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3 **Novel two-parameter flow cytometry (MIL4/ SSC followed by**
4 **MIL4/CT7) allows for identification of five fractions of guinea pig**
5 **leukocytes in peripheral blood and lymphoid organs**
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21 Abbreviations: BCG, bacillus Calmette and Guérin; MHC, major histocompatibility
22 complex; PBL, peripheral blood leukocytes; CTL, cytotoxic T lymphocyte; MIL4-
23 SSC^{large}CT7^{high}, MIL4^{side} scatter^{large}CT7^{high}; NIID, National Institute of Infectious
24 Diseases; FITC, fluorescein isothiocyanate; PE, phycoerythrin; mAb, monoclonal
25 antibody; APC, allophycocyanin; LN, lymph node; BM, bone marrow; PI, propidium
26 iodide; PAS, periodic acid Schiff; PMN, polymorphonuclear neutrophils.

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4

Abstract

Though the guinea pig has been an extremely useful animal model for a variety of diseases, the tools necessary to undertake a full-scale immunological analysis of the guinea pig have been lacking. For instance, traditional two-parameter forward/side scatter (FSC/SSC) flow cytometry, though effective in human and other animal models, is unable to adequately identify the distinct fractions of guinea pig peripheral blood leukocytes (PBL). We introduce here a new flow cytometric technique (MIL4/SSC followed by MIL4/CT7) which redresses this lack by identifying and characterizing five distinct fractions of PBL: neutrophils, lymphocytes, monocytes, eosinophils plus basophils, and the novel MIL4⁺SSC^{large}CT7^{high} population. The MIL4⁺SSC^{large}CT7^{high} cells possess cytoplasmic inclusion bodies of variable size that were positive for periodic acid Schiff (PAS). Their cell surface stained positive for the helper/inducer lymphocyte markers CD45, Thy-1, asialo GM1 and FcR, but negative for B cell markers, such as membrane-type IgM, CD8 and MHC class II. The novel flow cytometric technique also allowed us to establish that the five leukocyte fractions were found in PBL, splenocytes, thymocytes and lymph node cells. Cells which were positive for inclusion bodies comprised 16.6% of splenocytes, 9.9% of PBL and 4.3% of liver cells, but were comparatively rare in lymph node cells, thymocytes, and BM cells. The novel flow cytometric technique introduced here will allow a better understanding of the response of each type of guinea pig leukocyte and thereby shed light on the diseases with which they are associated.

1. Introduction

For more than 100 years, guinea pigs have been an important animal model for the study of *Mycobacterium tuberculosis* (*M. tuberculosis*), the vaccine strain *M. bovis* bacillus Calmette and Guérin (BCG) (Bloom et al., 1994) and other infectious agents (Griffith et al., 1991; Myers et al., 1992; Stanberry et al., 1995; Wicher et al., 2001; Woolf et al., 1991); carcinoma (van der Meijden et al., 1989); allergies (Broder et al., 1978; Daffonchio et al., 1989; Gulbenkian et al., 1990; Kallos et al., 1984; Samejima et al., 1988; Tohda et al., 2001); and various other biological phenomena (Wagner et al., 1976).

Though an animal model of considerable importance, guinea pigs pose some immunological limitations not seen in other models. For instance, in humans PBL leukocyte fractions of granulocytic, lymphoid or mononuclear phagocytic origins are easily identified (Abramson et al., 1977; Prchal et al., 1978) using traditional flow cytometry, and terminally differentiated cells within those fractions are known to function cooperatively as a major defense against foreign pathogens (Ginaldi et al., 1999; Silva et al., 1989; Paul et al., 1993; Paul et al., 1999; Zhang et al., 1992). However, those fractions cannot be clearly identified in guinea pigs using standard two-parameter forward/side scatter (FSC/SSC) flow cytometric analysis (Choong et al., 1995; Goto et al., 1989; Lanza et al., 1992).

Other obstacles to the identification of distinct PBL populations in guinea pigs include the lack of phenotypic characterization of cell surface markers due to the limited availability of anti-guinea pig antibodies (only ten clones are available), the paucity of genetic data on guinea pig MHC haplotypes, the lack of well-established animal strains, and the lack of data on the functional properties of guinea pig leukocytes, for example cytotoxic T lymphocyte (CTL) activity. Finally, guinea pig spleen cells do not always respond well to immunogens, underscoring the challenge of using these animals in immunologic studies.

