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Peritoneal sanctuary for human lymphopoiesis in SCID mice injected with human peripheral blood lymphocytes from Epstein-Barr virus-negative donors

The successful engraftment in SCID mice of intraperitoneally (i.p.) injected human lymphocytes (hu-PBL-SCID) and the failure of intravenously injected peripheral blood lymphocytes (PBL) directed the present study to investigate the early events of donor cell proliferation in the peritoneal cavity. We found focal lymphocyte engraftment together with histio-monocytic interleukin (IL)-6$^+$ cell phenotypes which must have been transferred with the human cell inoculum, which could explain certain immune functions observed in hu-PBL-SCID chimeras. Following i.p. injection of 10^8 PBL, human cells suspended in peritoneal fluid as well as those adherent to the serosal peritoneum and abdominal organs were investigated by immunocytology and immunohistology. Human cells were found to form foci consisting predominantly of proliferating human lymphoblastoid CD3$^+$ cells, which were mostly activated HLA-DR$^+$ CD8$^+$ lymphocytes. Among the lymphoid cells larger epithelioid-like cells were found to belong to the monocytic series and to stain strongly with anti-HLA-DR and anti-CD11c antibodies. Some of these cells were also positive with anti-ICAM and anti-IL-6. Congenic as well as allogeneic mouse PBL, injected i.p. into SCID mice, temporarily produced analogous foci, which shifted later on to foci similar in appearance to milky spots. However, the human cell foci appeared less compact, more closely resembling *in vitro*-culture soft agar colonies. It is possible that cytokines in the human histio-monocytic cells of the foci may have a feeder effect on the human lymphocytes and be a prerequisite for proliferation of human PBL in SCID mice. The observed early HLA-DR activation of human lymphocytes in the peritoneal foci could reflect triggering of immune reactions like xenogeneic graft-versus-host reactions in the peritoneal site, where the human CD11c$^+$ HLA-DR$^+$ histio-monocytic cells may act as antigen-presenting cells.

1 Introduction

Intraperitoneal (i.p.) transfer of human peripheral blood lymphocytes (PBL) in SCID mice has become an important xenotransplantation model (hu-PBL-SCID) for human-to-mouse lymphoid chimerism [1]. Intravenous cell transfer usually failed to establish chimerism. In addition we found surprisingly few human cells in SCID spleens and lymph nodes shortly after i.p. injection of high numbers (10^8) of human blood PBL. Early events of cell proliferation in the peritoneal cavity must, therefore, be regarded as being responsible for successful chimerism. Human cells in peritoneal lavages of hu-PBL-SCID have already been phenotyped by FCM [2]. To obtain information concerning local events that may help us to understand cell engraftment, the present study focuses on the immunohistology and -cytology of what appears to be a peritoneal sanctuary for lymphoid cells in SCID mice. Apart from human cells suspended in the peritoneal fluid, we found human lymphocytes adherent to serosal surfaces of the peritoneum in localized areas. The patterns of peritoneal settling of T and B cells and a third cell type, interleukin (IL)-secreting human histio-monocytic cells, have escaped immunocytology so far but may be significant for understanding lymphoid proliferation and immune functions in hu-SCID-PBL. These foci with human cells were compared with the foci which we found after intraperitoneal engraftment of allogeneic and syngeneic mouse lymphocytes.

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2 Material and methods

2.1 Mice

CB-17 scid/scid mice were originally obtained from H. Wagner, University of Ulm with the kind permission of M. J. Bosma. They were bred and maintained under pathogen-free conditions at our animal facility. They were housed in horizontal laminar flow cabinets and were fed with autoclaved food (Altromin 1314 fortified, Altromin, D-4937 Lage, FRG).

BALB/c mice and C57BL/6 (Thy-1.1), originally obtained from Jackson laboratories, were also kept at the GSF animal facilities.

2.2 Transplantation of human PBL

Healthy human blood donors were serologically tested for their EBV status at the Munich University Hospital Großhadern. The purification procedure of sterile human PBL prior to i.p. injection included density-gradient centrifuga-

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tion with Ficoll ($\varrho = 1.077$) and assessment of viability by Trypan blue exclusion. Aliquots of donor PBL consisted of approximately 750 µl of cell suspension containing 100×10^6 cells/mouse. In the experiment reported here, reconstitution was carried out using EBV$^-$ human PBL.

