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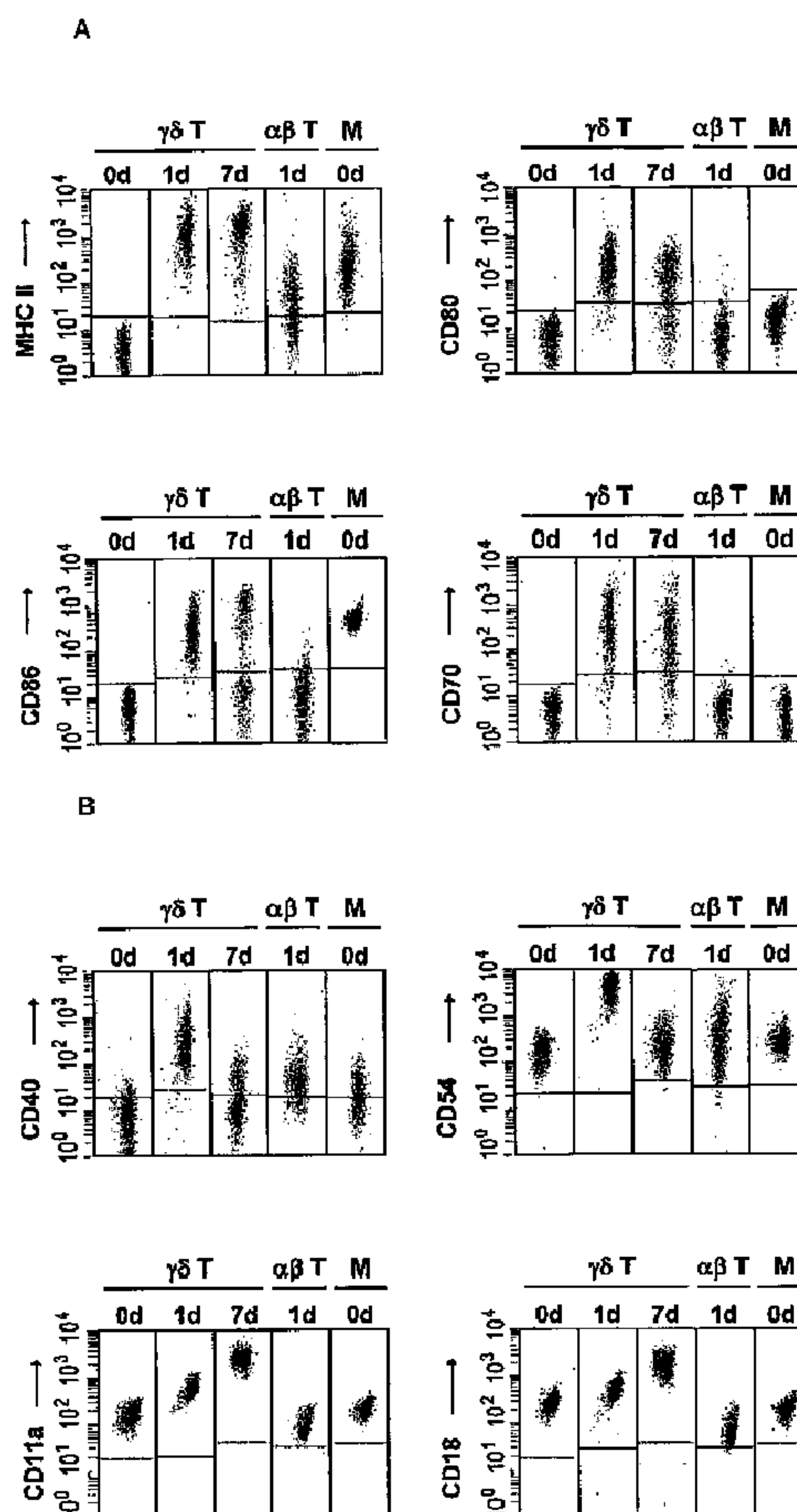
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(54) Titre : PREPARATION DE CELLULES T  $\gamma\delta$  HUMAINES PRESENTATRICES DE L'ANTIGENE ET LEUR  
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(54) Title: PREPARATION OF ANTIGEN-PRESENTING HUMAN  $\gamma\delta$  T CELLS AND USE IN IMMUNOTHERAPY



(57) Abrégé/Abstract:

The invention relates to a method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells, to the  $\gamma\delta$  T cells prepared by such a method, and to their use in immunotherapy, vaccination, vaccine development and diagnostics. Similar to dendritic cells



(57) **Abrégé(suite)/Abstract(continued):**

(DCs) in potency and efficacy, these human  $\gamma\delta$  T cells process antigens and present antigenic peptides to  $\alpha\beta$  T cells and induce antigen-specific responses (proliferation and differentiation) in naïve  $\alpha\beta$  T cells.  $\gamma\delta$  T cells are easily purified from peripheral blood, acquire "maturation" status (expression of essential adhesion, co-stimulatory and major histocompatibility complex molecules) within 1 day of in vitro culture under stimulation and induce strong primary and secondary T helper cell and cytotoxic T cell responses. The  $\gamma\delta$  T cells may be used in a method of treatment of tumors or chronic or recurrent infectious diseases, in identification of novel tumor or pathogen-derived antigens, and in the diagnosis of the immune competence of a patient.

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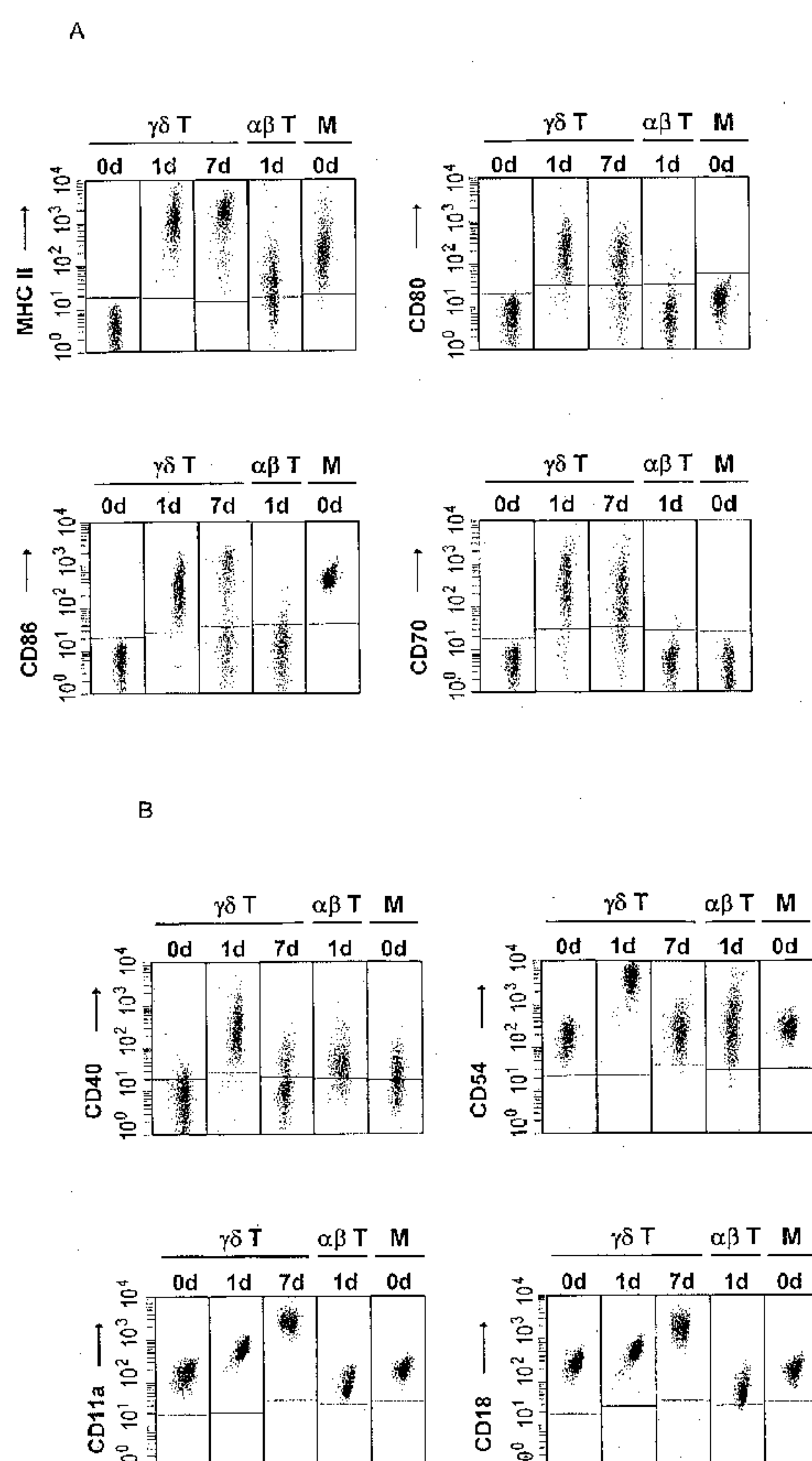
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(54) Title: PREPARATION OF ANTIGEN-PRESENTING HUMAN  $\gamma\delta$  T CELLS AND USE IN IMMUNOTHERAPY



**(57) Abstract:** The invention relates to a method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells, to the  $\gamma\delta$  T cells prepared by such a method, and to their use in immunotherapy, vaccination, vaccine development and diagnostics. Similar to dendritic cells (DCs) in potency and efficacy, these human  $\gamma\delta$  T cells process antigens and present antigenic peptides to  $\alpha\beta$  T cells and induce antigen-specific responses (proliferation and differentiation) in naïve  $\alpha\beta$  T cells.  $\gamma\delta$  T cells are easily purified from peripheral blood, acquire "maturation" status (expression of essential adhesion, co-stimulatory and major histocompatibility complex molecules) within 1 day of *in vitro* culture under stimulation and induce strong primary and secondary T helper cell and cytotoxic T cell responses. The  $\gamma\delta$  T cells may be used in a method of treatment of tumors or chronic or recurrent infectious diseases, in identification of novel tumor or pathogen-derived antigens, and in the diagnosis of the immune competence of a patient.

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## Preparation of antigen-presenting human $\gamma\delta$ T cells and use in immunotherapy

### Field of the invention

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The invention relates to a method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells, to the  $\gamma\delta$  T cells prepared by such a method, and to their use in immunotherapy, antigen identification and diagnosis of immune competence.

### 10 Background of invention

#### *The cellular components of the adaptive immune system*

The cellular components of the immune system are divided into the cells of the innate immune system and the cells of the adaptive (acquired or specific) immune system. Cells  
15 of the innate immune system include (among others) monocytes, granulocytes, natural killer cells in peripheral blood, and mast cells, macrophages and dendritic cells (DCs) in extravascular compartments including peripheral tissues, such as skin, airways, gastrointestinal and urogenital tracts, internal organs as well as secondary lymphoid tissues, such as spleen, lymph nodes (LNs) and Peyer's patches (PPs). The main  
20 functions of innate cells are a) provision of immediate protection by neutralizing and limiting dissemination of infectious particles, and by tumor cell clearing, b) immune surveillance of healthy tissues, and c) initiation of adaptive immune responses. Cells of the adaptive immune system include lymphocytes, such as T and B cells. They are distinguished from innate cells by the presence of clonotypic cell surface antigen  
25 receptors, referred to as T cell antigen receptor (TCR) and B cell antigen receptor (BCR). Each individual lymphocyte carries a distinct TCR or BCR that recognizes a particular antigen. The specificity of antigen recognition is determined by rearrangement of multiple variable TCR or BCR gene segments during T and B cell development and during antigen affinity maturation at the time of effector T and B cell generation. Naïve, antigen-  
30 inexperienced T cells in peripheral blood differ from each other in the antigen-selectivity of their TCRs, and individual naïve T cells become expanded in response to immune activation by agents containing the antigen they are specific for. Consequently, during adaptive immune responses a set of naïve T cells with TCRs specific for the potentially infectious agent becomes expanded *via* cell proliferation, and develops into a) effector  
35 T cells for immediate participation in the defence against the potentially infectious agent,

and into b) memory T cells for long-lasting protection against this particular potentially infectious agent. Effector T cells are short-lived, i.e. disappear during the resolution phase of the immune response, whereas the memory T cells are long-lived and are divided into memory T cell subsets according to their primary tissue residence or preferential recirculation routes (Moser et al., 2004). T cells are further divided into  $\alpha\beta$  T cells and  $\gamma\delta$  T cells (see below) according to the composition of the heterodimeric TCRs;  $\alpha\beta$ -TCRs are composed of  $\alpha$ - and  $\beta$ -protein chains, and  $\gamma\delta$ -TCRs are composed of  $\gamma$ - and  $\delta$ -protein chains. The majority (>80%) of all CD3<sup>+</sup> T cells in a normal, healthy person are  $\alpha\beta$  T cells. TCRs are associated with the invariant CD3 molecule that distinguishes T cells from B cells and all other types of immune cells. The majority of  $\alpha\beta$  T cells recognizes the antigen in a so-called major histocompatibility complex (MHC) molecules-restricted fashion. This is in contrast to BCRs in B cells that directly bind the nominal antigen in a MHC-non-restricted fashion. The term MHC-restriction refers to the mode by which the TCRs recognize their antigens and involves the presentation of antigenic peptides together with MHC molecules, as so-called MHC-peptide complexes, on antigen-presenting cells (APCs), including DCs (see below). There are two major classes of MHC molecules, MHC class I (MHC-I) and MHC class II (MHC-II), which trigger the TCRs of the two major subsets of  $\alpha\beta$  T cells, the CD8<sup>+</sup>  $\alpha\beta$  T cells and CD4<sup>+</sup>  $\alpha\beta$  T cells. The TCRs on CD4<sup>+</sup>  $\alpha\beta$  T cells recognize MHC-II-peptide complexes whereas the TCRs on CD8<sup>+</sup>  $\alpha\beta$  T cells recognize MHC-I-peptide complexes. In striking contrast, the major subset of  $\gamma\delta$  T cells in human peripheral blood does not express CD4 or CD8 and its TCRs do not require MHC-restriction for antigen recognition (see below).

#### *$\gamma\delta$ T cells*

$\gamma\delta$  T cells are a distinct subset of CD3<sup>+</sup> T cells featuring TCRs that are encoded by V $\gamma$ - and V $\delta$ -gene segments (Morita et al., 2000; Carding and Egan, 2002). They are further divided according to their primary residence in blood or tissues, the protein chain composition of their V $\gamma$ V $\delta$ -TCRs and their antigen selectivity. In humans, V $\delta$ 1<sup>+</sup>-TCR chain expressing  $\gamma\delta$  T cells (V $\delta$ 1<sup>+</sup> T cells) predominate in epithelial or epithelia-associated/mucosal tissues of the skin, airways, digestive and urogenital tracts, and several internal organs, and constitute a minor fraction (<20%) of  $\gamma\delta$  T cells in peripheral blood. The TCRs of V $\delta$ 1<sup>+</sup> T cells recognize lipid antigens presented by MHC-related CD1 molecules. Further, V $\delta$ 1<sup>+</sup> T cells respond to stress-associated proteins, including MHC-related molecules MICA and MICB, and heat-shock proteins. They are thought to provide a first-line defence against tumors and otherwise stressed cells and, in addition, are thought to contribute to wound

healing, tissue repair and autoimmunity. In human peripheral blood of healthy individuals  $\gamma\delta$  T cells make up 2-10% of total CD3<sup>+</sup> T cells, and the majority (>80%) of peripheral blood  $\gamma\delta$  T cells are V $\gamma$ 2V $\delta$ 2<sup>+</sup>-TCR chain-expressing  $\gamma\delta$  T cells (V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells) (Morita et al., 2000; Carding and Egan, 2002). They are highly selective for small non-peptide antigens of mostly microbial origin and do not require antigen presentation by classical MHC molecules, as is typical for peptide-selective  $\alpha\beta$  T cells (see above). V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cell antigens include prenyl-pyrophosphates, such as isopentenyl pyrophosphate (IPP), alkyl-amines and metabolites of a newly discovered isoprenoid biosynthesis pathway found in most human microbial pathogens and commensal bacteria (Morita et al., 2000; Eberl et al., 2003). Some of these V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cell antigens, such as IPP and alkyl amines, are also released by necrotic tissue cells. The homologue of human V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells, carrying homologous V $\gamma$ V $\delta$ -TCRs with selectivity for small non-peptide antigens of microbial origin, does also exist in higher primates, such as macaques, but does not exist in rodents, including mice and rabbits. It is not clear why the presence of V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells is limited to higher primates but it could be speculated that they evolved to satisfy the special need for cellular protection against a distinct, species-specific selection of microbes. V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells rapidly expand in response to the model antigen IPP or microbial extracts *in vitro* during tissue culture of peripheral blood V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells, or *in vivo* during vaccination experiments in higher primates, such as macaques (Chen and Letvin, 2003). Also, microbial infections in humans are frequently associated with tremendous expansion of peripheral blood V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells, reaching levels as high as >60% of total peripheral blood CD3<sup>+</sup> T cells. These findings support the notion that human V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells play an important role in immune processes during microbial infections (Morita et al., 2000; Carding and Egan, 2002; Chen and Letvin, 2003). Their unique selectivity for non-peptide antigens that are commonly found in microbes, including pathogens and commensal bacteria, suggest that the TCRs in V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells fulfil a similar function as toll-like receptors (TLR) that trigger activation and maturation of DCs and other APCs in response to diverse ligands of microbial origin.  $\gamma\delta$  T cells contribute to pathogen elimination by rapid secretion of chemokines that initiate the recruitment of cells of the innate immune system and proinflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ ) that stimulate antigen-presenting cells and enhance bacterial killing by granulocytes, macrophages and NK cells (Morita et al., 2000; Carding and Egan, 2002; Chen and Letvin, 2003). They also express natural killer cell receptors for killing of infected or neoplastic tissue cells. These findings support the notion that  $\gamma\delta$  T cells primarily fulfil innate functions, although secretion of pro-inflammatory cytokines, such as

TNF- $\alpha$ , is known also to contribute to local adaptive immune responses. On the other hand, evidence for direct involvement of  $\gamma\delta$  T cells in adaptive immune responses is not clear-cut. For instance, it is reported that CD1-restricted T cells induce maturing in DCs that present CD1-lipid complexes. Also, studies in mice demonstrated a not further explained role for  $\gamma\delta$  T cells in B cell responses, and human  $\gamma\delta$  T cells were shown to regulate B cell responses during *in vitro* co-cultures (Brandes et al., 2003). Finally, studies in macaques demonstrated that V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells were able to mount *in vivo* memory responses to *Mycobacterium bovis* antigens (Chen and Letvin, 2003). Collectively, these findings provide evidence that  $\gamma\delta$  T cells are able to interact with cells of the adaptive immune system, such as B cells and DCs. Most of these immunomodulatory functions were attributed to cytokine production by  $\gamma\delta$  T cells or were left unexplained. Importantly, none of these findings support a role for  $\gamma\delta$  T cells in antigen presentation.