To help redress these problems, we found cross-reactivity of porcine m-Ab MIL4 (Haverson et al., 1994) and here introduce a new flow cytometric technique (MIL4/SSC followed by MIL4/CT7 antibodies) capable of clearly differentiating five distinct fractions of guinea pig leukocytes: neutrophils; monocytes; lymphocytes; eosinophils plus basophils; and

the novel MIL4⁺side scatter^{large}CT7^{high} (MIL4⁺SSC^{large}CT7^{high}) leukocytes. We also used this technique to characterize the fractions and to normalize tissue distribution.

2. Materials and Methods

2.1 Animals

Female Hartley guinea pigs, 4 to 6 wks of age and weighing 350 to 400 g each, were obtained from Japan SLC, Inc., Shizuoka, Japan. Animals used in the studies were free of pathogenic bacteria and parasites. They were housed in accordance with the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science, 1987, in full compliance with the Law for the Humane Treatment and Management of Animals (in Japan). Animals were fed and maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases (NIID), Japan. Once approved by an institutional committee for animal experiments, these studies were conducted at the Animal Facility of Toyama Campus, NIID, Japan, in accordance with the requirements specifically stated in the Laboratory Biosafety Manual of the World Health Organization.

2.2. Antibodies

The anti-guinea pig antibodies used in this study are listed in Table I. Fluorescein isothiocyanate (FITC)-labeled T helper/inducer (CT7) cells, anti-CD8 cells (CT6, Samejima et al., 1988), anti-B cells (Msgp9) and anti-T cells (CT5, Broder et al., 1978), along with the purified anti-porcine neutrophil subset (MIL4, Haverson et al., 1994), anti-CD45 (IH-1) and phycoerythrin (PE)-labeled rabbit anti-mouse IgG-F(ab')₂ were obtained from Serotec, Ltd. (Oxford, UK). The MIL4 monoclonal antibody (mAb) was labeled with FITC and biotin by conventional methods. Streptavidin-allophycocyanin (APC) and streptavidin-cychrome was purchased from Pharmingen (San Diego, CA, USA). Antibodies to guinea pig MHC class II antigens (R27E7), IgM (31D2), leukocyte common antigen (LCA, H201), an additional T cell

antigen (H159), an additional T helper/inducer antigen (H155), Thy-1 (H154) and Fc receptors (20ED7) were kindly supplied by Dr. R. Burger, Department of Immunology, Robert Koch-Institute, Nordufer 20, D-13353, Berlin, Germany (Schafer et al., 1991; Steerenberg et al., 1991). Anti-asialo GM1 was obtained from Wako Pure Chemicals, Ltd. (Tokyo, Japan). Anti-human CD56 antibodies used in the study were NCAM16.2 (Becton Dickinson, San Jose, CA, USA), MY 31 (Becton Dickinson), B159 (Pharmingen), NK1-nbi-1, (Nichirei Bioscience Inc., Tokyo, Japan) and NKH-1 (Beckman Coulter, Florida, USA).

2.3. Cells and isolation of leukocyte subsets

Splenocytes, lymph node (LN) cells, thymocytes and liver cells were teased out by pressing organ fragments through a mesh filter cell strainer (Becton Dickinson). PBL, liver cells, bone marrow (BM) cells and splenocytes were hemolysed using a lysis buffer consisting of 0.15 M NH_4Cl , 0.1 mM EDTA-2Na, 1.0 mM KHCO_3 . After being washed twice, the cells were resuspended in PBS-staining buffer with 0.1% NaN_3 and 3% FCS.

2.4. Flow cytometric analysis of PBL and immune cells by their cell-surface staining with specific monoclonal antibodies

First, normal pig γ -globulin was purified by 33% by subjecting it to two courses of ammonium sulfate precipitation and then incubated with cells at a final concentration of 1mg/ml at 4°C for 10min to prevent nonspecific binding of the specific antibodies, except for asialo GM1 (Nakahara et al., 1980), FcR, and IgM staining. Then, we added a purified antibody and incubated for a further 30min before washing and adding a PE-conjugated rabbit anti-mouse IgG F(ab) at 4°C for 30min. FITC-conjugated and biotin-conjugated antibodies were incubated with the cells at 4°C for 30min. Finally, we incubated the cells with streptavidin APC and streptavidin cychrome at 4°C for 30min and washed with staining buffer. Dead cells were excluded by propidium iodide (PI) and viable cells were analyzed on a FACScalibur (Becton-Dickinson, San Jose, CA, USA) equipped with an argon and

semiconductor laser sets at 488 nm and 635 nm using Cell Quest software (Becton-Dickinson) .