2.3 Transplantation of mouse lymphocytes

Spleen cells (10^8 in 300 µl PBS) of either BALB/c (Thy-1.2) or of C57BL/6 (Thy-1.1) were injected i.p. into SCID mice.

2.4 Immunohistochemistry

The mesenterium, pancreas, stomach, diaphragm, abdominal wall and kidneys with surrounding tissue were removed on day 3 and at weekly intervals up to 8 weeks, starting 1 week after transplantation. Additional moribund transplanted mice were killed up to 12 weeks after injection of lymphocytes. Organs were snap-frozen, and 5-µm-thick cryostat sections were air-dried and fixed in acetone for 10 min. Incubations with antibodies and either normal rat or normal mouse serum (as negative control) lasted 60 min and were stopped by washing in Tris-buffered saline. Peroxidase activity was revealed with 3-amino-9-ethyl carbazole. The tissue sections were counterstained with hematoxylin. Abdominal lavage cells of killed SCID mice were also immunocytochemically investigated by binding to poly-L-lysine spots on multispot slides, which were prepared according to Bross [3], and modified by Kranz et al. [4, 5].

2.5 Antibodies

Antibodies used in immunohistochemistry and cytochemistry are summarized in Table 1. Mouse mAb were revealed with either peroxidase-labeled rat anti-mouse IgG (H + L) or with peroxidase-labeled goat anti-mouse IgG (H + L). Rat mAb were stained with peroxidase-labeled mouse anti-rat IgG (H + L). These commercial antibodies were from Jackson Immunoresearch (West Grove, CA).

3 Results

3.1 Early stage of engraftment of human PBL

On day 3 and 1–3 weeks after i.p. injection of 10^8 human peripheral blood lymphocytes, the majority of transferred cells can be found growing in the peritoneal cavity, on or under the serosal peritoneum of most SCID mice. Foci or flat layers (Fig. 1), or cauliflower-like structures (Fig. 2) of human cells can be distinguished that apparently prefer the serosal areas of the diaphragm, stomach, mesentery or pancreas, and are even found between pancreatic lobules or in perirenal tissue; they are found more rarely on the serosal layer surrounding the liver, spleen, and abdominal wall. These foci consist of a mixture of small and large cells. Human CD3$^+$ T cells (Fig. 1 a, b), mostly of the CD8$^+$ $T_{C/S}$ type (Fig. 2 a) and lymphoblastoid in appearance, prevail. CD4$^+$ $T_{H/D}$ lymphocytes are less numerous. Human Ig$^+$ B cells are rare. Most of the lymphoid cells are also activated expressing HLA-DR (Fig. 2 b). Many are Ki67$^+$ proliferating cells. Among the lymphocytic cells, larger cells with pale nuclei and broad cytoplasmic rims are present. Some resemble epithelioid cells. They are strongly labeled by anti-HLA-DR and by anti-CD11c (Fig. 1 c). Some of them are Ki67$^+$ proliferating cells. The pattern of staining allows classification of this cell type as belonging to the monohistiocytic series, *i.e.* a phenotype of monocytic appearance in the blood and of histiocytic appearance in tissues. They are strongly stained by anti-human ICAM, and many of them are also human IL-6$^+$ (Fig. 1 d).

Table 1. Antibodies used for immunocytochemistry and immunohistochemistry, source in parenthesis

Specificity	Target cells	
	Human	Mouse
CD3	UCHT1 (mouse)[a]	17A2 (rat)[b]
CD4/L3T4	Anti-Leu-3a (mouse)[c] 38/II/8 (rat)[d]	RmCD4-1[e]
CD8/Ly-2	Anti-Leu-2a (mouse)[c] 38/I/10 (rat)[d]	RmCD8-1[e]
CD11c	Anti-Leu-M5 (mouse)[c]	
Mac 1		M1/70 (rat)[f]
HLA-DR/Ia	IFH-Ia 7510 (mouse)[g]	M5/114 (rat)[h]
CD20	Coulter Clone B1 (mouse)[i]	
Nuclear Ki67	Ki67 (mouse)[k]	
Ig	µ chains (rabbit)[k] λ light chains (rabbit)[k] κ light chains (rabbit)[k]	IgG (H+L) (rat)[l] IgG (H+L) (goat)[l]
IL-1β	FIB3 (mouse)[m]	
IL-6	Anti-IL-6 (mouse)[n]	
ICAM	P3.58 (mouse)[o]	YN1/1.7.4-CRL 1878 Anti-ICAM (rat)[p]