Lymphocyte function is intimately related to the lymphocyte migration potential, as defined by the expression of chemokine receptors and adhesion molecules (Moser et al., 2004). Accordingly,  $\alpha\beta$  T cells are divided into a) naïve T cells expressing the LN-homing chemokine receptor CCR7 but lacking receptors for inflammatory chemokines, b) short-lived effector T cells bearing distinct combinations of chemokine receptors and inducible adhesion molecules that mirror the inflammatory conditions at the site of infection, and c) three subsets of resting, long-lived memory T cells. The distinction of T cell subsets according to their migration potential, i.e. their expression profile of cell surface chemokine receptors and adhesion molecules, correlates well with their state of differentiation and potential function in immune processes. Of note, the “profiling” of chemokine receptors and adhesion molecules is widely used for phenotypic and functional definition of leukocyte subsets, including DCs, and T and B cells (Moser et al., 2004).

The migration properties of human peripheral blood  $\gamma\delta$  T cells differ strikingly from those of human peripheral blood  $\alpha\beta$  T cells (Brandes et al., 2003). Most notably, the majority (>80%) of V $\gamma$ 2V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells (hereafter referred to as “ $\gamma\delta$  T cells”) lacks CCR7 and, thus, is excluded from secondary lymphoid tissues, but features an inflammatory migration profile (Brandes et al., 2003). In clear contrast, the majority (>70%) of  $\alpha\beta$  T cells in peripheral blood express CCR7, which agrees with their continuous recirculation through secondary lymphoid tissues (spleen, LNs, PPs) where they scan APCs for the presence of the appropriate MHC-peptide complexes. In case of ongoing adaptive immune responses, a selection of  $\alpha\beta$  T cells becomes activated during contact with APCs presenting their cognate antigens and differentiates into CCR7-negative effector cells with inflammatory homing potential. By contrast, peripheral blood  $\gamma\delta$  T cells feature an inflammatory

migration program for their immediate tissue mobilization in response to inflammatory chemokines produced at sites of infection. Upon activation, e.g. in response to microbial extract antigens or defined small non-peptide antigens (such as IPP), the migration profile in  $\gamma\delta$  T cells rapidly switches from an inflammatory to a LN-homing phenotype, as

5 evidenced by downmodulation of receptors for inflammatory chemokines and induction of CCR7 (Brandes et al., 2003). By contrast to  $\alpha\beta$  T cells,  $\gamma\delta$  T cells are relatively rare in LNs, which agrees with their distinct mode of activation that is fully independent of MHC-restricting APCs present at these locations (Brandes et al., 2003). The frequency of  $\gamma\delta$  T cells is increased in disease-associated LNs (notably in germinal centers), suggesting

10 that  $\gamma\delta$  T cells may contribute to the initiation of humoral (antibody) responses and possibly other adaptive immune processes.

Collectively, migration characteristics of human  $\gamma\delta$  T cells and their occasional presence in LNs suggest a role for these cells in the initiation of adaptive immune responses. However, this role is not further defined and there is no evidence that  $\gamma\delta$  T cells may

15 function as antigen-presenting cells.

#### *Dendritic cells (DCs)*

DCs form a distinct class of leukocytes, are derived from hematopoietic progenitor cells in the bone marrow, and primarily reside in extravascular sites that include

20 epithelial/mucosal tissues (skin, airways and gastrointestinal/urogenital tracts, among others) and secondary lymphoid tissues (spleen, LNs, PPs) (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). In peripheral blood, DCs or DC precursors make up less than 1% of mononuclear leukocytes. Distinct DC subsets differ in their tissue localization, as exemplified by interstitial DCs that primarily reside in soft

25 tissues bordering epithelia, Langerhans cells (LCs) present in the epidermis and plasmacytoid DCs with homing preferences for LNs. These DC subsets are fully differentiated non-proliferating cells with a limited life-span of several days to several weeks, indicating that they are continuously replaced under steady-state conditions by bone marrow-derived precursors. By contrast, human memory T cells survive for many

30 years and are maintained by means of steady-state (homeostatic) proliferation.

The principal function of tissue-resident DCs is the uptake and processing of local antigens, their relocation *via* afferent lymphatic vessels to draining LNs and the initiation of antigen-specific adaptive immune responses (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). DCs also induce tolerance when antigens are

35 presented to T cells under tolerogenic conditions, i.e. in the absence of pro-inflammatory T cell co-stimulation. Similarly, antigen-presenting B cells have been shown to induce

tolerance (Zhong et al., 1997). Break in the immunological tolerance against self-antigens is thought to be the frequent cause of autoimmune diseases and, thus, tolerogenic DCs presenting self-antigens are essential regulators of immune homeostasis. In healthy peripheral tissues DCs are present in their fully differentiated but “immature” state.

5 Immature DCs express a set of receptors for inflammatory chemokines for quick recruitment to local infection, inflammation or tissue damage. They themselves are poorly immunogenic, i.e. are not capable of inducing primary adaptive immune responses. Instead they are experts in antigen uptake (by means of receptor-mediated endocytic or fluid phase pinocytic mechanisms), antigen processing and peptide loading onto

10 intracellular MHC-I/II molecules and their cell surface presentation. Since immature DCs do not generally express the LN-homing receptor CCR7 it is not known at present how this type of DCs reaches the T cell areas in spleen, LNs and PPs. A multitude of maturation signals, including virus- or bacteria-derived stimuli that trigger toll-like receptors (TLRs), host cell-derived inflammatory mediators (interferon [IFN]- $\gamma$ , tumor

15 necrosis factor [TNF]- $\alpha$ , interleukin [IL]-1, prostaglandin E2 [PGE2], tissue growth factors, among others), and T cell co-stimulatory molecules (CD40-ligand/CD154), induce DC “maturation” (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). During the early phase of DC maturation, DCs secrete high levels of inflammatory chemokines for augmentation of the inflammatory response *via* recruitment of additional

20 immature DCs and cells of the innate immune system (monocytes, granulocytes, natural killer cells). Subsequently, the inflammatory migration program is gradually substituted by a LN-homing migration program characterized by substitution of receptors for inflammatory chemokines with CCR7. CCR7 is essential for efficient relocation of sensitized DCs from peripheral tissues to draining LNs in response to the two CCR7-

25 selective chemokines ELC/CCL19 and SLC/CCL21 present on lymphatic vessels and in the T cell area of spleen, LNs and PPs. Thus, CCR7 expression marks mature or maturing DCs. In addition to LN-homing properties, mature DCs feature stable cell surface expression of MHC-I/II-peptide complexes in large numbers as well as diverse co-stimulatory molecules that are required for proper stimulation of naïve (antigen-

30 inexperienced)  $\alpha\beta$  T cells (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). DCs are also referred to as “professional” APCs because they are capable of stimulating naïve  $\alpha\beta$  T cells during primary immune responses. Memory (antigen-experienced) T cells have a lower activation threshold and, thus, respond to less stringent stimulatory regimens. The functional duality of DCs that distinguishes between

35 the two states of differentiation, a) immature, antigen-processing DCs in peripheral tissues and b) relocated mature, antigen-presenting and co-stimulating DCs in the tissue-draining

5 LNs, is a hallmark of DC physiology and is tightly linked to local inflammation, infection or tissue damage. Finally, the outcome (quality and quantity) of the adaptive immune response is largely determined by the “mode” of response initiation. DCs are known to “instruct” naïve T cells within the T cell area of LNs and PPs about the type of immune response required for pathogen elimination (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). Accordingly, the inflammatory environment in the tissue directly influences DC maturation and, due to DC relocation, also determines T cell differentiation within draining LNs. Distinct effector fates of naïve T cell differentiation include specialized subsets of T helper cells (IFN- $\gamma$ /TNF- $\alpha$ -producing type 1 T helper [Th1] cells, IL-4/IL-5/IL-13-producing Th2 cells, among others), regulatory T cells and cytolytic T cells (CTLs). Effector T cells home to sites of inflammation, rapidly mount effector functions (cytokine secretion, lysis/killing of infected/tumor cells) and have a limited life-span. By contrast, memory T cells are the long-lived product of primary immunization and mount superior immune responses against recall antigens.

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#### *Dendritic cells in immunotherapy*

DCs are “nature’s adjuvant”, i.e. constitute the most expert cellular system for induction of protective immune responses, and, therefore, are being developed for use in human immunotherapy (Fong and Engleman, 2000; Steinman et al., 2003; Schuler et al., 2003; Figdor et al., 2004). Potential applications include cancer therapy, vaccination against pathogens (such as human immunodeficiency virus [HIV]-1 and hepatitis C virus) and treatment of autoimmune diseases. Current DC therapy protocols include

20 1. Isolation and purification of DC precursors from patients’ blood (bone marrow-derived CD34<sup>+</sup> hematopoietic precursors or peripheral blood CD14<sup>+</sup> or CD11c<sup>+</sup> cells).

25 2. Generation of DCs during *in vitro* cell culture.

3. *In vitro* antigen loading for peptide-presentation by mature DCs.

4. Treatment of patients with single or repeated injections of peptide-presenting DCs.

Currently, the application of DCs in immunotherapy faces several problems (Fong and Engleman, 2000; Steinman et al., 2003; Schuler et al., 2003; Figdor et al., 2004). In brief, DC precursors are scarce in peripheral blood and do not proliferate during *in vitro* culture, necessitating repeated manipulation with large blood samples from patients. DCs are functionally heterogeneous and may induce opposing or unwanted effects, e.g. immune suppression instead of effector T cell generation. Also, DCs are functionally instable and go through a preset sequence of irreversible differentiation steps ending in compromised (“exhausted”) immune functions. This causes great difficulties in generating functionally homogeneous DC preparations by *in vitro* manipulations. Finally, the generation of

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peptide-presenting DCs for use in immunotherapy is technically demanding, time consuming and costly.

5 Summary of invention

The present invention describes the simple isolation and *in vitro* preparation of antigen-presenting human  $\gamma\delta$  T cells and their use as efficient antigen-presenting cells (APCs) in immunotherapy. Similar to dendritic cells (DCs) in potency and efficacy, human  $\gamma\delta$  T cells process antigens and present antigenic peptides to  $\alpha\beta$  T cells and induce antigen-specific responses (proliferation and differentiation) in naïve  $\alpha\beta$  T cells.  $\gamma\delta$  T cells are relative frequent in peripheral blood (2-10% of CD3<sup>+</sup> T cells), are easily purified from peripheral blood by diverse simple techniques, acquire “maturation” status (expression of MHC-II, and essential adhesion and co-stimulatory molecules) within 1 day of *in vitro* culture under simple stimulatory conditions, maintain efficient antigen-presenting functions over 7 days or more of *in vitro* culture and induce strong primary and secondary T helper cell responses. Furthermore,  $\gamma\delta$  T cells are readily expanded during *in vitro* culture for storage and later use.

20 The invention relates to a method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells comprising selecting  $\gamma\delta$  T cells out of human peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of antigen-presenting functions, and applying the antigen to these cells (either before, during or after induction of antigen-presenting functions), to the efficient antigen-presenting human  $\gamma\delta$

25 T cells prepared by such a method, their use in immunotherapy and in the manufacture of a medicament for use in immunotherapy, to a method of treatment of tumors or chronic or recurrent infectious diseases with such efficient antigen-presenting human  $\gamma\delta$  T cells, to a method of vaccination against tumors or agents inducing infectious or non-infectious diseases with  $\gamma\delta$  T cell-targeting vaccines and to the use of such  $\gamma\delta$  T cell-targeting

30 vaccines in the preparation of a medicament, to a method of identification of novel tumor or pathogen-derived antigens, and to a method of diagnosing the immune competence of patients using such efficient antigen-presenting human  $\gamma\delta$  T cells.

Brief description of the figures

*Fig. 1 (1A and 1B). Stimulated  $\gamma\delta$  T cells exhibit numerous antigen-presentation, co-stimulation and adhesion molecules.*

- 5 Examination of cell surface molecules by flow cytometry is performed with  $\gamma\delta$  T cells ( $\gamma\delta$  T), either freshly isolated from peripheral blood or after stimulation with IPP and *in vitro* culture for 1 or 7 days, or with  $\alpha\beta$  T cells ( $\alpha\beta$  T) after 1 day stimulation with anti-CD3/CD28, or with freshly isolated peripheral blood monocytes (M). The numbers above the individual dot-blot refer to the time of *in vitro* culture; (0d) freshly isolated, (1d) 1 or  
 10 (7d) 7 days. Positivity is defined by staining with isotype-matched control antibodies, and horizontal lines represent the gates for 99% background stainings.

*Fig. 2. Stimulated  $\gamma\delta$  T cells express functional CCR7 and respond to the LN chemokine SLC/CCL21.*

- 15 The data are taken from Brandes et al., 2003. A) Chemotaxis responses are examined in  $\gamma\delta$  T cells after stimulation for 36 hours with IPP, and expressed as the fraction of cells (%), i.e. % of total input cells, that have migrated in response to the indicated chemokines. C = Control (blank). B) CCR7 expression is determined by flow cytometry, n = cell counts. Solid and broken lines refer to CCR7 expression on IPP-stimulated (see chemotaxis) and  
 20 resting peripheral blood  $\gamma\delta$  T cells, respectively; filled histogram depicts control staining with isotype antibody.

*Fig. 3. Rapid and extensive aggregate formation between stimulated  $\gamma\delta$  T cells and naïve  $\alpha\beta$  T cells.*

- 25 1 day stimulated  $\gamma\delta$  T cells are cultured together with autologous, naïve CD4<sup>+</sup>  $\alpha\beta$  T cells at the ratio of 1:5 for 3 hours ( $\gamma\delta$  T, 2 examples). As positive and negative control, mature monocyte-derived DCs (DC) and 1 day stimulated  $\alpha\beta$  T cells ( $\alpha\beta$  T), respectively, are mixed with autologous naïve  $\alpha\beta$  T cells. All cells are isolated from the same donor to exclude antigenic interactions. Naïve CD4<sup>+</sup>  $\alpha\beta$  T cells are loaded with CFSE for  
 30 identification by fluorescence microscopy. The phase-contrast and fluorescence images of live cells are taken with a Zeiss-Axiovert 35 inverted microscope and images are combined in overlays.

*Fig. 4. Stimulated  $\gamma\delta$  T cells induce potent TT-specific proliferation responses in resting  $CD4^+$   $\alpha\beta$  T cells.*

$\gamma\delta$  T cells are stimulated for 1 day in the presence of 20  $\mu\text{g/ml}$  TT, washed and then added to CFSE-labeled TT-specific  $\alpha\beta$  T cells at a ratio of 1:2. After 4 (4d) and 6 (6d) days of culture, the CFSE signals in  $CD4^+$  cells are examined by flow cytometry ( $\gamma\delta\text{T}(+\text{TT})$ ). As a positive control, monocyte-derived DCs are matured in the presence of 20  $\mu\text{g/ml}$  TT (DC(+TT)) and used to stimulate  $\alpha\beta$  T cells at a ratio 1:10 under the same conditions as TT-presenting  $\gamma\delta$  T cells. As negative control,  $\alpha\beta$  T cells are co-cultured with  $\gamma\delta$  T cells ( $\gamma\delta\text{T}(-\text{TT})$ ) and DCs (DC(-TT)), which are stimulated and matured, respectively, in the absence of TT. The horizontal bars above histograms indicate the positions of CFSE signals in undivided (non-responsive) cells; n = cell counts.

*Fig. 5. Proliferation of TT-specific  $CD4^+$   $\alpha\beta$  T cells requires contact with TT-presenting  $\gamma\delta$  T cells.*

Preparation of TT-presenting  $\gamma\delta$  T cells and CFSE-labeled responder cells as well as data analysis are performed exactly as described in Fig. 4. In a two-chamber tissue culture system, TT-presenting  $\gamma\delta$  T cells and TT-specific responder ( $CD4^+$   $\alpha\beta^+$  T) cells are added to the lower chamber (B), and responder cells alone are added to the upper chamber (A). The two chambers are separated by a porous membrane allowing the free exchange of soluble proteins but not intact cells. Horizontal bars above histograms indicate the gates for undivided CFSE-labeled cells.

*Fig. 6. TT-presenting  $\gamma\delta$  T cells induce TCR down-modulation in TT-specific  $CD4^+$   $\alpha\beta$  T cells.*

$\gamma\delta$  T cells and DCs are stimulated and matured, respectively, in the presence of increasing concentrations of TT, ranging from 0 (no TT added) to 1'000  $\mu\text{g/ml}$  and then cultured with resting TT-specific  $CD4^+$   $\alpha\beta$  T cells at a APC:responder cell ratio of 1:2. After 18 hours, the level of CD3 expression on  $\alpha\beta$  T cells is determined by flow cytometry and shown in % of mean fluorescence intensity (MFI) compared to cells without TT added. Preparation of TT-presenting  $\gamma\delta$  T cells and responder cells as well as data analysis are performed exactly as described in Fig. 4.