2.5. Morphological studies

Sorted cells were cytocentrifuged using a Cytospin-3 (Shandon Life Sciences International, Ltd., Runcorn, UK) and stained with May-Giemsa solution (Merck KGaA, Darmstadt, Germany) or periodic acid Schiff (PAS) (MUTO Pure Chemicals Co., Ltd., Tokyo, Japan), as described elsewhere.

2.6. Statistical analysis

Data analysis was carried out using the Stat View program (SAS Institute, Cary, NC) and data are expressed as the mean $\pm$ SD.

3. Results

3.1 Evaluation of guinea pig leukocytes by conventional flow cytometry

As noted above, one of the drawbacks of studying immunologic responses in guinea pigs is the difficulty of identifying distinct PBL populations by conventional assay methods (guinea pig PBL, 1A-a; and human PBL, 1B-a). We found that one reason for the difficulty in gating guinea pig PBL was a unique population of cells in guinea pigs (the gated cell population in 1A-b), not present in humans (no corresponding cells in the gated area in Fig. 1B-b), that stains highly positive for the helper/inducer marker CT7 and SSC (the gated cell population in Fig. 1A-b). When the SSC^{large}CT7^{high} population was deleted from guinea pig PBL in the cytometric analysis data, the three distinct populations of lymphocytes, gated with green circles; monocytes, gated with red circles; and granulocytes, gated with blue circles, could be clearly visualized using the conventional FSC/SSC flow cytometry of guinea pig PBL (Fig. 1A-c).

3.2. The unique PBL fraction is further identified as MIL4^{SSC^{large}CT7^{high}}

By screening human and mouse mAbs that cross-react with guinea pig cell surface antigens, we identified one antibody MIL4 that proved useful in differentiating guinea pig PBL into four subpopulations using two-parameter, consecutive MIL4/SSC flow cytometry (Fig. 2A). That antibody had previously been shown to recognize porcine neutrophils, eosinophils and basophils (Haverson et al., 1994). However, the CD antigen which was recognized by this antibody has not been defined (Haverson et al., 1994). By comparing the flow charts of guinea pig PBL obtained using a two-parameter MIL4/SSC analysis (Fig. 2A) with that obtained using a FSC/SSC analysis (Fig. 2B), we were able to identify four distinct PBL subpopulations: lymphocytes (green), monocytes (red), granulocytes (blue) and a novel MIL4⁺SSC^{large} population (orange). As shown in Figure 2D, we further separated the MIL4⁺SSC^{large} subpopulation (orange) by gating with MIL4/CT7 into MIL4⁺CT7^{high} cells (approximately 84±6%) of the MIL4⁺SSC^{large} cells and MIL4⁺CT7^{low} cells (approximately 16±3%, gated population of Fig2D). Thus, we separated guinea pig PBL into 5 distinct subpopulations.

3.3. Morphological confirmation of the five leukocyte fractions isolated by cell sorting

We first sorted the green, red and blue leukocyte fractions in Fig. 2A with a FACS Vantage and morphologically confirmed them as guinea pig lymphocyte, monocyte and granulocyte fractions, respectively, using a conventional May-Giemsa staining procedure (data not shown). Next, we further sorted the two leukocyte fractions in Fig. 2D with the cell sorter and used May-Giemsa staining to reveal that the MIL4⁺SSC^{large}CT7^{low} population, the gated subpopulation in Fig. 2D, was comprised of eosinophils and basophils (Fig. 3A). However, another MIL4⁺SSC^{large}CT7^{high} population, unique to guinea pigs, generally contained an inclusion body in the cytoplasm (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 $\text{MIL4}^{\text{SSC}^{\text{large}}\text{CT7}^{\text{high}}}$ leukocytes in the non-gated population of Fig. 2D were found to be identical with the gated $\text{SSC}^{\text{large}}\text{CT7}^{\text{high}}$ 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 ($11.09 \pm 9.42\%$ of neutrophils of the blue cell fraction in Fig. 2B), monocyte ($30.45 \pm 11.22\%$ of monocytes of the red cell fraction in Fig. 2B) and lymphocyte ($1.27 \pm 0.75\%$ 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 subtypes of leukocytes including the novel $\text{MIL4}^{\text{SSC}^{\text{large}}\text{CT7}^{\text{high}}}$ cells (Fig. 3C).