a) P. C. L. Beverly, London, GB.
b) McDonald, Lausanne, Schweiz.
c) Becton Dickinson, Mountain View, CA, USA.
d) R. Schuh, München, FRG.
e) K. Reinecke, München, FRG.
f) Serotec, Oxford, GB.
g) U. Kummer, München, FRG.
h) Boehringer, Mannheim, FRG.
i) Coulter Immunology, Hiallah, FL, USA.
k) Dakopatts, Glostrup, Denmark.
l) Jackson Immunoresearch, West Grove, CA, USA.
m) Dianova, Hamburg, FRG.
n) Genzyme, Cambridge, CA, USA.
o) J. Johnson, München, FRG.
p) ATCC, Rockville, MD, USA.

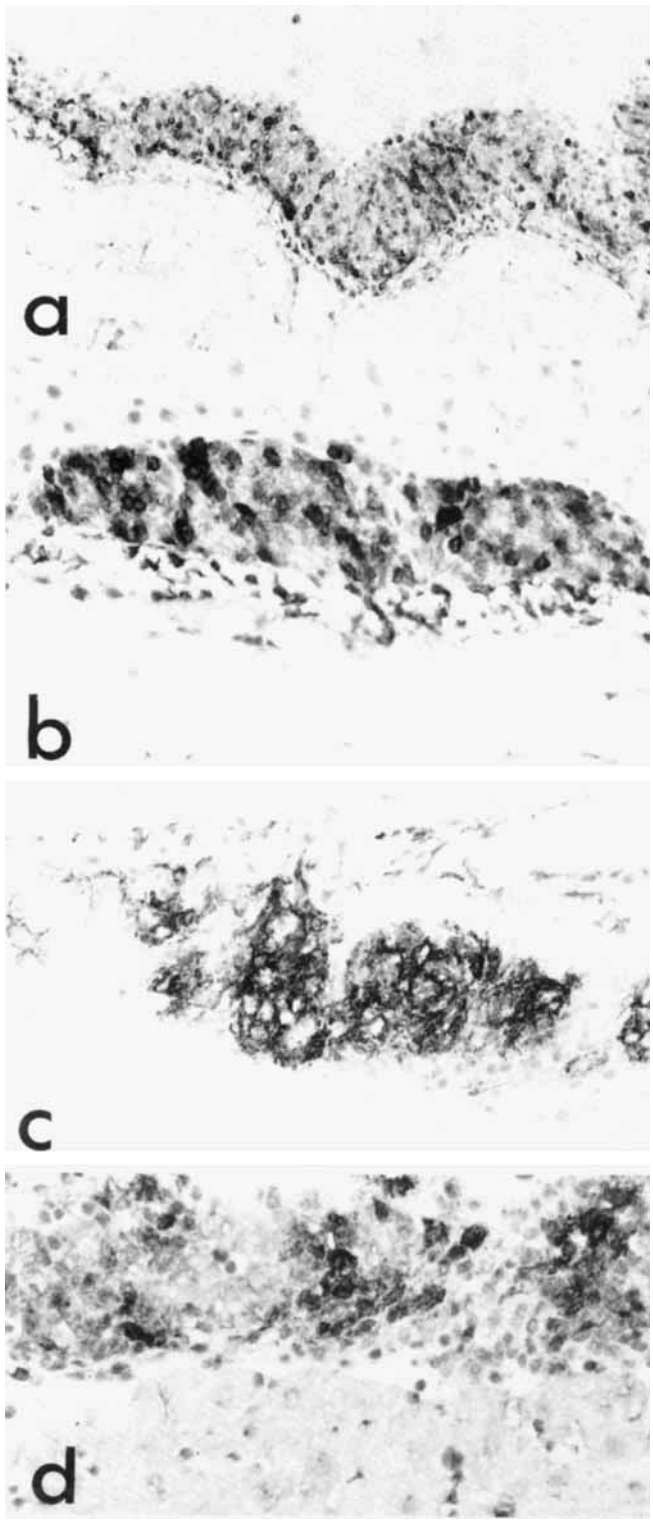

Figure 1. Flat layers of human cells adherent to the peritoneal layer in SCID mice injected with human PBL: (a, b) human $CD3^+$ cells; (c) larger human $CD11c^+$ cells with histiocytic appearance between the human lymphocytes; and (d) anti-$IL\text{-}6^+$ reaction in histiocytic cells. a × 110, b–d × 280.