*Fig. 7. TT-presenting  $\gamma\delta$  T cells induce the expression of activation markers on TT-specific  $CD4^+$   $\alpha\beta$  T cells.*

The level of the activation markers CD25, ICOS and OX40 on TT-specific  $CD4^+$   $\alpha\beta$  T cells (responder cells) is determined by flow cytometry after culture for 5 days with  $\gamma\delta$  T cells that are stimulated in the presence (+TT) or absence (-TT) of 10  $\mu\text{g/ml}$  TT (see Fig. 4). Open and filled histograms show the fluorescence stainings with specific and control isotype antibodies, respectively; n = cell counts. Horizontal bars represent the gates for marker-positive cells, and the numbers above the histograms indicate the degree of positivity, expressed as percent positive cells (%) and mean fluorescence intensity (MFI).

*Fig. 8. Heterologous EBV-B cells and TT-specific  $CD4^+$   $\alpha\beta$  T cells do not functionally present TT to TT-specific responder cells.*

CP.EBV cells, responder cells (TT-specific  $CD4^+$   $\alpha\beta$  T cells) and  $\gamma\delta$  T cells (as positive control) are cultured for 1 day in the presence of 20  $\mu\text{g/ml}$  TT, washed, irradiated and then added to TT-specific  $CD4^+$   $\alpha\beta$  T cells at a ratio of 1:2. After 3 (3d) and 8 (8d) days of culture, the CFSE signals in  $CD4^+$  cells are examined by flow cytometry. CP.EBV and responder cell preparation, CFSE-labeling and flow cytometry are described in "Examples".

*Fig. 9. Stimulated  $\gamma\delta$  T cells are also efficient in processing and presenting complex protein antigens (PPD).*

$\gamma\delta$  T cells and DCs are stimulated/matured in the presence (+PPD) or absence (-PPD) of 20  $\mu\text{g/ml}$  PPD and analyzed for induction of proliferation in resting autologous PPD-specific  $CD4^+$   $\alpha\beta$  T cells. As additional negative control, PPD-specific  $CD4^+$   $\alpha\beta$  T cells are cultured in the absence of  $\gamma\delta$  T cells or DCs but in the presence of 20  $\mu\text{g/ml}$  PPD. The experimental set-up is identical to the one used in Fig. 4.  $\gamma\delta$  T cells and DC preparation, CFSE-labeling of PPD-specific  $CD4^+$   $\alpha\beta$  T cells and flow cytometry are described in "Examples".

*Fig. 10. TSST-1-loaded  $\gamma\delta$  T cells induce proliferation in autologous naïve  $CD4^+$   $\alpha\beta$  T cells.*

Stimulated  $\gamma\delta$  T cells ( $\gamma\delta$  T), stimulated  $CD4^+$   $\alpha\beta$  T cells ( $\alpha\beta$  T), mature DCs (DC) and freshly isolated blood monocytes (M) are loaded with 10 ng/ml TSST-1 and mixed with freshly isolated, CFSE-labeled naïve  $CD4^+$   $\alpha\beta$  T cells at a ratio of 1:5. After 4 days of culture the proliferation responses in  $V\beta 2^+$ - $\alpha\beta$  T cells ( $V\beta 2$ ) are determined by flow

cytometry. Vertical and horizontal lines in the dot-blots define the gates for  $V\beta 2^+$ - $\alpha\beta$  T cells and divided cells; the left-upper and left-lower quadrants show divided  $V\beta 2^+$ -cells and divided  $V\beta 2^{\text{neg}}$ -cells, respectively, and the right-upper and right-lower quadrants show undivided  $V\beta 2^+$ -cells and undivided  $V\beta 2^{\text{neg}}$ -cells; the numbers refer to the percent of total  
 5 cells present within the individual quadrants. Cell preparation, TSST-1-loading and flow cytometry are performed according to "Examples".

*Fig. 11. TSST-1-loaded  $\gamma\delta$  T cells demonstrate potent APC functions (titration of TSST-1).* Stimulated  $\gamma\delta$  T ( $\gamma\delta$  T) and  $\alpha\beta$  T cells ( $\alpha\beta$  T), dendritic cells (DC) and monocytes (M) are  
 10 loaded with increasing concentrations of TSST-1 and mixed with CFSE-labeled naïve  $CD4^+$   $\alpha\beta$  T cells at a ratio of 1:5. After 4 days of culture the number of divided  $V\beta 2^+$ - $\alpha\beta$  T cells per culture well ( $V\beta 2^+$  T) are determined by flow cytometry (see upper-left quadrants in Fig. 10). The antigen-presenting cells tested are those shown in Fig. 10 and, in addition, stimulated  $\gamma\delta$  T cells that are cultured for 7 days and then loaded with varying  
 15 concentrations of TSST-1 ( $\gamma\delta$  T 7d). Each data point plus error bar represents the mean  $\pm$  standard deviation (SD) of duplicate values from 2 separate experiments and data are representative of 3 independent experiments. Cell preparation, TSST-1-loading and flow cytometry are performed according to "Examples".

20 *Fig. 12. TSST-1-loaded  $\gamma\delta$  T cells demonstrate potent APC functions (titration of APCs).* Stimulated  $\gamma\delta$  T cells and mature DCs are loaded with either 1  $\mu\text{g/ml}$  or 100  $\text{ng/ml}$  TSST-1 and tested at various dilutions (1:5 to 1:200) of APC: $\alpha\beta$  T cells (APC:  $\gamma\delta$  T cells or mature DCs) for induction of proliferation in naïve  $CD4^+$   $\alpha\beta$  T cells. Cell preparation, TSST-1-loading and flow cytometry are performed according to "Examples".

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*Fig. 13 (13A and 13B). TSST-1-loaded  $\gamma\delta$  T cells induce T helper cell differentiation in naïve  $CD4^+$   $\alpha\beta$  T cells.*

Stimulated  $\gamma\delta$  T cells,  $\alpha\beta$  T cells or mature DCs loaded with 10  $\text{ng/ml}$  TSST-1 are mixed with naïve  $CD4^+$   $\alpha\beta$  T cells, and  $V\beta 2^+$  responder cell proliferation is examined as  
 30 described in Fig. 10. After 4 days of culture, expression of the memory marker CD45RO and the extent of cell division are determined by flow cytometry in  $V\beta 2^+$  responder cells. After 21 days of culture, when the cells return to a resting, non-proliferating state, cells are stimulated by PMA/ionomycin and examined by flow cytometry for the production of the intracellular cytokines IL-4 and IFN- $\gamma$  (Fig. 13 B). The numbers in the left-upper, right-  
 35 upper and right-lower quadrants define the fraction (percent of total cells) of Th2, Th0 and

Th1 cells generated, respectively, and the numbers above the individual dot-blots refer to the ratio of APCs to responder cells at the beginning of the cell cultures. Cell preparation, TSST-1-loading and determination of cytokine production are described in "Examples".

5 *Fig. 14. Induction of proliferation in naive CD4<sup>+</sup> αβ T cells by TSST-1-loaded γδ T cells is cell contact-dependent.*

Stimulated γδ T cells are loaded with 100 ng/ml TSST-1 and CFSE-labeled naive CD4<sup>+</sup> αβ T cells are cultured alone (A) or together (B) with γδ T cells at an APC:responder cell ratio of 1:5. The two-chamber culture system and the CFSE flow cytometry data analysis  
10 is described in Fig. 5.

*Fig. 15. γδ T cells induce primary CD8<sup>+</sup> T cell responses.*

Mixed-leukocyte responses with naïve, CD8<sup>+</sup> αβ T cells and heterologous IPP-stimulated γδ T cells (circles), LPS-matured, monocyte-derived DCs (squares) or superantigen-stimulated αβ T cells (triangles) at decreasing APC:responder cell ratios (representative of  
15 6 experiments). Proliferation responses in CFSE-labeled CD8<sup>+</sup> responder cells were assessed by flow cytometry data analysis as described in Fig. 5.

*Fig. 16. γδ T cells induce differentiation of naive CD8<sup>+</sup> T cells into cytotoxic T cells.*

20 CD8<sup>+</sup> T cells derived from mixed-leukocyte responses, as described in Fig. 15, were examined after 14 days of culture for cytolytic activity. The cytotoxicity assay included effector cells, i.e. CD8<sup>+</sup> T cells derived from mixed-leukocyte responses, and CFSE-labeled target cells. The mixture of target cells contained true target cells (heterologous CD4<sup>+</sup> T cells) and negative control target cells (autologous CD4<sup>+</sup> T cells) at a 1:1 ratio.  
25 True and negative control target cells were labeled with different concentrations of CFSE before mixing in order to discriminate between the two target cell subsets.  
(A) Flow cytometric analysis of the target cell mixture in the absence of effector cells shows the negative control (C) and true target (T) cell populations.  
(B) After 12 h of co-culture of CD8<sup>+</sup> T cells and target cells at ratios of 30:1, 10:1, 3:1, and  
30 1:1, the degree of target cell lysis was assessed by measuring the decrease in number in the true target cell population (arrow). The numbers by the arrows refer to percent specific killing and relate to loss of cell counts with high CFSE fluorescence (true target cells) as compared to control cell counts with low CFSE fluorescence.

Left column: CD8<sup>+</sup> T cells were derived from mixed-leukocyte responses with IPP-stimulated γδ T cells as APCs.  
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Right column: CD8<sup>+</sup> T cells were derived from mixed-leukocyte responses with mature DCs as APCs.

*Fig. 17.  $\gamma\delta$  T cells express high levels of the endocytic cell surface proteins DEC205 (CD205) and CD11b.*

5  $\gamma\delta$  T cells freshly isolated from peripheral blood ( $\gamma\delta$  T) and immature DCs (DC) were analyzed for CD205 and CD11b expression by flow cytometry as described in Fig. 7. Vertical and horizontal lines in the dot-blots figure define the gates for CD205 or CD11b-expressing cells, and the right-upper quadrants in the figure depict cells double-positive  
10 for CD205 and CD11b; the numbers refer to the percent of total cells present within the individual quadrants. Positivity is defined by staining with isotype-matched control antibodies, and horizontal or vertical lines represent the gates for 99% background stainings.

15 Detailed description of the invention

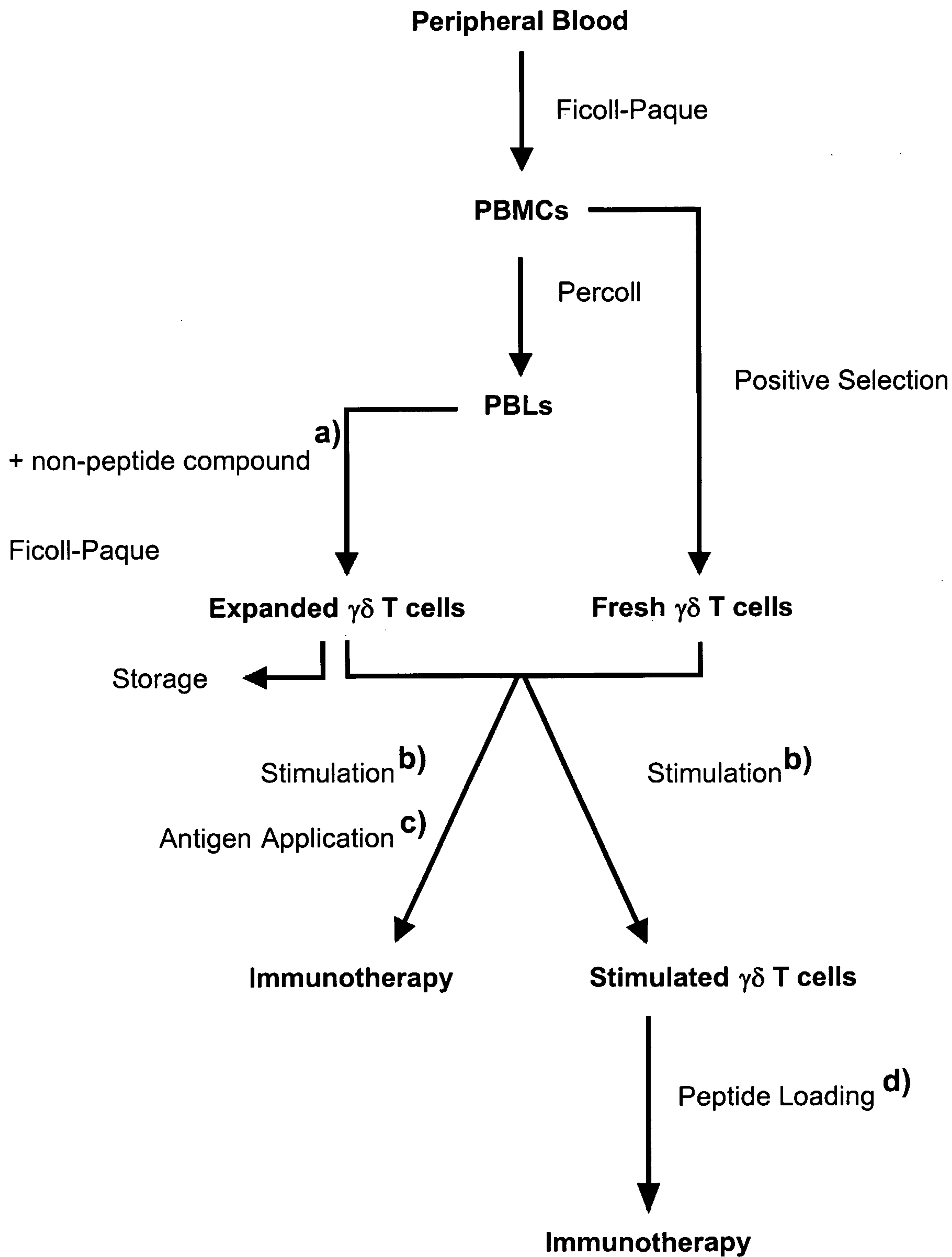
The flow diagram of the Scheme summarizes the method of invention for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells ( $V\gamma 2V\delta 2^+$   $\gamma\delta$  T cells, hereafter referred to as " $\gamma\delta$  T cells") by isolation and *in vitro* treatment of human peripheral blood  $\gamma\delta$  T cells.  
20 Although protocols for isolation and *in vitro* proliferation of  $\gamma\delta$  T cells are known as such, the particular combination of stimulation and antigen application or peptide loading has not been described. Starting material is human peripheral blood that is processed by differential centrifugation or immunoabsorption to yield expanded or freshly isolated  $\gamma\delta$  T cells, respectively, at high purity. Short-term (e.g. 24 hours) stimulation in  
25 combination with antigen application or peptide loading results in  $\gamma\delta$  T cells with efficient antigen-presenting function for use in immunotherapy.

a)  $\gamma\delta$  T cells are readily expanded during culture of peripheral blood lymphocytes (PBLs) in the presence of isopentenyl pyrophosphate (IPP) or other small molecular weight non-peptide compounds with selectivity for  $\gamma\delta$  T cells as listed hereinbelow (Morita et al., 2000;  
30 Eberl et al., 2003). IPP or other small molecular weight non-peptide compounds with selectivity for  $\gamma\delta$  T cells are also used to induce antigen-presenting functions in selected  $\gamma\delta$  T cells as described hereinbelow. After 10-21 days in culture the vast majority of live, expanded cells are ( $V\gamma 2V\delta 2^+$ )  $\gamma\delta$  T cells, which are separated from dead cells by Ficoll-  
35 Paque centrifugation and used immediately for antigen-presenting cell (APC) generation

or stored in liquid nitrogen. Alternatively, as described in "Examples",  $\gamma\delta$  T cells are directly isolated from peripheral blood mononuclear cells (PBMCs) by means of positive selection.

Scheme: Preparation of efficient antigen-presenting human  $\gamma\delta$  T cells

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b) Isolated fresh or expanded  $\gamma\delta$  T cells are stimulated for a short period (e.g. 12 to 96 hours) with ( $V\gamma 2V\delta 2^+$ )  $\gamma\delta$  T cell-selective compounds or phytohemagglutinin (PHA) or other stimuli as listed hereinbelow for induction of antigen-uptake, of presentation function and of expression of co-stimulatory molecules.

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c)  $\gamma\delta$  T cells are treated before, during or after stimulation (b) with antigens resulting in antigen-presenting  $\gamma\delta$  T cells. This antigen application includes a variety of independent approaches, such as (among others) addition of complex extracts or defined proteins from tumors, microbes or viral-infected cells or DNA/RNA encoding such pathogen-derived proteins, either in the form of purified nucleic material or packaged in expression vectors or attenuated viruses for transfection/transduction of  $\gamma\delta$  T cells and endogenous expression and processing of microbe-, pathogen- or tumor cell-derived antigens.

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d) Instead of adding antigen (in the form of protein or DNA/RNA) to the  $\gamma\delta$  T cells during the short-term stimulation procedure (combinations of b and c), these steps may be performed sequentially. Stimulated  $\gamma\delta$  T cells are then "loaded" for a short period of time with defined peptide antigens that do not require intracellular proteolytic processing.

