+} T cells. The data presented herein also show that the stimulated individual CD4^{+} or CD8^{+} T cell could co-express OX40L along with OX40.

The reason why repeated activation was necessary for high levels of OX40L expression by the human T cells *in vitro* remains to be elucidated. In the murine system, it has been reported that bulk splenic T cells from normal adult mice do not express detectable levels of OX40L after stimulation with anti-CD3 with or without anti-CD28 stimulation either [13]. This is in contrast to the murine TCR-transgenic T cells that express OX40L readily after antigenic stimulation within a few days [27, 29]. Thus, it is likely that the expression of OX40L by bulk T cells, but not single TCR-expressing T cells, is under a tight control during the initial stage of T cell activation in normal mice and humans. Under physiological conditions, OX40L expression by T cells might be programmed to occur at a later stage of immune responses in order to maintain induced effector memory helper T cells [29].

Our present data suggest that the cytokine environment in the cultures, in which T cell stimulation is carried out, significantly affects OX40L expression. We found that IL-12 was inhibitory for the expression of OX40L by T cells (Fig.1, 2) Because the percentages of OX40^{+} T cells were higher in IL-12-treated T cells than those untreated or IL-4-treated, and anti-OX40 blocking mAb reversed the inhibitory effect of IL-12 on OX40L expression (Fig.4), it seems likely that IL-12 reduced the OX40L expression by T cells through enhanced production of functional OX40 molecules. Again, the effect of IL-12 on OX40L expression by human T cells is in contrast to the murine TCR-transgenic T cells. In this mouse system, the T cells preferentially express OX40L when they are stimulated in the presence of IL-12 via the activation of the STAT-4 pathway, and IL-4 severely down-modulates OX40L expression [27, 28]. Also in contrast to human cases, these TCR-transgenic T cells express enhanced levels of OX40 after treatment with IL-4. These discrepancies in OX40L and OX40 induction may be ascribed to species

-specific differences or simply due to a difference in the materials tested, i.e., bulk T cells from normal adults vs. naïve T cells from the TCR-transgenic mice. Experiments using naïve human T cells stimulated in the presence of anti-IL-4 (Th1-rich) or bulk T cells from normal mice may provide clues to such issues.

Even though there are some discrepancies between the human and murine systems, it is now apparent that endogenous OX40L molecules can be expressed on the activated T cells. Thus, functional OX40L molecules expressed at the cell surface may immediately interact with functional OX40 molecules expressed on the same T cell or adjacent T cells. The fate of OX40L on the T cells is unknown, and thus studies are required to address this issue in more detail in the future. It is possible that some human OX40L may be down-modulated via a post-transcriptional and cell to cell contact-dependent mechanisms as the murine T cell cases as shown by Soroosh et al [29]. Our present data indicate that some OX40L and OX40 molecules remained on the T cell surface without being bound by OX40 and OX40 molecules, respectively (Fig.2). In addition, some OX40L molecules on T cells may be transferred to adjacent cells via an OX40-dependent and/or independent manner [35].

The present study also revealed that the patterns of OX40L and OX40 expression were quite different between $CD4^+$ and $CD8^+$ T cell subpopulations. Thus, while the $CD4^+$ T cells tend to preferentially express OX40, the $CD8^+$ T cells appear to preferentially express OX40L (Fig.1, and 4). This difference was not merely phenotypic but also functional, because functional OX40L molecules that could bind soluble OX40 were abundant in $CD8^+$ T cells and the OX40 molecules expressed by $CD4^+$ T cells could bind sOX40L (Fig.2). One possible mechanism for this may be that the molecular ratios of OX40L and OX40 synthesized in these subpopulations are different. This phenotypic difference indicates that a communication between $CD4^+$ and $CD8^+$ T cells via OX40L-OX40 interaction is also possible. Since the OX40L-OX40 bindings have been shown to mediate cell to cell adhesion, it is highly possible that the OX40L-OX40 interaction may facilitate cross-talk between the two T cell populations after activation. In the murine system, Soroosh et al. [29] hypothesized that OX40L expressed by $CD4^+$ T cells can provide autonomous OX40 signals through T cell to T cell interactions which contribute to the longevity of antigen-specific $CD4^+$ T cells and thus lead to the generation and survival of $CD4^+$ memory T cells. The other functions suggested in the murine sytem include deviation of a Th1 response to a Th2 response [28], and control of Th2 proliferation [27]. Another possible speculation is that in addition to the immune-stimulating roles, the OX40L/OX40 interaction between activated T-T cells may serve as a feedback mechanism for the regulation of immune responses by rendering the $OX40^+$ T cells unresponsive to further OX40L signals from APC following saturation or masking of functional OX40. In addition, interaction of OX40L/OX40 on T-T cells may result in apoptosis of the cells involved in select environments since OX40 signal alone or together with TNF has been shown to enhance apoptosis of

OX40-transfectants [36, our unpublished data]. In addition to the signals generated by OX40 engagement, it is clear that engagement of OX40L also delivers a signal to the APC, which results in increased production of IL-12 by human DCs [10] leading to enhanced murine B cell differentiation [8]. Thus, it is very interesting to examine whether cross-linking of OX40L generates such signals to the T cells. Soroosh et al. [29] mentioned that cross-linking OX40L on T cells with a recombinant soluble OX40 or anti-OX40L mAb did not promote T cell proliferation, but rather suppressed it. Our preliminary experiments also failed to demonstrate any blocking or enhancing effects by anti-OX40L or anti-OX40 mAb on cell proliferation, cytokine production or T cell survival *in vitro* (data not shown).