In addition to these cells of human origin, the foci may contain small numbers of mouse cells. Mouse $Ly\text{-}2^+$ $T_{C/S}$ cells or mouse $L3T4^+$ $T_{H/D}$ cells are rarely found among the human cells. Mouse Mac 1^+ cells occur either as single cells or in more numerous quantities (Fig. 2d). They seem

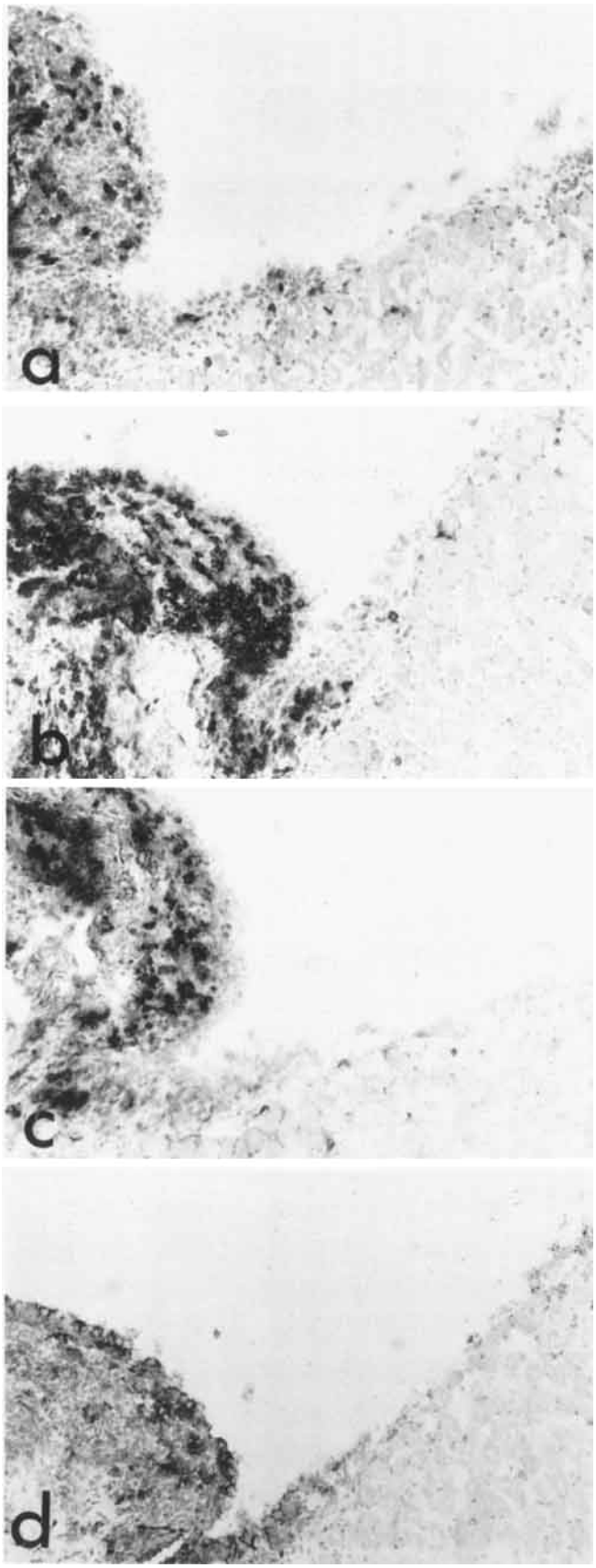

Figure 2. Cauliflower-like focus changing into flat layer after injection of human PBL: (a) human $CD8^+$ cells; (b) $HLA\text{-}DR^+$ cells; (c) human Ig^+ cells; and (d) mouse Mac 1^+ cells surrounding the focus against the peritoneal cavity. a–d × 110.

to prefer areas where human CD11c$^+$ cells do not occur, particularly along the border areas of the foci. Proportions of the different human and mouse cell types may vary between individual hu-PBL-SCID. Also, the localization of the different cell types within the foci is irregular.