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In addition to IPP, alternative small molecular weight non-peptide antigens with selectivity for ( $V\gamma 2V\delta 2^+$ )  $\gamma\delta$  T cells considered in step (a) are (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), ethyl pyrophosphate (EPP), farnesyl pyrophosphate (FPP), dimethylallyl phosphate (DMAP), dimethylallyl pyrophosphate (DMAPP), ethyl-adenosine triphosphate (EPPPA), geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP), isopentenyl-adenosine triphosphate (IPPPA), monoethyl phosphate (MEP), monoethyl pyrophosphate (MEPP), 3-formyl-1-butyl-pyrophosphate (TUBAg 1), X-pyrophosphate (TUBAg 2), 3-formyl-1-butyl-uridine triphosphate (TUBAg 3), 3-formyl-1-butyl-deoxythymidine triphosphate (TUBAg 4), monoethyl alkylamines, allyl pyrophosphate, crotyl pyrophosphate, dimethylallyl- $\gamma$ -uridine triphosphate, crotyl- $\gamma$ -uridine triphosphate, allyl- $\gamma$ -uridine triphosphate, ethylamine, isobutylamine, sec-butylamine, iso-amylamine and nitrogen containing bisphosphonates. IPP is preferably applied as presented by B cells or substitutes.

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Positive selection is, for example, performed by adding antibodies to human  $V\gamma 2V\delta 2$ -TCRs to human peripheral blood mononuclear cells, e.g. 1-3 hours incubation, followed by magnetic cell sorting. Alternatively, positive selection may be performed by adding

antibodies to human V $\gamma$ V $\delta$ -TCRs to human peripheral blood mononuclear cells (pan- $\gamma\delta$  T cell selection) followed by magnetic cell sorting.

(V $\gamma$ 2V $\delta$ 2<sup>+</sup>)  $\gamma\delta$  T cell-selective compounds useful as stimuli for induction of antigen-presenting functions in Step (b) are IPP and other non-peptide compounds as listed above for step (a), e.g. 4-hydroxy-3-methyl-but-2-enyl pyrophosphate or other related microbial metabolites. Alternatively, PHA or other substitutes are useful as stimuli for induction of antigen-uptake, of presentation function and of expression of co-stimulatory molecules.

10 Stimulated cells may, if desirable, be irradiated in order to inhibit  $\gamma\delta$  T cell proliferation.

Examples of antigens applied as proteins in step (c) and/or (d) are those currently used in immunotherapy protocols employing DCs as antigen-presenting cells and are related to the therapeutic targets, e.g. tumor cells, infectious agents (microbes including viruses, bacteria, yeasts, and parasites), and pathogen-associated toxins. Such antigens include (but are not limited to) tumor-associated antigens (tumor-specific metabolic, structural and cell surface proteins, and the like); virus-associated antigens (virus-encoded envelope, structural, metabolic and enzymatic proteins, e.g. HIV gp120 proteins of different viral clades, HIV Tat, HIV proteases, e.g. envelope, structural, metabolic and enzymatic proteins derived from hepatitis viruses, influenza viruses, human cytomegalovirus, polio viruses, rabies viruses, herpes viruses, among others); bacteria-associated antigens (bacteria-encoded cell wall, structural, metabolic and enzymatic proteins, and the like, e.g. those derived from *Mycobacteria* (e.g. *M. tuberculosis*, *M. leprae*), *Listeria monocytogenes*, *Pneumococci*, *Staphylococci* (e.g. *S. aureus*), *Streptococci* (e.g. *S. pyogenes*, *S. pneumoniae*), *Vibrio cholerae*, *Clostridium tetani*, among others); yeast and fungi-associated antigens (yeast/fungi-encoded cell wall, structural, metabolic and enzymatic proteins, and the like, e.g. those derived from *Candida albicans*, *Aspergillus fumigatus*, among others); and pathogen-derived toxins (bacteria enterotoxins, e.g. staphylococcal enterotoxins, toxic shock syndrome toxin, tetanus toxins, among others).

30 Examples of antigens applied as proteins also include those related to bacteria causing increased resistance to antibiotic treatment and those microorganisms causing life-threatening diseases world-wide (e.g. *Plasmodia* (for example *P. falciparum*, *P. vivax*, *P. malariae*), *Leishmania*, *Trypanosoma*, *Entamoeba*, *Schistosoma*, *Filaria*, among others). Antigens applied in step (c) in the form of RNA/DNA include those currently used in blood and tissue cell transfection protocols and include (but are not limited to) those encoding

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proteins from tumor cells, infectious agents (microbes including viruses, bacteria, yeasts, and parasites), and pathogen-associated toxins, as listed above.

“Efficient” as used in “efficient antigen-presenting human  $\gamma\delta$  T cells” means that the antigen-presenting functions of such cells are comparable to the corresponding antigen-presenting functions of DCs. “Efficient” is e.g. at least 10% as effective as with DCs under comparable conditions, the difference being a result of different cell morphology, including cell shape and surface area.

Short-term stimulated  $\gamma\delta$  T cells uniformly express the chemokine receptor CCR7, which is a prerequisite for homing of APCs to lymph nodes and Peyer’s patches and, consequently, is a critical factor for initiation of adaptive immune responses. Resting peripheral blood  $\gamma\delta$  T cells do not express MHC-II molecules and, therefore, are not capable of presenting peptide antigens in a tolerogenic fashion. This issue of “safety” puts  $\gamma\delta$  T cells apart from other APCs, such as DCs and B cells, with known tolerance induction properties. Applications of antigen-presenting  $\gamma\delta$  T cells include, among others, the induction and/or improvement of immune responses against tumors in the treatment of tumor patients and against microbes and viruses in the treatment of patients with chronic infections or with inappropriate immune competence. In addition, antigen-presenting  $\gamma\delta$  T cells can be used for identification of novel tumor and otherwise pathogen-derived antigens with strong immunogenic properties for potential application in immunotherapy. Eventually, the application of  $\gamma\delta$  T cells to monitor the adaptive immune competence (status of immune competence) of immune-suppressed individuals (among others) is described.

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The invention is now described in more detail with reference to the accompanying figures, which give ample proof of the efficiency of the method of the invention.

Fig. 1 describes the expression of cell surface molecules on  $\gamma\delta$  T cells, immediately after isolation from peripheral blood or after stimulation and culture for 1 or 7 days. For comparison, the same cell surface molecules are also examined on 1 day stimulated  $\alpha\beta$  T cells and freshly isolated monocytes. The cell surface molecules shown in Fig. 1 include MHC-II; the co-stimulatory molecules CD80 and CD86, the two selective ligands for the receptor CD28 present on naïve and memory T cells; CD70, the ligand for CD27; CD40, the receptor for CD154/CD40-ligand as well as the adhesion molecules

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CD54/ICAM-1, CD11a/ $\alpha$ L-integrin, and CD18/ $\beta$ 2-integrin, which are involved in cell-to-cell contact. These data demonstrate that freshly isolated  $\gamma\delta$  T cells have moderate levels of adhesion molecules but lack MHC-II and co-stimulatory molecules that are essential for the activation and differentiation of naïve T cells. By contrast, short-term (1 day) stimulation of  $\gamma\delta$  T cells results in very high levels of expression of MHC-II and co-stimulatory molecules and further enhance expression of adhesion molecules. Of note, and with the exception of CD40 and CD54, the levels of these cell surface molecules are maintained or further enhanced during culture of  $\gamma\delta$  T cells for 7 days. In clear contrast, the same series of molecules (with the exception of CD86 on monocytes) are moderately expressed or absent on  $\alpha\beta$  T cells and monocytes.

The numbers in Table 1 summarize the results from an extensive investigation of the expression of cell surface molecules on  $\gamma\delta$  T cells and, for comparison, on  $\alpha\beta$  T cells, monocytes and mature DCs. The list includes the examples shown in Fig. 1 as well as additional cell surface molecules. The numbers indicate the average expression levels, expressed as the mean of the mean fluorescence intensity (MFI), and the corresponding standard deviations (SD). Note the striking positivity of MHC-II (HLA-DR), co-stimulatory and adhesion molecules on activated  $\gamma\delta$  T cells that resembles mature DCs but exceeds by far the levels seen in activated  $\alpha\beta$  T cells and monocytes.

Fig. 2 and Table 2 correspond to previous results published by Brandes et al., 2003, and are included here to describe additional features of activated human  $\gamma\delta$  T cells that are pertinent to this invention. Table 2 lists the expression levels of chemokine receptors that differ clearly between freshly isolated peripheral blood  $\gamma\delta$  T cells and stimulated  $\gamma\delta$  T cells.  $\gamma\delta$  T cells in peripheral blood express chemokine receptors and adhesion molecules (shown in Fig. 1) for rapid recruitment to sites of inflammation and infection whereas short-term activation of  $\gamma\delta$  T cells partially inhibits these inflammatory migration properties (i.e. downmodulation of CCR2 and CCR5) and instead rapidly induces CCR7 expression for efficient homing to T cell areas of spleen, LNs and PPs.

Table 1. Expression levels of cell surface molecules on human  $\gamma\delta$  T cells.

|               | <u>fresh <math>\gamma\delta</math> T cells</u><br><b>MFI <math>\pm</math> SD (n) <sup>a</sup></b> | <u>1 day <math>\gamma\delta</math> T cells</u><br><b>MFI <math>\pm</math> SD (n)</b> | <u>7 days <math>\gamma\delta</math> T cells</u><br><b>MFI <math>\pm</math> SD (n)</b> | <u>1 day <math>\gamma\delta</math> T cells</u><br><b>MFI <math>\pm</math> SD (n)</b> | <u>fresh monocytes</u><br><b>MFI <math>\pm</math> SD (n)</b> | <u>8 h LPS DC</u><br><b>MFI <math>\pm</math> SD (n)</b> |
|---------------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------|---------------------------------------------------------|
| <b>HLA-DR</b> | 8 $\pm$ 13 (4)                                                                                    | 1738 $\pm$ 229 (4)                                                                   | 2426 $\pm$ 1416 (4)                                                                   | 368 $\pm$ 200 (8)                                                                    | 743 $\pm$ 298 (4)                                            | 1873 $\pm$ 142 (3)                                      |
| <b>CD80</b>   | 0 $\pm$ 0 (3)                                                                                     | 157 $\pm$ 74 (3)                                                                     | 115 $\pm$ 30 (4)                                                                      | 3 $\pm$ 22 (8)                                                                       | 2 $\pm$ 2 (6)                                                | 109 $\pm$ 90 (3)                                        |
| <b>CD86</b>   | 2 $\pm$ 1 (4)                                                                                     | 499 $\pm$ 249 (4)                                                                    | 412 $\pm$ 256 (6)                                                                     | 89 $\pm$ 89 (12)                                                                     | 333 $\pm$ 130 (5)                                            | 299 $\pm$ 325 (2)                                       |
| <b>CD70</b>   | 0 $\pm$ 0 (4)                                                                                     | 293 $\pm$ 191 (3)                                                                    | 422 $\pm$ 229 (3)                                                                     | 11 $\pm$ 5 (6)                                                                       | 0 $\pm$ 0 (4)                                                | 0 $\pm$ 0 (2)                                           |
| <b>CD54</b>   | 179 $\pm$ 44 (3)                                                                                  | 3589 $\pm$ 646 (3)                                                                   | 511 $\pm$ 116 (3)                                                                     | 949 $\pm$ 529 (6)                                                                    | 602 $\pm$ 194 (3)                                            | 1272 $\pm$ 605 (2)                                      |
| <b>CD11a</b>  | 274 $\pm$ 90 (3)                                                                                  | 651 $\pm$ 86 (2)                                                                     | 2663 $\pm$ 919 (2)                                                                    | 154 $\pm$ 8 (2)                                                                      | 411 $\pm$ 179 (4)                                            | 4 (1)                                                   |
| <b>CD18</b>   | 233 $\pm$ 60 (3)                                                                                  | 547 $\pm$ 18 (2)                                                                     | 1174 $\pm$ 420 (2)                                                                    | 129 $\pm$ 11 (2)                                                                     | 283 $\pm$ 34 (4)                                             | 5 (1)                                                   |
| <b>CD40</b>   | 10 $\pm$ 8 (3)                                                                                    | 684 $\pm$ 41 (2)                                                                     | 46 $\pm$ 7 (3)                                                                        | 108 $\pm$ 13 (4)                                                                     | 69 $\pm$ 33 (3)                                              | 493 $\pm$ 179 (2)                                       |
| <b>CD11b</b>  | 55 $\pm$ 11 (5)                                                                                   | 141 $\pm$ 63 (6)                                                                     | 117 $\pm$ 182 (4)                                                                     | 11 $\pm$ 6 (8)                                                                       | n.d. <sup>b</sup>                                            | 325 $\pm$ 118 (2)                                       |
| <b>CD11c</b>  | 8 $\pm$ 2 (2)                                                                                     | 100 $\pm$ 82 (3)                                                                     | 168 $\pm$ 42 (2)                                                                      | 46 (1)                                                                               | n.d.                                                         | 453 $\pm$ 141 (2)                                       |
| <b>CD50</b>   | 381 (1)                                                                                           | 347 $\pm$ 88 (2)                                                                     | 347 $\pm$ 88 (2)                                                                      | n.d.                                                                                 | n.d.                                                         | 30 $\pm$ 18 (2)                                         |
| <b>CD83</b>   | 0 $\pm$ 0 (2)                                                                                     | 85 $\pm$ 79 (2)                                                                      | 4 $\pm$ 1 (2)                                                                         | n.d.                                                                                 | n.d.                                                         | 33 $\pm$ 22 (2)                                         |

<sup>a</sup> Cells are stimulated and examined by flow cytometry for cell surface expression of indicated molecules as described in "Examples".  
MFI = mean fluorescence intensity; SD = standard deviation; (n) indicates the number of independent experiments.  
<sup>b</sup> n.d. stands for not determined.

**Table 2.** Expression of chemokine receptors in human  $\gamma\delta$  T cells.

|              | <u>Fresh <math>\gamma\delta</math> T cells</u><br>% of positivity <sup>a</sup><br>mean $\pm$ S.D. (n) | <u>Activated <math>\gamma\delta</math> T cells</u><br>% of positivity<br>mean $\pm$ S.D. (n) |
|--------------|-------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|
| <b>CXCR3</b> | 66.5 $\pm$ 8.2 (10)                                                                                   | 65.9 $\pm$ 18.9 (9)                                                                          |
| <b>CXCR4</b> | 53.1 $\pm$ 13.8 (7)                                                                                   | 57.2 $\pm$ 21.6 (6)                                                                          |
| <b>CXCR5</b> | 2.3 $\pm$ 1.8 (7)                                                                                     | 2.0 $\pm$ 2.9 (5)                                                                            |
| <b>CCR1</b>  | 20.6 $\pm$ 10.7 (5)                                                                                   | 24.5 $\pm$ 16.9 (4)                                                                          |
| <b>CCR2</b>  | 23.0 $\pm$ 7.2 (9)                                                                                    | 14.1 $\pm$ 5.3 (7)                                                                           |
| <b>CCR4</b>  | 13.3 $\pm$ 10.2 (12)                                                                                  | 29.9 $\pm$ 10.8 (12)                                                                         |
| <b>CCR5</b>  | 61.2 $\pm$ 10.4 (12)                                                                                  | 2.5 $\pm$ 5.4 (9)                                                                            |
| <b>CCR6</b>  | 20.1 $\pm$ 11.1 (6)                                                                                   | 15.2 $\pm$ 14.7 (4)                                                                          |
| <b>CCR7</b>  | 18.9 $\pm$ 9.7 (23)                                                                                   | 77.6 $\pm$ 14.7 (14)                                                                         |

<sup>a</sup> The data are taken from Brandes et al., *Blood* (2003)

5 Fig. 2 illustrates the migration properties of short-term activated  $\gamma\delta$  T cells, as assessed by *in vitro* chemotaxis analysis (Brandes et al., 2003). The data clearly demonstrate the responsiveness toward the CCR7 ligand SLC/CCL21 and the strongly reduced responsiveness toward the CCR5 (and CCR1, CCR3) ligand RANTES/CCL5 in activated  $\gamma\delta$  T cells. The data further demonstrate that changes in the level of cell surface  
10 chemokine receptors are directly mirrored in migration responses to the corresponding chemokines.

Collectively, the results summarized in Figs. 1-2 and Tables 1-2 underscore the fact that stimulated but not resting (freshly isolated)  $\gamma\delta$  T cells from peripheral blood express many  
15 essential factors, including adhesion and co-stimulatory molecules, and chemokine receptors, required for LN-homing and stimulation of naïve T cells. The activation-induced modulation of these migration- and APC-related parameters compares well with the activation-induced changes occurring in DCs (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). Furthermore, these data imply that stimulated  
20 human  $\gamma\delta$  T cells act as potent APCs comparable to DCs.

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Rapid, extensive and antigen-independent cell cluster formation is characteristic for DC-T cell interactions and is a prerequisite for antigen-dependent stimulation and differentiation of naïve T cells. Fig. 3 documents that stimulated  $\gamma\delta$  T cells also induce tremendous cell clustering with naïve (resting)  $CD4^+$  T cells within 3 hours of culturing. Of note, these clusters form in the absence of antigen, suggesting that adhesion and co-stimulatory molecules are responsible for this effect. The  $\gamma\delta$  T cell-mediated cluster formation is at least as robust as the one observed with mature DCs. By contrast, 1 day activated  $\alpha\beta$  T cells or freshly isolated monocytes (not shown) are much less efficient, which agrees with the reduced levels of adhesion and co-stimulatory molecules on these cells. These data provide further evidence that short-term activated  $\gamma\delta$  T cells have potent APC function.