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

Since nearly 50% of the unique $\text{MIL4}^{\text{SSC}^{\text{large}}\text{CT7}^{\text{high}}}$ 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 $\text{SSC}^{\text{high}}\text{MIL4}^{\text{CT7}^{\text{high}}}$ guinea pig cells (right-gated subpopulation of Fig. 2D), only leu19 (MY31, BD) proved to be cross-reactive, reacting with $6.3 \pm 2.4\%$ 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 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 II). As shown in Fig. 5A and Table II, 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 spleen, respectively (Table II 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 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 et al., 1966; Debout et al., 1984; Debout et al., 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

1 two guinea pig leukocyte fractions. An identification of eosinophils and basophils of guinea
2 pigs will be beneficial to the study of immediate-type hypersensitivity reactions in guinea pig
3 models. Therefore, the identification of five distinct subtypes of guinea pig leukocytes,
4 including one unique MIL4⁺SSC^{large}CT7^{high} cell type, paves the way for examining the role of
5 each of these cell types in the infections and inflammatory conditions that can be uniquely
6 studied in guinea pigs.

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Table 1

Panel of anti-guinea pig mAbs used for flow cytometry^{a)}

Clone	Specificity	Ig class
Msgp9	Pan B-cell	IgG1
31D2	IgM	IgG
R27E	MHC Class II	IgG
20ED7	FcR	IgG
CT5	Pan T-cell	IgG1
H159	Pan T-cell	IgG
CT7	CD4 (T helper/inducer)	IgG1
H155	T helper/inducer	IgG
H154	Thy-1	IgG
CT6	CD8 (T suppressor/cytotoxic)	IgG1
MIL4	Porcine neutrophils and eosinophils	IgG1
Asialo GM1	Mouse and rat NK cells	Polyclonal rabbit Ig
IH-1	LCA (leukocyte common antigen)	IgG1
H201	LCA (leukocyte common antigen)	IgG

^{a)} Anti-guinea pig mAbs R27E, 31D2, 20ED7, H159, H155, H154 and H201 were the kind gift of Dr. R. Burger, Robert Koch Institute, Berlin, Germany. Asialo GM1 was obtained from Wako Pure Chemicals, LTD, with the rest obtained from Serotec.

1 Table 2
 2 Mean percentage of leukocyte fractions in various tissues of normal guinea pigs obtained by
 3 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
L N	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

- 1 ^{a)} Leukocytes of guinea pigs were gated into 5 fractions by flow cytometry using MIL4/SSC
- 2 followed by MIL4/CT7. The MIL4⁻ SSC^{large} fraction was separated into CT7⁺ and CT7⁻ cells.
- 3 Data are expressed as the mean $\pm$ SD.
- 4

Figure Legends

FIGURE 1. Comparison of guinea pig and human PBL by two-parameter flow cytometric assays using light scatter and anti-CT7 (helper/inducer) mAb. Viable cells were gated by FSC/SSC after excluding dead cells using propidium iodide. A. Separation of guinea pig PBL fractions: (a) Unsuccessful separation of guinea pig PBL by conventional FSC/SSC two-parameter flow cytometry. (b) Another separation of the PBL by CT7/SSC two-parameter flow cytometry. (c) Distribution of PBL after removal of the $SSC^{high}CT7^{high}$ population which was located in the gated population of Fig. 1A-b. B. Separation of human PBL fractions: Two-parameter flow cytometric separation of PBL by (a) FSC/SSC and (b) CD4/SSC. Each gated color population from humans was equivalent to the color indicated by SSC and CD4: lymphocytes (green), monocytes (red), and granulocytes (blue). Gate in Fig. 1B-b contained a $SSC^{high}CD4$ -positive population.

FIGURE 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 Fig. 2A 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 $MIL4\text{-}SSC^{large}$ 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 $MIL4\text{-}SSC^{large}$ 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.

FIGURE 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 μ m. (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.

FIGURE 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.

FIGURE 5. Distribution pattern of leukocyte fractions in various guinea pig tissues. Cells isolated from 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

- 1 guinea pig leukocyte fractions are shown for spleen (A), lymph node (B), thymus (C), bone
- 2 marrow (D) and liver (E). Dead cells were excluded using propidium iodide and viable cells
- 3 were analyzed.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5