3.2 Later stage of peritoneal engraftment of human PBL

From 4 weeks post injection onwards, larger foci may show a granuloma-like appearance (Fig. 3). The central homogeneous cell-free zone of the foci is surrounded by a layer of HLA-DR$^+$, CD11c$^+$, and ICAM$^+$ (Fig. 3b) epithelioid histiocytic cells. They express HLA-DR and human CD11c. In the peripheral zone, human, mostly CD8$^+$ lymphocytes are present (Fig. 3a). Also, several loosely arranged HLA-DR$^+$ cells of the mono-histiocytic series can be found which are human IL-6$^+$ and sometimes also human IL-1$^+$. In SCID mice with substantial human blood T cell chimerism (> 10%), the peritoneal fluid regularly also contained human cells. Up to 90% of cells in the peritoneal lavage fluid were CD3$^+$ and HLA-DR$^+$ cells.

3.3 Peritoneal engraftment of congenic or allogeneic mouse PBL

To determine whether our observations in the peritoneum are characteristic of hu-SCID-PBL chimeras, a peritoneal settlement of syngeneic, more specifically of congenic BALB/c or allogeneic mouse (C57BL/6-Thy-1.1) spleen lymphocytes injected i.p. into SCID mice was studied immunohistochemically. We found foci of mouse cells on the peritoneal serosa (Fig. 4). Most of them were localized on the mesenterium, but foci on the pancreas were also frequent. They contain numerous B lymphocytes (Fig. 4a) and also donor-type T cells (Fig. 4b). From about day 10 post i.p. injection of mouse spleen lymphocytes, the foci

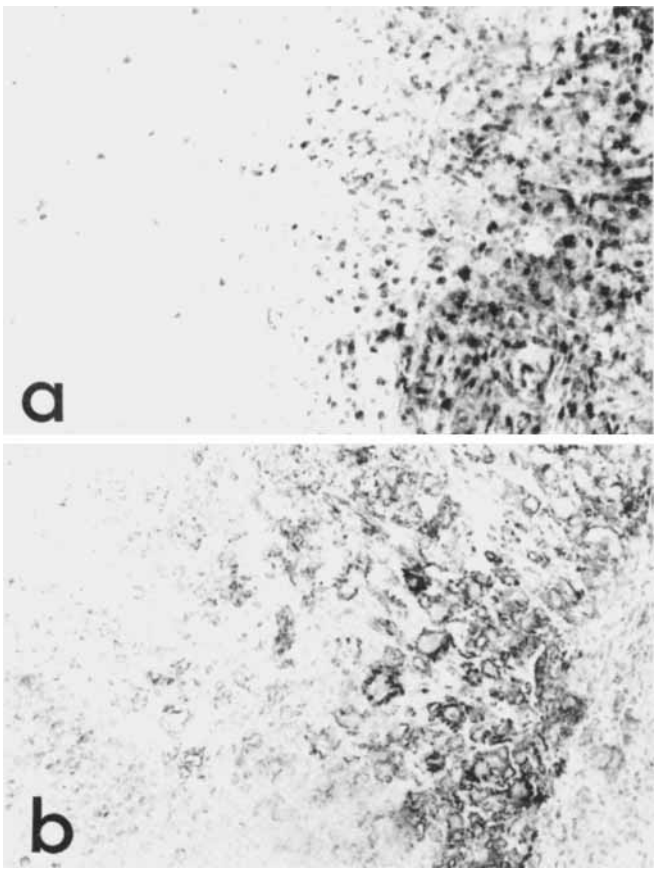

Figure 3. Older, granuloma-like focus with necrotic center in a hu-PBL-SCID chimera (a) containing a few CD8$^+$ human lymphocytes, (b) surrounded by human ICAM$^+$ histocytic cells. a, b × 110.