Activated  $\gamma\delta$  T cells are able to take up, process and present antigen for triggering responses in antigen-specific  $CD4^+$   $\alpha\beta$  T cell lines. Fig. 4 shows proliferation of tetanus toxoid (TT)-specific  $CD4^+$   $\alpha\beta$  T cells in response to short-term stimulated, TT-presenting  $\gamma\delta$  T cells or, as control, TT-presenting DCs. The TT-specific  $CD4^+$   $\alpha\beta$  T cell line is derived from the same donor who provided the  $\gamma\delta$  T cells. Resting TT-specific  $CD4^+$   $\alpha\beta$  T cells are loaded with CFSE and proliferation of responder cells is determined by measuring the reduction of the CFSE signals in cultured cells by means of flow cytometry (see "Examples"). During cell division the CFSE content is distributed onto the two daughter cells such that each round of cell division is characterized by 50% reduction in CFSE signals. This type of analysis allows the determination of a) the fractions of non-responding *versus* proliferating cells (input/maximal *versus* reduced CFSE signals), b) the cellular subsets with distinct rounds of cell division, and c) the fraction of cells at the beginning of the experiment that has responded to the APCs. This information cannot be obtained by performing the  $^3H$ -thymidin-incorporation assay, which is an alternative method for the determination of cell proliferation. Fig. 4 shows that  $\gamma\delta$  T cells are capable of inducing proliferation in TT-specific  $CD4^+$  T cells and that this response fully depends on the antigen, since  $\gamma\delta$  T cells that have been stimulated in the absence of TT are inactive. It further shows that between day 4 and day 6 of culture the  $CD4^+$  T cells continue to proliferate, as evidenced by the further reduction of CFSE signals (shift to the left of fluorescence signals in Fig. 4). Of note,  $\gamma\delta$  T cells are similarly potent as mature DCs in this response, which demonstrates that short-term stimulated  $\gamma\delta$  T cells have potent antigen-presenting functions.

Fig. 5 illustrates the fact that the TT-specific proliferation responses require cell contact between TT-presenting  $\gamma\delta$  T cells and responding TT-specific  $CD4^+$   $\alpha\beta$  T cells (responder cells). The responder cells that are separated by a porous membrane from co-cultures containing TT-presenting  $\gamma\delta$  T cells and responder cells do not proliferate. The porous membranes prevent the exchange of cells but do not prevent the exchange of soluble mediators between the two culture compartments. Therefore, the results in Fig. 5 also demonstrate that cytokines and growth factors produced during the co-culture of TT-presenting  $\gamma\delta$  T cells and responder cells have no effect on the proliferation of responder cells that are cultured separately.

10

TCR triggering is accompanied by TCR internalization, resulting in the downmodulation of cell surface TCR and accessory molecules, such as CD3. Fig. 6 documents that TT-presenting  $\gamma\delta$  T cells induce TCR downmodulation in TT-specific responder cells (see Fig. 4), as assessed by loss of cell surface CD3. Consequently,  $\gamma\delta$ T cells have the capacity to take up and process TT and to present TT-derived peptides in the form of MHC-II-peptide complexes to TT-specific responder cells in a manner sufficient for TCR engagement. Since activated but not resting peripheral blood  $\gamma\delta$  T cells express cell surface MHC-II molecules, the antigen-presentation function is closely associated with  $\gamma\delta$  T cell activation. Fig. 6 also shows that TCR downmodulation is a function of the density of TT-peptides on stimulated  $\gamma\delta$  T cells, which is controlled by varying the amount of TT added during  $\gamma\delta$  T cell stimulation. Obviously, the more TT-derived MHC-II-peptide complexes are present on stimulated  $\gamma\delta$  T cells the more TCRs on responder cells become engaged and downmodulated. The different potency in TCR downmodulation between  $\gamma\delta$  T cells and DCs is likely due to striking differences in cell morphology, since the cell surface area in mature, antigen-presenting DCs is >10-fold larger than in stimulated  $\gamma\delta$  T cells (Miller et al., 2004).

25

In addition to proliferation, TT-presenting  $\gamma\delta$  T cells induce the expression of activation markers in TT-specific  $CD4^+$   $\alpha\beta$  T cells. Fig. 7 shows the upregulation on the cell surface of responder cells of T cell activations markers, including CD25, ICOS and CD134/OX40, which are *de novo* expressed or enhanced in response to TCR triggering. As in induction of T cell proliferation, high-level expression of these activation markers fully depend on TT-peptide presentation by short-term stimulated  $\gamma\delta$  T cells, since these activation markers are not induced with  $\gamma\delta$  T cells, which are stimulated in the absence of TT.

35

Fig. 8 shows that heterologous Epstein Barr Virus (EBV)-immortalized B cells and autologous responder cells lack TT-presenting function. One protocol for stimulation of  $\gamma\delta$  T cells includes the use of irradiated, IPP-presenting cells that are either autologous B cells or, for convenience of *in vitro* experimentation, EBV-B cell lines, i.e. the heterologous CP.EBV line. Fig. 8 demonstrates that TT-treated CP.EBV cells fail to induce the proliferation of TT-specific  $CD4^+$   $\alpha\beta$  T cells, indicating that CP.EBV material did not contribute to the strong proliferative responses obtained with TT-peptide presenting  $\gamma\delta$  T cells. Also, Fig. 8 illustrates the poor TT-presenting function of the  $\alpha\beta$  T cell line on its own.

10

Similar effects as those shown in Figs. 4-8 with TT are obtained with the complex/undefined antigen *Mycobacterium tuberculosis* purified protein derivative (PPD). Fig. 9 shows the proliferation responses of PPD-specific  $CD4^+$   $\alpha\beta$  T cells after 4 days or 8 days of culture following stimulation with PPD-peptide presenting  $\gamma\delta$  T cells or, alternatively, PPD-peptide presenting DCs as APCs. Again, the experiments are performed under autologous conditions, i.e. the APCs, ( $\gamma\delta$  T cells and DCs) and responder cells (PPD-specific  $CD4^+$   $\alpha\beta$  T cell line) are derived from the same donor. As documented for TT, the proliferation responses are PPD-specific and do not differ substantially between  $\gamma\delta$  T cells and DCs. Also, the  $\alpha\beta$  T cell line itself fails to induce proliferation in PPD-specific responder cells. At equal concentrations of antigen  $\gamma\delta$  T cells and DCs induce more vigorous responses with TT as compared to PPD, which is due to the differences in the complexity between TT ( $M_r$  [TT]: 150 kDa) and PPD ( $M_r$  [PPD]:  $\geq 10'000$  kDa). Complex antigens, such as PPD or whole microorganisms, are highly diverse in the repertoire of antigenic peptides with the consequence that individual APCs present discrete MHC-peptide complexes at low levels. In agreement, TCR downmodulation is much less evident in cloned, PPD-specific  $CD4^+$  T cells during co-culture with PPD-presenting APCs than what is observed in the TT-system (Fig. 6).

25

Collectively, the experiments with TT-specific and PPD-specific responder cells document the finding of the present invention that stimulated (but not resting)  $\gamma\delta$  T cells derived from human peripheral blood have potent antigen uptake, antigen presentation and T cell stimulation functions. The potency and efficacy of these  $\gamma\delta$  T cells functions are remarkable and equal those obtained with DCs.

30

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The characteristic of efficient APCs, such as DCs, is their ability to induce primary adaptive immune responses that involve the stimulation of naïve (antigen-inexperienced) T cells and their differentiation into antigen-specific effector T cells with the capacity to produce cytokines or to kill target cells (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). Fully differentiated memory T cells have reduced thresholds of activation, and TCR triggering in the absence of co-stimulation is sufficient to initiate effector functions. The experimental results shown in the following figures prove that stimulated  $\gamma\delta$  T cells also have efficient antigen-presenting functions comparable to those of DCs. Therefore, instead of antigen-experienced  $CD4^+ \alpha\beta$  T cell lines (Fig. 4-9), autologous freshly isolated naïve  $CD4^+ \alpha\beta$  T cells are used as potential responder cells.

Fig. 10 shows the extent of proliferation in CFSE-labeled, naïve  $CD4^+ \alpha\beta$  T cells in response to toxic shock syndrome toxin (TSST-1)-loaded, short-term stimulated  $\gamma\delta$  T cells and TSST-1-loaded mature DCs, as opposed to TSST-1-loaded stimulated  $\alpha\beta$  T cells or TSST-1-loaded freshly isolated monocytes. TSST-1 binds to MHC-II molecules on APCs and is selective for  $\alpha\beta$ -TCRs containing the  $V\beta 2$ -chain. 4-10% of peripheral blood  $CD3^+$  T cells are  $V\beta 2^+$  and respond with high affinity to TSST-1-presenting APCs. As demonstrated in Fig. 10, TSST-1-loaded  $\gamma\delta$  T cells are expert APCs in induction of proliferation in naïve  $V\beta 2^+$ -T cells, as evidenced by the reduction of CFSE signals, and this response is much more prominent in naïve  $CD4^+ \alpha\beta$  T cells bearing  $V\beta 2^+$ -TCRs than those bearing  $V\beta 2^{neg}$ -TCRs. Eventually, after completion of cell expansion most of the resulting memory T cells express  $V\beta 2^+$ -TCRs (see also Table 3). Importantly, the proliferation responses equal the ones obtained with TSST-1-loaded DCs. Under these conditions, TSST-1-loaded  $\alpha\beta$  T cells or monocytes are completely inactive, indicating that the level of TSST-1-presentation and/or co-stimulation in these cells is not sufficient to induce efficient primary T cell responses.

**Table 3.**  $\alpha\beta$  T cell expansion and Th cell differentiation during long-term culture (21 days)

| <b>APCs<sup>a</sup></b> | <b>Ratio</b> | <b>Antigen<sup>b</sup></b> | <b>ng/ml</b> | <b>V<math>\beta</math>2<sup>+</sup> (%)<sup>c</sup></b> | <b>Th0 (%)<sup>d</sup></b> | <b>Th1 (%)</b> | <b>Th2 (%)</b> |
|-------------------------|--------------|----------------------------|--------------|---------------------------------------------------------|----------------------------|----------------|----------------|
| $\gamma\delta$ T        | 1:5          | TSST-1                     | 10           | 32                                                      | 18                         | 25             | 16             |
| $\gamma\delta$ T        | 1:1          | TSST-1                     | 10           | 70                                                      | 2                          | 73             | 1              |
| $\gamma\delta$ T        | 1:5          | TSST-1                     | 100          | 99                                                      | 9                          | 46             | 6              |
| $\gamma\delta$ T        | 1:5          | TSST-1                     | 1000         | 93                                                      | 13                         | 50             | 5              |
| DC                      | 1:5          | TSST-1                     | 10           | 80                                                      | 6                          | 84             | <1             |
| DC                      | 1:5          | TSST-1                     | 100          | 83                                                      | 2                          | 86             | <1             |
| DC                      | 1:5          | TSST-1                     | 1000         | 67                                                      | 1                          | 82             | <1             |
| –                       |              | PHA                        | 1000         | 9                                                       | n.d. <sup>e</sup>          | n.d.           | n.d.           |

<sup>a</sup> APCs are either 1 day stimulated  $\gamma\delta$  T cells or mature DCs that are loaded with TSST-1 and added to naïve CD4<sup>+</sup>  $\alpha\beta$  T cells at the ratio of 1:5 or 1:1.

<sup>b</sup> TSST-1 is the antigen used to load the APCs; alternatively, naïve CD4<sup>+</sup>  $\alpha\beta$  T cells are directly activated with PHA in the absence of APCs.

<sup>c</sup> The percent V $\beta$ 2<sup>+</sup> responder cells is determined by flow cytometry as described in Fig. 10.

<sup>d</sup> The fraction in percent of cytokine polarized Th cells is determined by measuring intracellular cytokines as described in Fig. 13.

<sup>e</sup> Data not determined.

In addition to co-stimulatory molecules, the parameters that largely determine the kinetics and extent of proliferation in naïve CD4<sup>+</sup>  $\alpha\beta$  T cells are the density of MHC-II-TSST-1 complexes on APCs and the ratio between APCs and responder cells. Fig. 11 shows the proliferation of V $\beta$ 2<sup>+</sup> naïve responder cells in response to increasing concentrations of TSST-1 that are used to load the different types of APCs. Before addition to naïve CD4<sup>+</sup>  $\alpha\beta$  T cells, the APCs are washed to get rid off excess TSST-1 (see “Examples”). The list of autologous APCs includes 1 day stimulated or 7 day stimulated  $\gamma\delta$  T cells, mature DCs, freshly isolated monocytes and 1 day stimulated  $\alpha\beta$  T cells. 1 day stimulated  $\gamma\delta$  T cells induce T cell proliferation responses at TSST-1 loading concentrations as low as 1 ng/ml and are equally efficient as DCs in terms of maximal responses. The approx. 10-fold higher potency of DCs may be due to their greatly enlarged cell surface area (Miller et al., 2004), which allows more frequent or extensive contacts with responder cells (see also

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Fig. 6). Of note, substantial proliferation responses are still obtained with stimulated  $\gamma\delta$  T cells that are expanded during culture for 7 days before loading with TSST-1. Obviously,  $\gamma\delta$  T cells maintain APC functions over extended periods of time, which is in agreement with the observed conservation of adhesion and co-stimulatory molecules (see also Fig. 1 and Table 1). Monocytes and  $\alpha\beta$  T cells are >100-fold less potent than  $\gamma\delta$  T cells. These data demonstrate that stimulated  $\gamma\delta$  T cells have efficient antigen-presenting functions.

In the experiment shown in Fig. 12, instead of titration of TSST-1 during APC loading, the ratio of APCs to naïve responder cells is varied between 1:5 to 1:200 while keeping the TSST-1 loading concentration at 100 ng/ml or 1  $\mu$ g/ml. Proliferation responses of naïve  $V\beta 2^+$   $CD4^+$   $\alpha\beta$  T cells are determined as in Fig. 11. There is no obvious difference between 1 day stimulated  $\gamma\delta$  T cells and DCs, and at highest APC dilution (1:200) the proliferation responses still range between 28-40 % of maximal responses. These data further illustrate the proficiency of  $\gamma\delta$  T cells as efficient APCs.

Primary immune responses involve the differentiation of naïve  $CD4^+$  T cells into polarized Th1, Th2 or Th0 cells with the capacity to produce type 1 (IFN- $\gamma$ ), type 2 (IL-4) or type 0 (IFN- $\gamma$  + IL-4) cytokines, respectively. Naïve  $CD4^+$  T cells have the capacity to differentiate into either one of these polarized Th cells. T cell polarization is determined by the co-stimulatory environment provided by APCs at the time of naïve T cell priming. Efficient APCs not only induce proliferation of naïve T cells but also support their differentiation into effector cells. Fig. 13 demonstrates that stimulated  $\gamma\delta$  T cells are fully capable of presenting TSST-1 in the proper context of co-stimulation for the generation of effector Th cells. 4 days after priming with TSST-1-loaded  $\gamma\delta$  T cells, most  $V\beta 2^+$  naïve  $CD4^+$  T cells have responded by proliferation and expression of the memory marker CD45RO. After 21 days of culture the majority of cells return to a resting state, uniformly express CD45RO and consist of  $V\beta 2^+$  T cells (Table 3). Importantly, the majority of cells produce cytokines typical of either Th1, Th2 or Th0 cells, and Th1 polarization is further enhanced by increasing the ratio of TSST-1-loaded  $\gamma\delta$  T cells and responder cells to 1:1. Again, the more prominent effect seen with DCs may be due to morphological criterion as discussed above (Miller et al., 2004) (see also Figs. 6, 9). By contrast, antigen-unselective activation (phytohemagglutinin) does not result in selective expansion of  $V\beta 2^+$  T cells (Table 3), and stimulated  $\alpha\beta$  T cells fail to induce CD45RO expression and proliferation of naïve  $CD4^+$   $\alpha\beta$  T cells (Fig. 13).

As seen with TT-specific and PPD-specific CD4<sup>+</sup> αβ T cells (see e.g. Fig. 5), induction of responses in naïve CD4<sup>+</sup> αβ T cells is fully dependent on cell-to-cell contact with TSST-1-loaded γδ T cells (Fig. 14). Stimulated γδ T cells induce strong antigen-specific responses in naïve CD4<sup>+</sup> αβ T cells and support their proliferation and differentiation in a manner typical for efficient APCs.

CD4<sup>+</sup> T cells recognize MHC class II-peptide complexes on APCs whereas CD8<sup>+</sup> T cells recognize MHC class I-peptide complexes on APCs. MHC class I molecules are ubiquitously expressed on blood and tissue cells; therefore, all cells in the body are potential target cells for CD8<sup>+</sup> T cells. Of note, CD8<sup>+</sup> T cells are crucial players in the defense against viral infections and tumors and, frequently, successful vaccination depends on the generation of antigen-selective, cytotoxic CD8<sup>+</sup> T cells. Fig. 15 documents that Vδ2<sup>+</sup> T cells are highly potent APCs in the induction of primary CD8<sup>+</sup> T cell responses. Naïve, untouched CD8<sup>+</sup> αβ T cells are used as responder cells in proliferation assays containing IPP-stimulated γδ T cells, mature DCs or superantigen-stimulated αβ T cells as APCs (all from the same donor). γδ T cells fully match or are even better as DCs in induction of CD8<sup>+</sup> T cell proliferation, and αβ T cells are inferior APCs. Fig. 16 documents that γδ T cells induce the differentiation of cytotoxic effector T cells. The data illustrate that γδ T cells are indistinguishable from DCs in driving the differentiation of naïve CD8<sup>+</sup> T cells into alloantigen-specific, cytotoxic T cells. Collectively, TCR-stimulated Vδ2<sup>+</sup> T cells induce strong proinflammatory responses in both naïve CD4<sup>+</sup> and CD8<sup>+</sup> αβ T cells in a “professional” APC-like manner.

γδ T cells also express endocytic receptors, such as the C-type lectin DEC-205 (CD205) and the integrin subunit CD11b with selectivity for multiple protein and non-protein ligands. CD205 and CD11b are known to be highly expressed on DCs (Banchereau and Steinman, 1998; Steinman et al., 2003; Banchereau et al., 2004). Fig. 17 documents that γδ T cells, similar to DCs, inherently express high levels of these endocytic receptors. In analogy to novel methods of antigen-delivery to DCs, these data demonstrate that antigens, including protein vaccines for tumors and infectious agents, can also be targeted to γδ T cells for *in vivo* antigen processing and presentation.