Finally, although some reagents that trigger human OX40L on a number of non-T cell lineages such as the CD11c$^+$ DC [37], NK cells [21] and mast cells [22] have been reported, it should be emphasized that TGF-β1 played a prominent role in inducing the expression of functional OX40L on both human CD4$^+$ and CD8$^+$ T cells within 3 days after activation (Figs.5, 6 and 7). Since TGF-β1 is known to induce IL-17 to generate Th17 cells [38], it is interesting to test whether the OX40L$^+$ cells are related to Th17 cells or not. Preliminary data obtained in our laboratory indicated that TGF-β1 exerted its OX40L-inducing effect on T cells as early as 12 hours after T cell activation even in cultures of T cells that had been previously treated with mitocycin-C (data not shown), suggesting that TGF-β1 directly stimulates activated T cells to express OX40L without a requirement for either IL-17 or cell proliferation. The biological function of this TGF-β1-induced OX40L is not known at present. However, it can be speculated that the OX40L/OX40 interaction may be partly involved in multiple functions of TGF-β1 [39] on immune responses including generation of regulatory T cells [40] and autonomous promoting survival of peripheral T cells [41]. It is interesting to examine whether such OX40L-expressing T cells express Foxp3 or not. However, at present, we are unable to test this due to the fact that our anti-OX40L mAbs used to stain the cells is not detectable on the cell surface following treatment of the OX40L expressing cells with the permeabilization buffer provided with the human Foxp3 staining kit (eBioscience, Inc., San Diego, CA, USA , data not shown). Further studies are in progress to elucidate the precise immunological roles of OX40L expression by human T cells.

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FIGURE LEGENDS

Fig.1. OX40L expression by human activated T cells. PBMCs were stimulated with anti-CD3/CD28 immunobeads in the presence of IL-2 together with either IL-12 (+IL-12) or IL-4 (+IL-4) every 3 days. T cells 3 days after the first stimulation (1st) and third stimulation (3rd) were subjected to a multicolor staining using 5A8-FITC, B-7B5-Cy5 and either CD4-PE or CD8-PE. Flow cytometry data on OX40L and OX40 expression by T cells of $CD4^+$ or $CD8^+$ gate were shown as dot blots. Numbers in the dot blot graphs indicate percent positive cells. Data shown is representative of 3 similar experiments.

Fig.2. Expression of functional OX40L and OX40 on repeatedly activated T cells. Aliquots of cultures of PBMCs re-stimulated three times every 3 days for a total of 9 days with anti-CD3/28 immunobeads in the presence of IL-2 together with either IL-12 (+IL-12) or IL-4 (+IL-4) were incubated with either biotinylated recombinant soluble forms of OX40 or OX40L along with either CD4-PC5 or CD8-PC5 mAb for gating. Binding of the soluble proteins was detected by using streptavidin-PE. The bars and the numbers displayed in the histogram graphs show positive area and percent positive cells. The control profile used to define the bar for the positive area was obtained on aliquots of the cultured cells untreated with the soluble proteins. Data shown is representative of 3 similar experiments.

Fig.3. Anti-OX40 mAb (W4-54) blocks OX40-OX40L interaction.

(a) Molt4 cells expressing OX40 (Molt4/OX40) or those expressing OX40L (Molt4/OX40L) were pre-incubated with 5 μg/ml W4-54 or negative control mAb (Mo-8) for 30 mimutes on ice. These cells were then incubated with biotinylated sOX40L or sOX40 without washing for additional 30 mimutes on ice followed by staining with streptavidin-PE. The top and bottom panels depict the sOX40L binding to Molt4/OX40 cells and sOX40 binding to Molt4/OX40L cells, respectively. The thin line represents background staining with streptavidin-PE, and while the dotted line represents staining with sOX40L or sOX40 in the presence of the negative control mAb. The dark thick line represents staining in the presence of the W4-54 blocking antibody. Data shown is representative of 3 similar experiments.

(b) In order to test the ability of W4-54 to prevent OX40 and OX40L transfer by cell to cell contact, Molt4/OX40L cells and Molt4/OX40 cells were each mixed either with the mock-transfected Molt4 cells or mixed with each other in the presence of 5 μg/ml of W4-54 blocking antibody or for purposes of control the Mo-8 mAb on ice for 30 mimutes. These mixed population of cells were then stained with anti-OX40L-FITC (5A8) and a different clone of anti-OX40-Cy5 (B-7B5). Representative data from 3 experiments are shown. Note that when Molt4/OX40L cells and Molt4/OX40 cells were co-cultured in the control mAb, the Molt4/OX40 cells acquired OX40L, and at the same time, the Molt4/OX40L cells