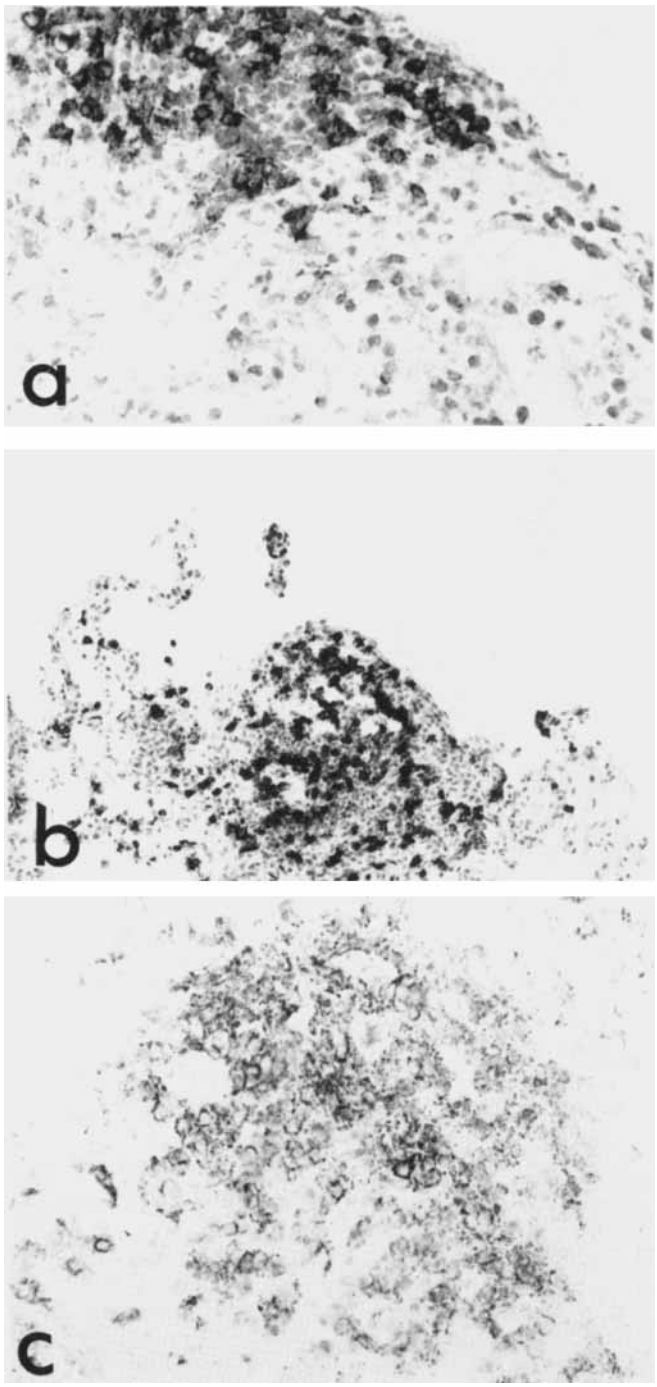

Figure 4. Peritoneal foci in SCID mice after injection of allogeneic mouse spleen lymphocytes: (a) Flat mouse B cell-containing focus on the surface of peritoneal serosa; (b) Thy-1$^+$ mouse T cells in a cauliflower-like peritoneal focus; (c) Ia$^+$ cells in a milky spot-like lymphatic focus between the serosal layers of the mesenterium. a, c × 280, b × 110.

containing donor cells had sunk into the mesentery between the serosal layers or under the serosa covering abdominal organs. They were well vascularized and similar in appearance to lymphatic tissue. There were small areas in the foci where B lymphocytes predominated and other tiny regions where postcapillary venules were surrounded by T lymphocytes. Both L3T4$^+$ and Ly-2$^+$ cells were observed. Mac 1$^+$ cells were rare. In SCID mice injected with allogeneic spleen lymphocytes, many of the donor cells were activated Ia$^+$ cells (Fig. 4c).

4 Discussion

While establishment of SCID-hu chimerism by co-implantation of human fetal tissue has been followed immunohistologically [6, 7], cell engraftment in hu-PBL-SCID has been studied by testing human cells obtained from peritoneal lavage [8]. The additional use of immunohistology allows not only the localization of cells within tissues but also the detection of rare cell types, including their cytokines, which may escape FCM. In addition, we compared our findings to the engraftment of i.p. injected allogeneic and congenic lymphocytes which had also not been studied so far but showed differences which may help to understand certain pecularities of the hu-PBL-SCID model.

The peritoneal serosa and its underlying tissue has a high affinity for lymphatic tissue, best known as milky spots in the mesentery of young animals. In SCID mice injected i.p. with mouse lymphocytes, mouse cells initially became attached to the peritoneal serosa where they grew as flat or cauliflower-like structures also containing cells of the monocytic series. Soon, however, donor lymphocytes migrated into the mesentery underneath the serosal coating, or even between pancreatic lobules, and grew there in the typical pattern of milky spots. The latter are characterized by T cells in the neighborhood of postcapillary venules and B cells which are aggregated in irregular foci.