These experimental details prove that the method of the invention for the preparation of efficient antigen-presenting human γδ T cells comprising selecting γδ T cells out of human

peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of antigen-presenting functions, and applying the antigen to the stimulated cells provides ACPs comparable to DCs.

- 5 In particular, the invention concerns such a method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells wherein selecting  $\gamma\delta$  T cells is performed by magnetic cell sorting with antibodies to human  $V\gamma V\delta$ -T cell receptors. Alternatively, selecting  $\gamma\delta$  T cells is performed by culturing freshly isolated peripheral blood lymphocytes in the presence of structurally defined small molecular weight non-peptide compounds that induce the
- 10 selective expansion of  $V\gamma 2V\delta 2^+$ -T cell receptor chain-expressing  $\gamma\delta$  T cells, for example in the presence of IPP, e.g. as presented by B cells or substitutes.

The invention further concerns the particular method wherein the stimulus for induction of efficient antigen-presenting functions is a small molecular weight non-peptide compound

15 or a substitute or phytohemagglutinin, and the particular method wherein the antigen is applied in the form of defined proteins, undefined protein mixtures, or crude or enriched extracts from tumor and infected cells, for example wherein the antigen applied is a pathogen- or tumor cell- derived peptide, or a pathogen-derived protein which is applied in the form of DNA or RNA encoding it under conditions allowing endogenous expression of

20 said pathogen-derived protein, in particular in the form of purified DNA or RNA or a delivery vector containing such DNA or RNA. If the antigen is in the form of a DNA or RNA conditions are selected such that said DNA or RNA may be properly expressed.

Application of an antigen may occur before, during or after  $\gamma\delta$  T cell stimulation for induction of antigen-presenting functions. If an antigen is applied as a peptide (protein

25 fragment), its application may also be after stimulation in a separate step termed "peptide loading".

The efficient antigen-presenting human  $\gamma\delta$  T cells prepared according to the invention as described hereinbefore may be used in immunotherapy. Such use may be similar to the

30 known use of DCs in immunotherapy, thereby overcoming the drawbacks of the use of DCs such as scarcity in peripheral blood, inability to proliferate *in vitro*, heterogeneity and functional instability.

In particular, the efficient antigen-presenting human  $\gamma\delta$  T cells prepared according to the

35 invention as described hereinbefore may be used for the manufacture of a medicament (pharmaceutical composition) for use in immunotherapy.

The present invention relates also to pharmaceutical compositions that comprise the efficient antigen-presenting human  $\gamma\delta$  T cells prepared according to the invention as described hereinbefore, and that can be used especially in the treatment of the diseases mentioned hereinbefore and hereinafter. Compositions for parenteral administration, such as intravenous, intramuscular, subcutaneous, mucosal or submucosal administration, to humans are especially preferred. The compositions comprise the cells together with a pharmaceutically acceptable carrier. The dosage of the cells of the invention depends upon the disease to be treated and upon the age, weight, and individual condition, the individual pharmacokinetic data, and the mode of administration. The pharmaceutical compositions comprise from approximately 0.01% to approximately 50% of the cells of the invention. Unit dose forms are, for example, ampoules or vials.

Preference is given to the use of isotonic aqueous suspensions. The pharmaceutical compositions may comprise excipients, for example preservatives, stabilizers, wetting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers and are prepared in a manner known *per se*, for example by means of conventional mixing processes. The manufacture of injectable preparations is usually carried out under sterile conditions, as is the filling, for example, into ampoules or vials, and the sealing of the containers.

The invention further relates to a method of treatment of tumors or chronic or recurrent infectious diseases wherein efficient antigen-presenting human  $\gamma\delta$  T cells are injected into a patient in need thereof. In particular the method involves single or repeated applications of said  $\gamma\delta$  T cells, e.g. pharmaceutical compositions containing same, by intradermal, subcutaneous, intramuscular, intravenous, mucosal or submucosal routes.

In the method of treatment of tumors  $\gamma\delta$  T cells are used, which have been stimulated in the presence of defined tumor proteins or crude (undefined) tumor cell extracts or, alternatively, which have been obtained using treatment with recombinant RNA/DNA technologies that are routinely used for transfection or transduction of live blood or tissue cells. Preferably, if defined tumor peptides for direct loading onto cell surface MHC molecules are known, then such peptides are added to stimulated  $\gamma\delta$  T cells, incubated, washed and immediately used for therapy. It is important to emphasize that the number of  $\gamma\delta$  T cells used per administration as well as the route and frequency of  $\gamma\delta$  T cell administration depend on the efficacy in immune response induction by the individual

tumor antigen used as well as the type and location of the tumor present in the individual patient. Preferred protocols are, for example,  $1-20 \times 10^6$  cells/0.5-2 ml per administration with 1 to 6 follow-up administrations with the same or lower amounts of cells at two-weeks to two-months intervals.

5

In the method of treatment of chronic or recurrent infectious diseases  $\gamma\delta$  T cells are used which have been stimulated in the presence of defined infectious agents, preferably in attenuated form, or crude (undefined) infected cell extracts or, alternatively, which have been obtained using treatment with recombinant RNA/DNA technologies that are routinely  
10 used for transfection or transduction of live blood or tissue cells. The preferred treatment protocol corresponds to the one described above.

The invention further relates to a method of vaccination against tumors or chronic or recurrent infectious diseases wherein efficient antigen-presenting human  $\gamma\delta$  T cells, to  
15 which non-infectious and non-tumorigenic antigens have been applied, are injected into a patient to be vaccinated. For vaccination purposes the same procedures are applied as described hereinbefore but wherein  $\gamma\delta$  T cells are used to which non-infectious and non-tumorigenic antigens have been applied. The preparation of vaccine antigen-presenting  $\gamma\delta$  T cells and administration of these cells for the vaccination of patients against tumor  
20 antigens or infectious agents follows the description of tumor immunotherapy (see above). Preferred protocols are those currently employed in DC-based vaccination treatments. The immune status of such treated patients, i.e. the quality (efficacy, kinetics, etc.) of the vaccine responses, is examined as described below.

25 The invention further relates to another method of (prophylactic or therapeutic) vaccination against tumors or vaccination against agents inducing infectious or non-infectious diseases, comprising the administration of  $\gamma\delta$  T cell-targeting vaccines to individuals. Preferably, such  $\gamma\delta$  T cell-targeting vaccines are hybrid compounds composed of vaccine agents and  $\gamma\delta$  T cell-targeting molecules.  $\gamma\delta$  T cell-targeting molecules are antibodies or  
30 ligands specific for endocytic receptors on  $\gamma\delta$  T cells, including but not limited to CD11b and CD205. Vaccine agents are proteins or related molecules against which an immune protection is desired.

Administration of  $\gamma\delta$  T cell-targeting vaccines entails the repeated treatment of individuals with  $\gamma\delta$  T cell-targeting vaccines by means of injections, oral administrations or any other protocol of vaccine delivery yielding optimal immune protection.

- 5 The invention further relates to a method of identification of novel tumor or pathogen-derived antigens comprising selecting  $\gamma\delta$  T cells out of human peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of efficient antigen-presenting functions, applying fractions of an undefined protein mixture, or crude or enriched extracts from tumor and infected cells, or RNA or DNA libraries derived from  
10 tumors and infectious agents, testing for *in vitro* activation of autologous naïve  $\alpha\beta$  T cells, and comparing the activation results of different antigen fractions.

In this method,  $\gamma\delta$  T cells are used as *in vitro* screening tools for the identification of novel and improved antigens for use in therapeutic and prophylactic vaccinations. Isolating,  
15 selecting and stimulating  $\gamma\delta$  T cells are performed as described in the method of preparation of efficient antigen-presenting human  $\gamma\delta$  T cells. In the step of applying antigens, fractions of crude antigen preparations (e.g. cell extracts or undefined mixtures) or RNA/DNA libraries derived from tumors and infectious agents are used. Such prepared  $\gamma\delta$  T cells are then tested for activation of naïve  $\alpha\beta$  T cells from the same donor, i.e.  
20 autologous  $\alpha\beta$  T cells. Read-outs in these *in vitro* immune response assays are proliferation and cytokine production in  $\alpha\beta$  T cells, or any other simple measurement of  $\alpha\beta$  T cell activation. Culture conditions are preferably those described above for  $\alpha\beta$  T cell responses to TSST-1-presenting  $\gamma\delta$  T cells in Figs. 10-14 and the "Examples". When comparing activation results from different antigen fractions, improved  $\alpha\beta$  T cell responses  
25 indicate that the antigen sources are "enriched" in terms of immunogenicity. Corresponding "enriched" fractions are further processed, and the whole cycle of experimental steps repeated with the further fractionated antigens. Eventually, repeated fractionation of "enriched" antigen sources (protein or DNA library fractionation) leads to single proteins with maximal immunostimulatory functions. Such novel proteins may be  
30 further manipulated by proteolytic cleavage, and cleavage mixtures analyzed accordingly for the generation of (small) immunogenic peptides for direct loading onto APCs.

The invention further relates to a method of diagnosing the immune competence of a patient comprising selecting  $\gamma\delta$  T cells out of the patient's peripheral blood mononuclear  
35 cells, treating the selected cells with a stimulus for induction of efficient antigen-presenting

functions, applying the antigen for which the immune competence has to be determined, and testing for *in vitro* activation of autologous  $\alpha\beta$  T cells.

In this method,  $\gamma\delta$  T cells and their impact on antigen-specific memory  $\alpha\beta$  T cells from the same donor are used as *in vitro* tools for the diagnosis of the immune competence of a patient with regard to a particular antigen and for determination whether a vaccination has been successful. Isolating, selecting and stimulating  $\gamma\delta$  T cells are performed as described in the method of preparation of efficient antigen-presenting human  $\gamma\delta$  T cells. In the step of applying antigens, the particular antigen for which immune competence has to be determined, is applied to stimulated  $\gamma\delta$  T cells. Culture conditions and read-outs for determination of  $\alpha\beta$  T cell responses (*in vitro* immune response assays) are preferably those described above for  $\alpha\beta$  T cell responses to TSST-1-presenting  $\gamma\delta$  T cells in Figs. 10-14 and the "Examples". The decisive difference is that memory  $\alpha\beta$  T cells instead of naïve  $\alpha\beta$  T cells are monitored for enhanced immune responses (proliferation, cytokine production, etc.) during stimulation with antigen-presenting  $\gamma\delta$  T cells. Successful immunotherapy (vaccination) leads to the generation of antigen-specific effector/memory T cells, which will give many fold improved immune responses during *in vitro* monitoring of immune status as compared to naïve  $\alpha\beta$  T cells. These assays are preferentially performed with bulk (unfractionated)  $\alpha\beta$  T cells, since antigen-specific memory  $\alpha\beta$  T cells are highly enriched in successfully vaccinated individuals.

### Examples

#### 25 *Abbreviations*

|                                |                                                                     |
|--------------------------------|---------------------------------------------------------------------|
| DC                             | dendritic cell                                                      |
| LN                             | lymph node                                                          |
| PP                             | Peyer's patch                                                       |
| TCR                            | T cell antigen receptor                                             |
| 30 BCR                         | B cell antigen receptor                                             |
| MHC                            | major histocompatibility complex                                    |
| APC                            | antigen-presenting cell                                             |
| $V\delta 1^+$ T cells          | $V\delta 1^+$ -TCR chain expressing $\gamma\delta$ T cells          |
| $V\gamma 2V\delta 2^+$ T cells | $V\gamma 2V\delta 2^+$ -TCR chain expressing $\gamma\delta$ T cells |
| 35 IPP                         | isopentenyl pyrophosphate                                           |

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|    |               |                                                               |
|----|---------------|---------------------------------------------------------------|
|    | TT            | <i>Clostridium tetani</i> tetanus toxin                       |
|    | PPD           | <i>Mycobacterium tuberculosis</i> purified protein derivative |
|    | TSST-1        | <i>Staphylococcus aureus</i> toxic shock syndrome toxin 1     |
|    | PHA           | phytohemagglutinin                                            |
| 5  | IFN- $\gamma$ | interferon- $\gamma$                                          |
|    | TNF- $\alpha$ | tumor necrosis factor- $\alpha$                               |
|    | IL            | interleukin                                                   |
|    | FACS          | fluorescence-activated cell sorter                            |
|    | MFI           | mean fluorescence intensity                                   |
| 10 | SD            | standard error                                                |
|    | CFSE          | carboxyfluorescein diacetate succinimidyl ester               |

### 1. Cell isolation and generation

#### *$\gamma\delta$ T cells*

15 Human peripheral blood mononuclear cells (PBMCs) are isolated from heparin-treated donor blood buffy coats or fresh blood by Ficoll-Paque centrifugation according to standard protocols (Brandes et al., 2003). Out of PBMCs,  $\gamma\delta$  T cells are positively selected with antibodies to human V $\gamma$ V $\delta$ -TCRs using the magnetic cell sorting system from Miltenyi Biotec. In this way, 50 ml of fresh blood routinely yields 2-5 x10<sup>6</sup> cells with a purity of 98-  
20 99 %  $\gamma\delta$  T cells.

#### *Naïve $\alpha\beta$ T cells*

Untouched, naïve CD4<sup>+</sup> or CD8<sup>+</sup>  $\alpha\beta$  T cells (98-99% purity) are isolated from PBMCs by negative magnetic cell sorting with specific antibodies to V $\gamma$ V $\delta$ -TCR, CD1c, CD14, CD16, CD19, CD25, CD45RO, CD56, HLA-DR plus CD4 or CD8, respectively, followed by  
25 fluorescence-activated cell sorting of cells that stain negative for these markers.

#### *$\alpha\beta$ T cell lines*

From PBMCs CD4<sup>+</sup>  $\alpha\beta$  T cells are positively selected with antibodies to human CD4 by means of the magnetic cell sorting. CD4<sup>+</sup>  $\alpha\beta$  T cells are stimulated with TT- or PPD-presenting autologous, irradiated (30 Gy) PBMCs at a ratio 1:100 in the first and 1:10 in  
30 the following cycles, and are expanded in IL-2 containing medium. Antigen-specificity is verified by a CFSE-based proliferation assay (see below) after three cycles of antigenic selection and expansion.

#### *B cells*

B cells are isolated by negative magnetic cell sorting out of PBMCs. B cells are either  
35 used directly for  $\gamma\delta$  T cell stimulation (see below), or B cell lines are generated by EBV-

induced transformation following standard protocols and then used for  $\gamma\delta$  T cells stimulation.

#### *Monocytes*

Monocytes are isolated by positive magnetic cell sorting from PBMCs with antibodies to human CD14, and stringent washing of the separation columns before elution of magnetically trapped cells results in enrichment of CD14<sup>high</sup> monocytes.

#### *DCs*

Monocyte-derived DCs are generated by culturing CD14<sup>high</sup> cells in medium containing 10% FCS in the presence of IL-4 (10 ng/ml) and GM-CSF (25 ng/ml). After 6-7 days of culture the majority of cells is immature, as assessed by cell surface staining for HLA-DR, CD1a, CD14, CD80, CD83, CD86 and CCR7. DC maturation is induced by further culture for 8 hours in the presence of 100 ng/ml LPS (from *Salmonella abortus equi*) (Langenkamp et al., 2000). DC maturation is confirmed by flow cytometry staining for cell surface DC maturation markers HLA-DR, CD80, CD83, CD86 and CCR7.

### 2. T cell stimulation

#### *$\gamma\delta$ T cells*

50  $\mu$ M isopentenyl pyrophosphate (IPP) presented by heterologous or autologous EBV-transformed B cell lines or primary B cells at a dilution of 1:10 is used to activate resting (freshly isolated)  $\gamma\delta$  T cells as described (Brandes et al., 2003).  $\gamma\delta$  T cells are cultured in medium supplemented with 8% human serum in the presence of IL-2 (20 or 200 IU/ml).

#### *$\alpha\beta$ T cells*

Positive selected  $\alpha\beta$  T cells are activated on plates coated with 10  $\mu$ g/ml anti-CD3 antibody (OCT3) plus 250 ng/ml anti-CD28 antibody (28.2) or, alternatively, with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) plus 1  $\mu$ g/ml ionomycin or, alternatively, with 1  $\mu$ g/ml PHA, and cultured in medium supplemented with 8% human serum in the presence of IL-2 (200 IU/ml).

### 3. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling

Cells are washed with PBS<sup>-</sup> and labeled with 2.5  $\mu$ M CFSE (Molecular Probes, Eugene, OR) in PBS<sup>-</sup> supplemented with 1% FCS for 4 min at room temperature. Labeling is stopped by repeated washing of the cells with ice-cold PBS<sup>-</sup> supplemented with 5% FCS. The CFSE-labeled cells are immediately used in cell activation and proliferation assays.

#### 4. In vitro antigen-presentation assays

##### *Antigen presentation to $\alpha\beta$ T cell lines*

Blood  $\gamma\delta$  T cells are activated by the IPP-presenting and irradiated (100 Gy) heterologous EBV-B cell line CP-EBV (see above) for 24 to 60 hours in the presence of 10-20  $\mu\text{g/ml}$  TT (Berna Biotech, Bern, Switzerland) or 20  $\mu\text{g/ml}$  PPD (Statens Serum Institut Copenhagen, Denmark). Monocyte-derived DCs are cultured with TT or PPD for the same period of time and matured for the last 8 hours (see above). After irradiation (26 and 40 Gy for  $\gamma\delta$  T cells and DCs, respectively) and intensive washing, these APCs are used to stimulate TT- and PPD-specific  $\text{CD4}^+$   $\alpha\beta$  T cells clones at a ratio of 1:5 (if not indicated otherwise). At various time points of culture responder cells are examined by flow cytometry for expression of activation markers (HLA-DR, ICOS, and CD25), TCR internalization (loss of cell surface CD3) and cell proliferation (reduction in CFSE fluorescence signals).