In SCID mice injected with human PBL from EBV$^-$ donors, a somewhat different pattern of donor cell settlement and aggregation in the peritoneal cavity could be observed, which is probably due to species difference. Early attachment of human cells to the peritoneal surface was also seen. However, human cells shifted less regularly and completely under the serosal surface. Sinking of the cells could be observed, for instance, between the pancreatic lobules or in the diaphragm. The cellular composition of the peritoneal surface foci in hu-PBL-SCID consisted of many donor lymphocytes and CD11c$^+$ cells. In SCID mice reconstituted with mouse cells the foci contained B, T cells and Mac 1$^+$ cells. The latter belong to the monocyte/macrophage lineage with the morphology of histiocytic cells.

Among the donor-type lymphocytes in the foci of hu-PBL-SCID chimeras, T cells, particularly of the CD8$^+$ type, predominated, whereas in SCID mice reconstituted with mouse lymphocytes, B cells were most frequent. This may be due to the different percentages of B lymphocytes in human PBL (about 10%) and in mouse spleen cell suspensions (about 60%–70%). The peritoneal surface of foci in hu-PBL-SCID were, however, barely vascularized. Their cellular appearance was more similar to soft agar colonies than to lymphatic tissue. The foci expressing ICAM may be held together by cell adhesion, but show comparatively little tissue-like organization. Such relatively loose cell connections are probably the reason why fewer peritoneal foci could be found following a peritoneal lavage. It is conceivable that the compactness and structure of the cell foci may influence cell chimerism and may explain why, in contrast to our allogeneic chimeras T cell chimerism varied considerably and was absent in about one third of more than 50 hu-PBL-SCID (Hoffmann-Fezer et al., manuscript in preparation).

On the other hand the presence of cytokine-producing human histio-monocytic cells, which we found in hu-PBL-SCID foci, may reflect more than what goes on in an *in vitro* soft agar cell colony. They may stimulate surrounding human lymphocytes. IL-1 and IL-6 are known to have some overlapping biological activities. IL-6 is a B cell stimulatory factor as well as a T cell-activating factor or a T cell co-stimulant, which can provide, at least in part, the second signal that is required in addition to antigen or mitogen for T cell activation. IL-6 is able to augment proliferation of monocyte-depleted preparations of stimulated peripheral blood T lymphocytes. Among its other functions, IL-1 promotes proliferation of T lymphocytes, augments the capacity of accessory antigen-presenting cells to activate T cell-dependent immune response, and is a potent inducer of IL-6 [9]. We suppose that a feeder effect of autologous histiocytic cells is necessary for the proliferation of lymphocytes injected into the peritoneal cavity.

Tary-Lehmann [2] described an initial drop and subsequent proliferation of human PBL in the peritoneal cavity. We found the donor lymphocyte population migrating through the diaphragm via lymphatic clefts to be almost devoid of histiocytic cells. Donor lymphocytes did not become more numerous in the peripheral host organs before they had proliferated in the peritoneal sanctuary, from where they migrated into the circulation. Opportunity for an initial i.p. proliferation may explain why i.p., in contrast to i.v., injected human PBL develop hu-SCID chimerism [10, 11].

Older foci had almost completely lost their lymphocyte proliferative capacity. They became granuloma like in appearance, despite the presence of IL6$^+$ and IL-1$^+$ histiocytic cells. Although most of the few human lymphocytes in the splenic white pulp do not express activation markers during the first weeks after injection of human PBL, many lymphocytes in the peritoneal foci already express HLA-DR. They resemble human lymphocytes found in the spleens of hu-PBL-SCID from 4 weeks on after cell transfer, at a time when we observe xenogeneic graft-versus-host reactions in hu-PBL-SCID with substantial T cell chimerism (Hoffmann-Fezer et al., manuscript in preparation). It is, therefore, possible that early lymphocyte activation in the peritoneal foci reflects the triggering of GVHD in the peritoneal sanctuary where we immunohistologically identify CD11c$^+$ cells, which may serve as antigen-presenting cells.

Thus, the peritoneal cavity of SCID mice appears to be the privileged site for induction of even phylogenetically distant xenogeneic lymphopoiesis. As the present study documents, it also harbors xenogeneic cells of the histiomonocytic series. It remains to be determined whether this

makes it also the place of initial immune cell cooperation, where T cells are triggered and B cells are restimulated for the production of human Ig, which is found circulating in the blood [1].

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