##### *Priming of naïve $\text{CD4}^+$ $\alpha\beta$ T cells*

Following stimulation of  $\gamma\delta$  T cells and  $\alpha\beta$  T cells or following maturation of monocyte-derived DCs or following isolation of monocytes from PBMCs, these potential APCs are pulsed for 1 hour at 37°C with various concentrations of toxic shock syndrome toxin (TSST-1) (Toxin Technology, Sarasota, FL). Then, the potential APCs are irradiated with 12 Gy (or 40 Gy for DCs), extensively washed and mixed (routinely at a ratio 1:5) with CFSE-labeled naïve  $\text{CD4}^+$   $\alpha\beta$  T cells. Generally, 96-well round bottom plates contain  $8 \times 10^3$  APCs and  $4 \times 10^4$  CFSE-labeled responder cells in culture medium without exogenous cytokines. Cell proliferation is analyzed after 4 days by flow cytometry.

In Th cell differentiation assays, 100 IU/ml IL-2 is added on day 5 of culture, and cells are expanded during subsequent 10-16 days until responder cells cease to proliferate and return to a resting state (Langenkamp et al., 2000). At day 21, Th cell differentiation is examined by measuring intracellular cytokine production. Cells are stimulated for 6 hours with PMA/ionomycin in the presence of 10  $\mu\text{g/ml}$  Brefeldin A (Sigma-Aldrich), then fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin in PBS containing 2% FCS, stained with antibodies to IL-2, IFN- $\gamma$ , IL-4 and V $\beta$ 2-TCR, and analyzed by flow cytometry.

##### *Priming of naïve $\text{CD8}^+$ $\alpha\beta$ T cells*

In mixed-leukocyte responses, irradiated, IPP-stimulated  $\gamma\delta$  T cells, superantigen-activated  $\alpha\beta$  T cells or LPS-matured DCs are co-cultured with heterologous CFSE-labeled naïve  $\text{CD8}^+$   $\alpha\beta$  T cells. Generally, 96-well round bottom plates contain  $4 \times 10^4$  CFSE-labeled responder cells and APCs in the range of  $4 \times 10^4$  cells to 4 cells per well, corresponding to APC:responder cell ratios of 1:1 to 1:10'000. Cell proliferation is analyzed after 6 days of culture by flow cytometry.  $\text{CD8}^+$  effector cell generation is

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evaluated, after 14 days of mixed-leukocyte responses with naïve CD8<sup>+</sup> αβ T cells, in a cytolytic assay, involving co-culture for 12 h with a mixture of heterologous (true targets) and autologous (negative control) CD4<sup>+</sup> T cells labeled with high (1 μM) and low dose (0.05 μM) of CFSE, respectively, followed by flow cytometry analyzing CFSE signals.

- 5 Reduction in the counts of true target cells indicates antigen-specific killing by CD8<sup>+</sup> effector cells whereas reduction in the counts of negative control cells indicates unspecific target cell killing by CD8<sup>+</sup> effector cells.

### 5. Culture media

- 10 The medium used throughout is RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 μg/ml penicillin/streptomycin, 5x10<sup>-5</sup> M 2-mercaptoethanol and either 10% FCS (Hyclone Laboratories, Logan, UT, or GIBCO BRL) or 8% human serum (Swiss Red Cross, Bern, Switzerland). Human recombinant IL-2 is produced using the myeloma-based expression system.

15

### 6. Flow cytometry

#### *Cell preparation*

- Cells are washed twice in ice-cold PBS<sup>-</sup> supplemented with 2% FCS and 0.01% sodium-acid. After blocking for 10 min with 10 mg/ml human immunoglobulin, cells are sequentially incubated for 20 min on ice with primary antibodies specific for diverse cellular proteins or isotype-matched control antibodies, washed, and in case of untagged primary antibodies, further incubated with fluorescence tag-labeled secondary reagents. After final washing, cells-associated fluorescence is measured with a FACSCalibur (Becton Dickinson, San Jose, CA), and the recorded data re analyzed by the
- 20
- 25 CellQuestPro software (Becton Dickinson).

#### *Antibodies*

- Source of antibodies: Mouse mAbs anti-CD1a (HI149), CD3 (UCHT1), CD4 (RPA-T4), CD8 (HIT8α), CD11b (D12), CD14 (MΦP9), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD25 (M-A251), CD40 (5C3), CD45RA (HI100), CD45RO (UCHL-1), CD50 (TU41), CD54 (HA58), CD56 (B159), CDw70 (Ki-24), CD80 (L307.4), CD83 (HB15e), CD86 (2331; FUN1), CD134 (L106), CD205 (MG38), HLA-DR (G46-6), pan-VγVδ-TCR (11F2), IL-2 (MQ1-17H12), IL-4 (8D4-8), IFNγ (B27), and IL-10 (No 20705A) from BD PharMingen, San Diego, CA; mouse mABs anti-CD1a (NaI/34-HLK) and CD19 (HD37) from DAKO Diagnostics, Glostrup, Sweden; mouse mAB anti-TCRVβ2 (MPB2D5) from Immunotech, Marseille, France; mouse mAB anti-CD138 (B-B4) from Diaclone, Besançon, France;
- 30
- 35 mAB anti-CD102 (B-T1) from Leinco Technologies, St. Louis, MO; mouse mAbs anti-

CD11a (TS1-22) and CD18 (TS1-18) from R. Pardi, Milano, Italy; mouse mAb anti-ICOS (F44) from R.A. Kroczeck, Berlin, Germany; rat mAb anti-CCR7 (3D12) from M. Lipp, Berlin, Germany. These are used for flow cytometric analysis or cell isolation.

The following secondary Ab, conjugates and control Ab are used: RPE-conjugated goat anti-mouse IgG from Sigma-Aldrich, St. Louis, MO; RPE-conjugated donkey anti-rat IgG from Jackson ImmunoResearch Laboratories, West Grove, PA; RPE as well as RPE-Cy5 conjugated streptavidine (SA) from DAKO; APC conjugated SA from BD PharMingen; mouse control IgG1 (MOPC21) from Sigma-Aldrich; other isotype-matching control Abs from BD PharMingen.

10

### 7. Immunotherapy of tumors or chronic/recurrent infections

For the preparation of stimulated, tumor antigen-presenting  $\gamma\delta$  T cells, 50-150 ml of peripheral blood is drawn from tumor patients (or patients with chronic/recurrent infections, see below) and  $\gamma\delta$  T cells isolation and antigen loading is performed as described above. Alternatively, such freshly isolated  $\gamma\delta$  T cells are expanded by *in vitro* culture under  $V\gamma 2V\delta 2^+$ -TCR-stimulatory conditions (see methods of  $\gamma\delta$  T cell activation with IPP above) in the presence of 20-1000 IU/ml IL-2 and then stored in liquid nitrogen for later use in the preparation of tumor (or vaccine, see below) antigen-presenting  $\gamma\delta$  T cells. Importantly, instead of defined tumor/vaccine proteins, many other ways of antigen delivery to  $\gamma\delta$  T cells for the preparation of APCs are possible, including (among others) addition of crude (undefined) tumor cell extracts or extracts from infected cells (see below) or, alternatively, the treatment of  $\gamma\delta$  T cells by recombinant RNA/DNA technologies that are routinely used for transfection or transduction of live blood or tissue cells. Also, if defined tumor (or vaccine, see below) peptides for direct loading onto cell surface MHC molecules are known, then such peptides at 0.1-10  $\mu$ g/ml are added to stimulated  $\gamma\delta$  T cells at  $1-10 \times 10^6$  cells/ml, which are then incubated at 20-37°C for short period of time, washed 2-times with isotonic phosphate-buffered saline solution and immediately used for therapy. It is important to emphasize that the number of  $\gamma\delta$  T cells used per administration as well as the route and frequency of  $\gamma\delta$  T cells administration depend on the efficacy in immune response induction by the individual tumor (or vaccine, see below) antigen used as well as the type and location of the tumor present in the individual patient. Protocols follow those currently employed in DC-based immunotherapies (Fong and Engleman, 2000; Steinman et al., 2003; Schuler et al., 2003; Figdor et al., 2004), i.e.  $1-20 \times 10^6$  cells/0.5-2 ml per administration with 1 to 6 follow-up administrations with the same or lower amounts of cells at two-weeks to two-months intervals.

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CLAIMS

1. A method for the preparation of efficient antigen-presenting human  $\gamma\delta$  T cells comprising selecting  $\gamma\delta$  T cells out of human peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of antigen-presenting functions, and applying the antigen for uptake and presentation by these cells.  
5
2. The method of claim 1 wherein selecting  $\gamma\delta$  T cells is performed by magnetic cell sorting with antibodies to human V $\gamma$ V $\delta$ -T cell receptors by standard protocols.  
10
3. The method of claim 1 wherein selecting  $\gamma\delta$  T cells is performed by culturing freshly isolated peripheral blood lymphocytes in the presence of structurally defined small molecular weight non-peptide compounds that induce the selective expansion of V $\gamma$ 2V $\delta$ 2<sup>+</sup>-T cell receptor chain-expressing  $\gamma\delta$  T cells.  
15
4. The method of claim 3 wherein the structurally defined small molecular weight non-peptide compound is isopentenyl pyrophosphate.  
20
5. The method of claim 1 wherein the stimulus for induction of antigen-presenting functions is a small molecular weight non-peptide compound.
6. The method of claim 5 wherein the structurally defined small molecular

weight non-peptide compound is isopentenyl pyrophosphate.

7. The method of claim 5 wherein the structurally defined small molecular weight non-peptide compound is 4-hydroxy-3-methyl-but-2-enyl pyrophosphate.  
5
8. The method of claim 1 wherein the stimulus for induction of antigen-presenting functions is phytohemagglutinin.
- 10 9. The method of claim 1 wherein the antigen applied is in the form of defined proteins, undefined protein mixtures, or crude or enriched extracts from tumor or infected cells.
- 15 10. The method of claim 1 wherein the antigen applied is a pathogen- or tumor cell-derived peptide.
11. The method of claim 9 wherein the antigen is a pathogen-derived protein, which is applied in the form of DNA or RNA encoding it under conditions allowing endogenous expression of said pathogen-derived proteins.  
20
12. The method of claim 11 wherein the DNA or RNA is purified DNA or RNA or a delivery vector containing such DNA or RNA.
13. Efficient antigen-presenting human  $\gamma\delta$  T cells prepared by the method of

claim 1.

14. Use of efficient antigen-presenting human  $\gamma\delta$  T cells for the manufacture of a medicament for immunotherapy or vaccination.
- 5
15. Use of efficient antigen-presenting human  $\gamma\delta$  T cells for the manufacture of a medicament for treating tumors or chronic or recurrent infectious diseases.
- 10 16. The use according to claim 15 wherein the medicament is formulated for single or repeated applications of said  $\gamma\delta$  T cells by intradermal, subcutaneous, intramuscular, intravenous, mucosal or submucosal injection.
- 15 17. Use of efficient antigen-presenting human  $\gamma\delta$  T cells for the manufacture of a medicament for vaccinating against tumors or agents inducing chronic or recurrent infectious diseases.
- 20 18. Use of efficient antigen-presenting human  $\gamma\delta$  T cell-targeting vaccines for the manufacture of a medicament for immunotherapy or vaccination.
19. Use of  $\gamma\delta$  T cell-targeting vaccines for the manufacture of a medicament for vaccinating against tumors or agents inducing infectious or non-infectious diseases.

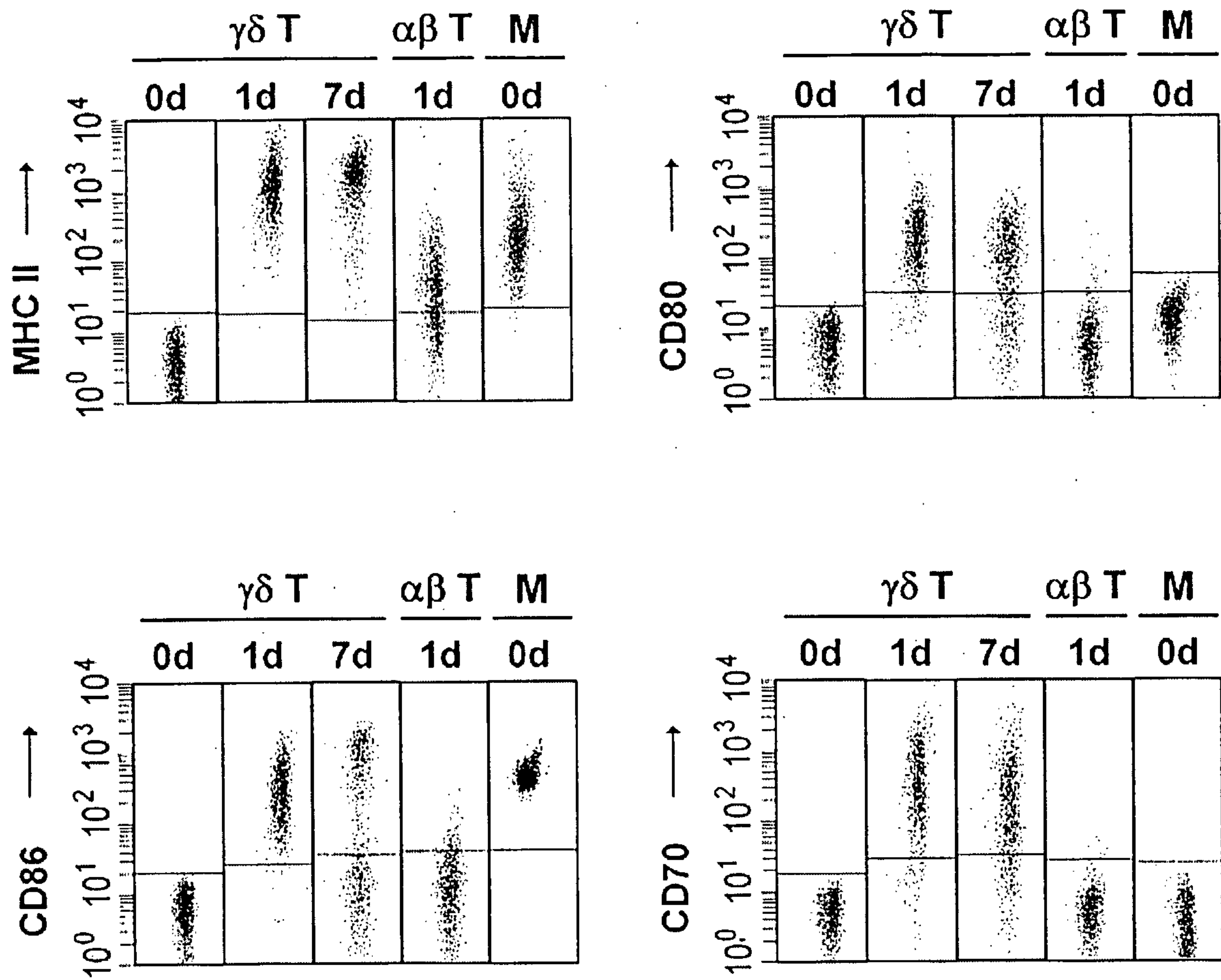
20. The use according to claim 19 wherein  $\gamma\delta$  T cell-targeting vaccines are hybrid compounds composed of vaccine agents and  $\gamma\delta$  T cell-targeting molecules.
- 5
21. The use according to claim 20 wherein  $\gamma\delta$  T cell-targeting molecules are antibodies or ligands specific for endocytic receptors on  $\gamma\delta$  T cells.
22. The use according to claim 20 wherein  $\gamma\delta$  T cell-targeting molecules are antibodies or ligands specific for CD1 1b and CD205.
- 10
23. The use according to claim 20 wherein vaccine agents are proteins against which an immune protection is desired.
- 15
24. The use according to claim 20 wherein administration of  $\gamma\delta$  T cell-targeting vaccines entails repeated injections or oral administrations.
- 20
25. A method of identification of novel tumor-associated or pathogen-derived antigens comprising selecting  $\gamma\delta$  T cells out of human peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of antigen-presenting functions, applying fractions of an undefined protein mixture, or crude or enriched extracts from tumor and infected cells, or RNA or DNA libraries derived from tumors and infectious agents, testing for *in vitro* activation of autologous naïve  $\alpha\beta$  T cells, and

selecting protein samples with increased antigenicity.

26. A method of diagnosing the immune competence of a patient comprising selecting  $\gamma\delta$  T cells out of the patient's peripheral blood mononuclear cells, treating the selected cells with a stimulus for induction of antigen-presenting functions, applying the antigen for which the immune competence has to be determined, and testing for *in vitro* activation of autologous  $\alpha\beta$  T cells.

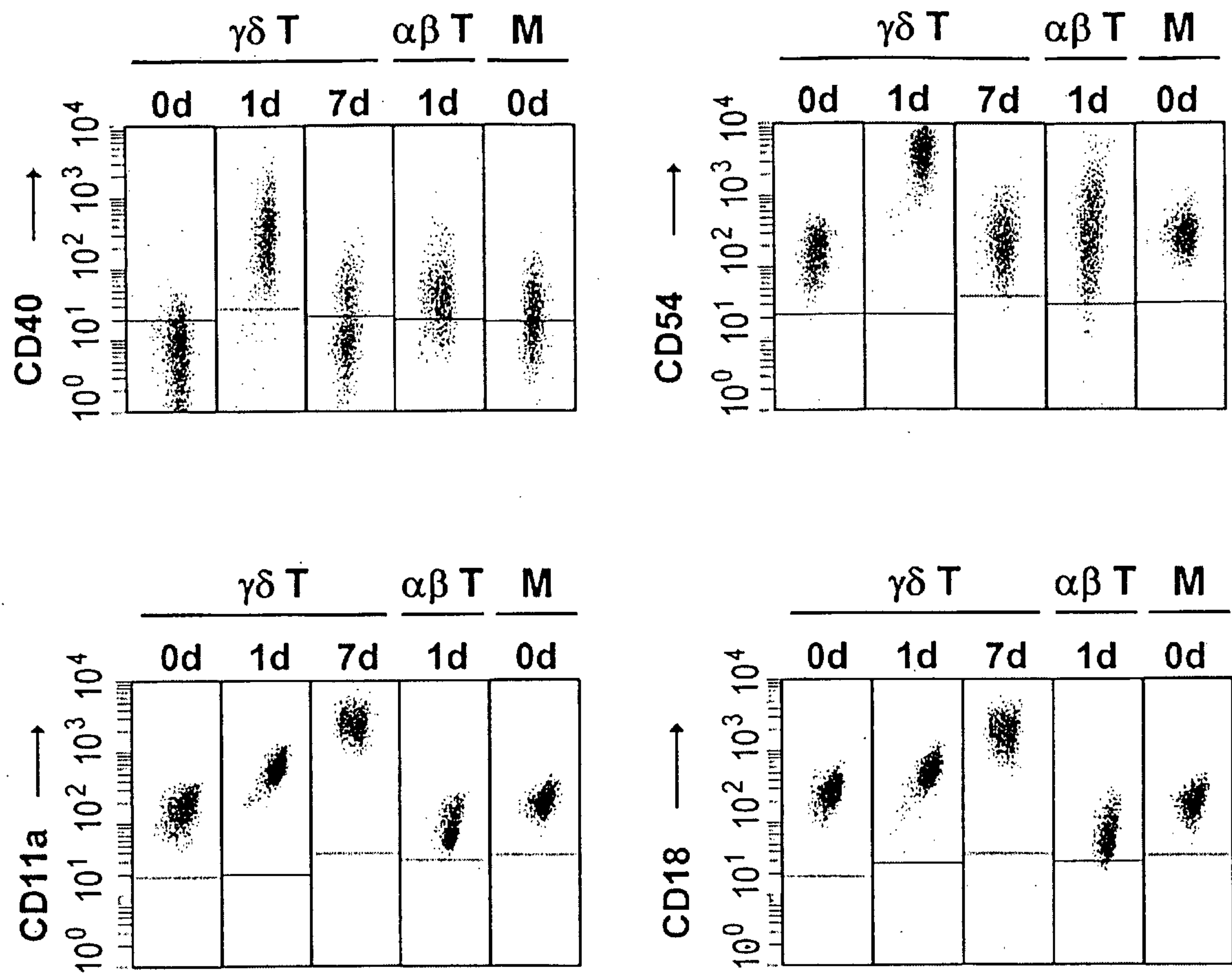
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Fig. 1A



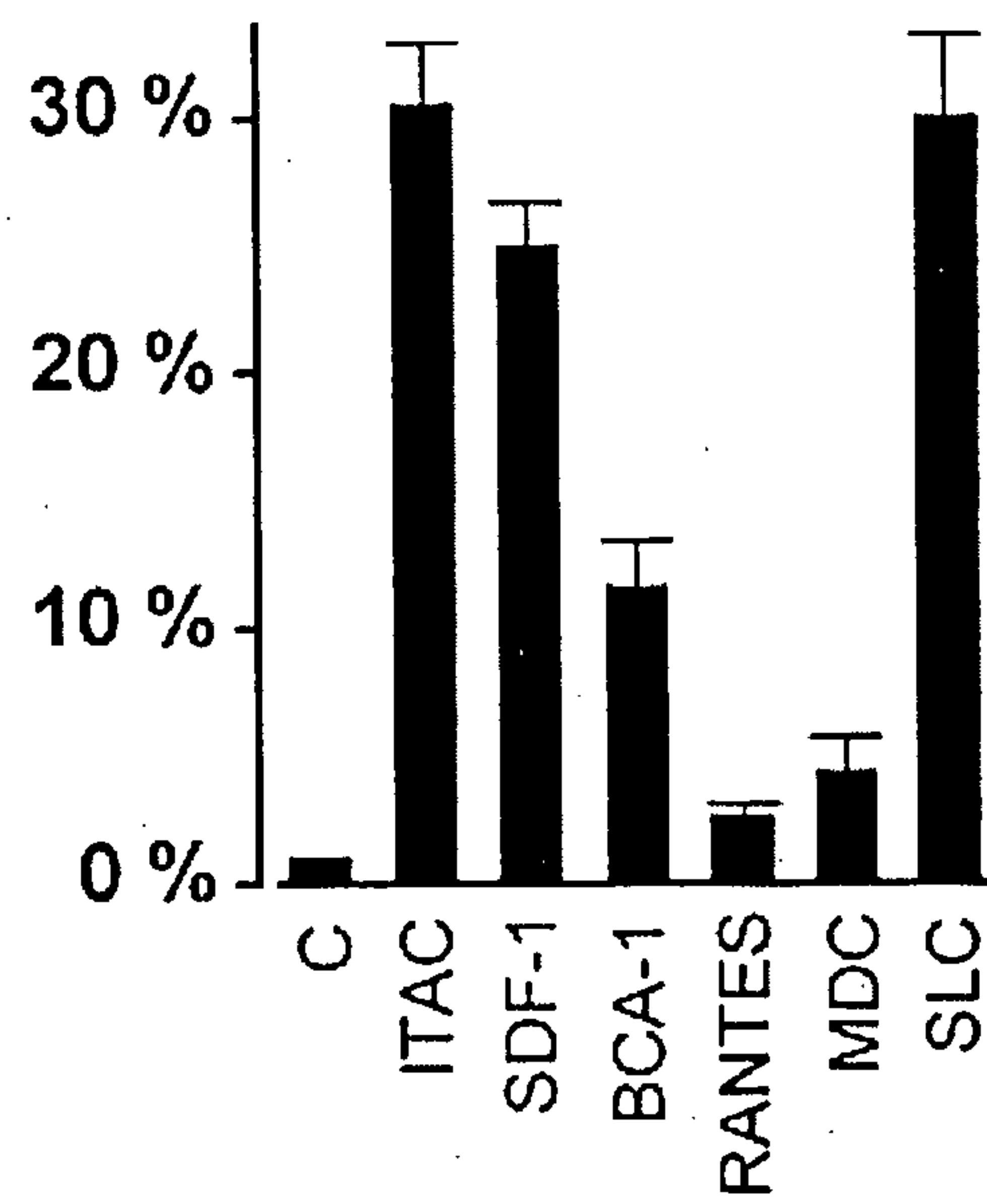
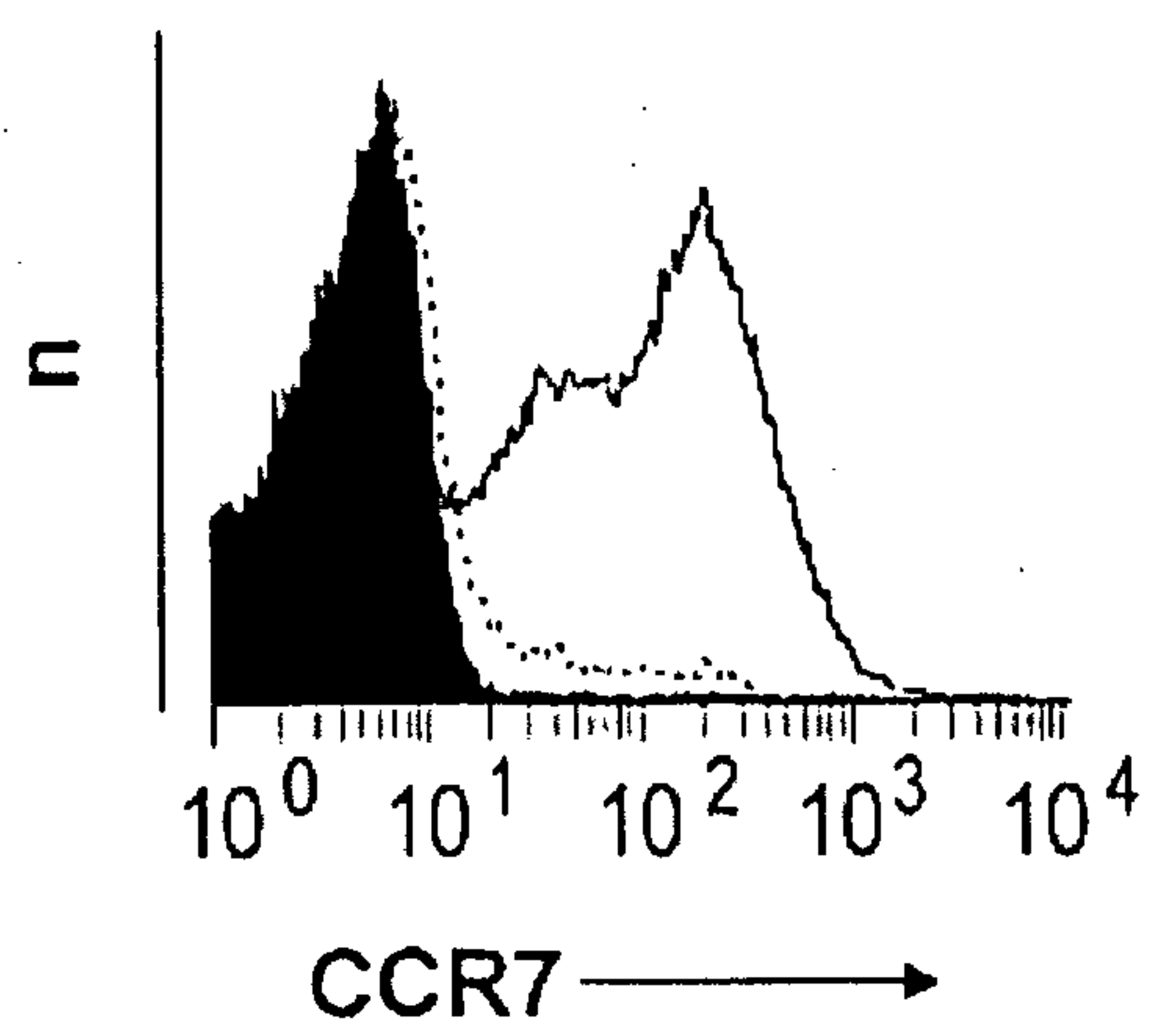
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Fig. 1B



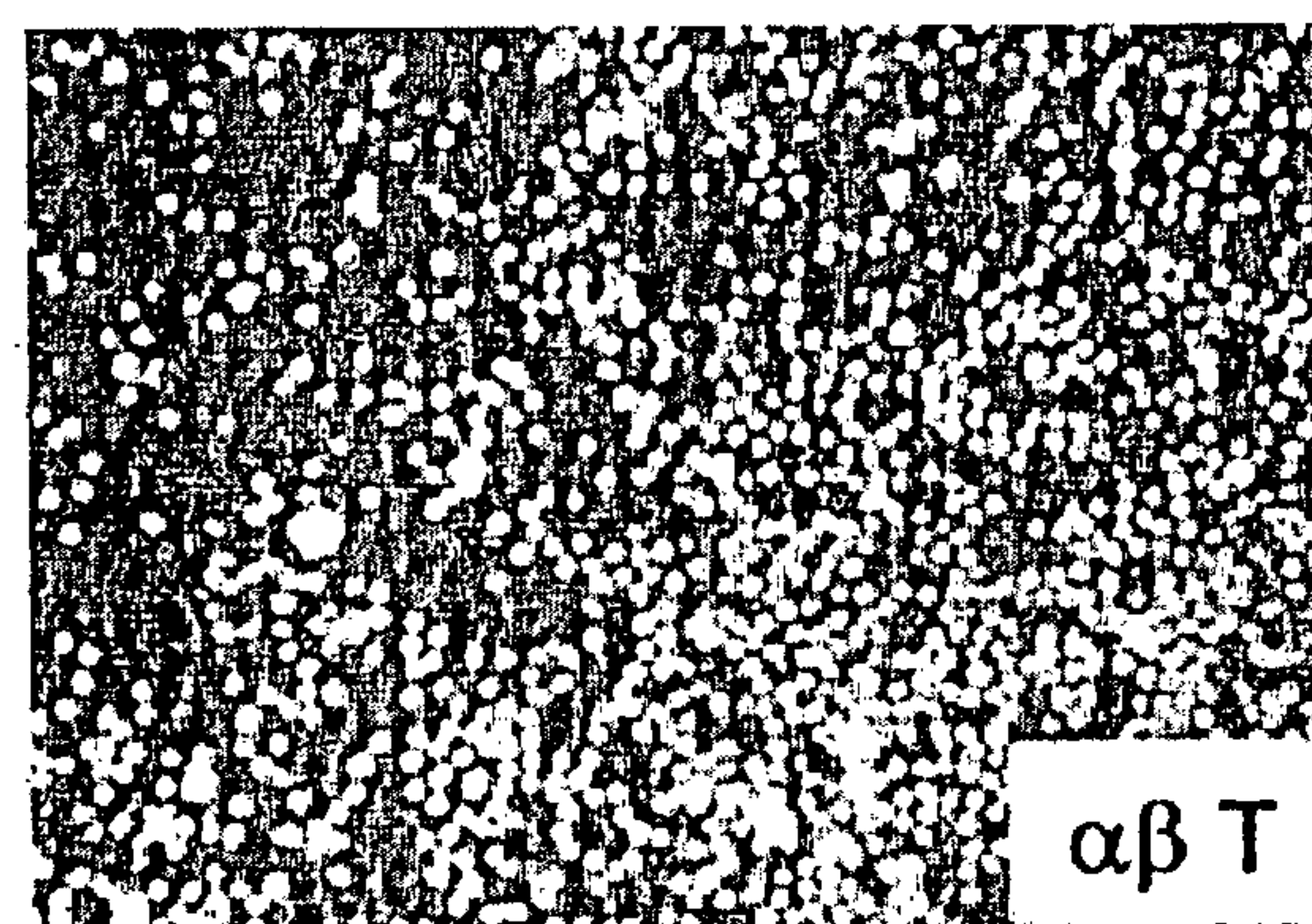
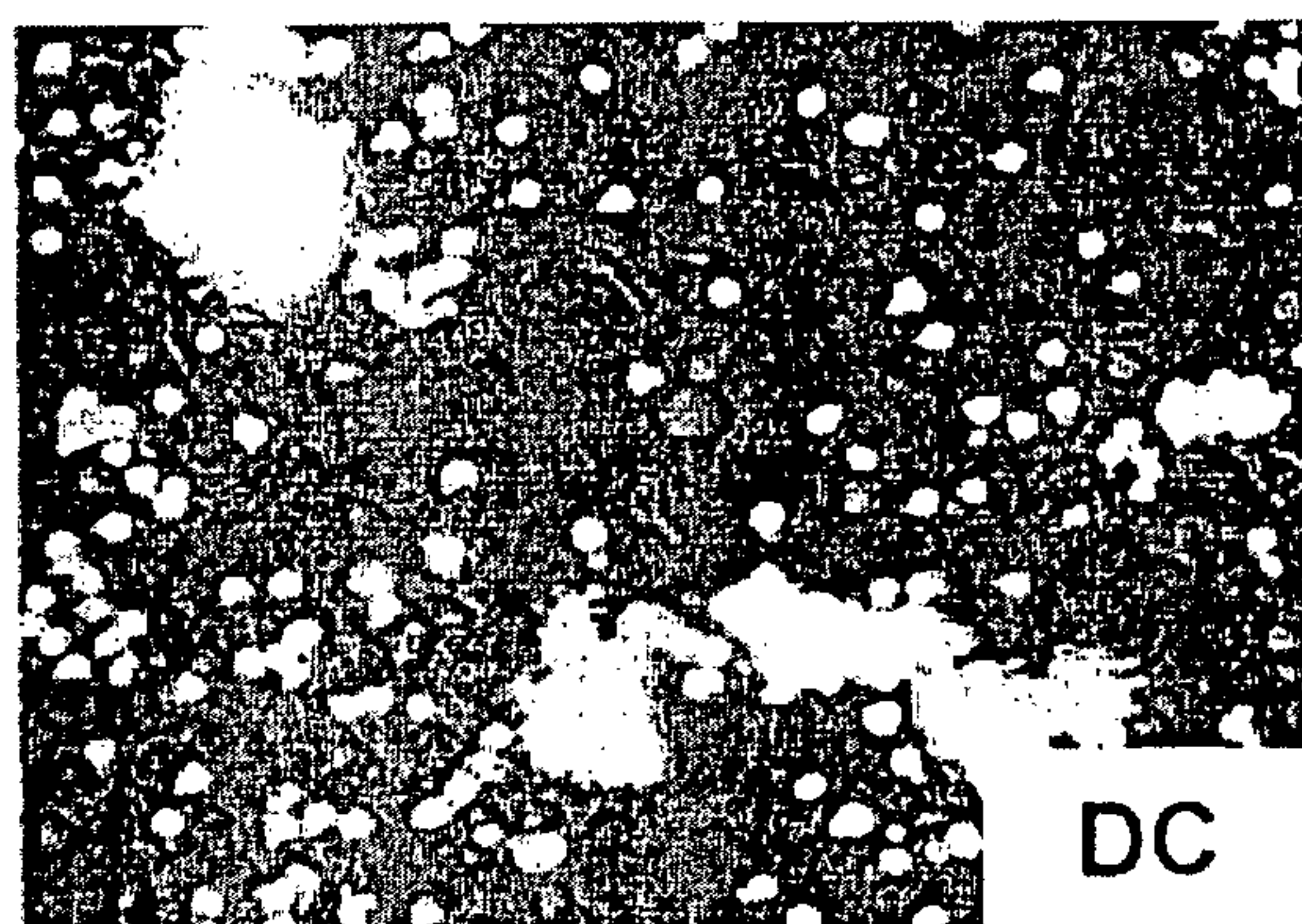
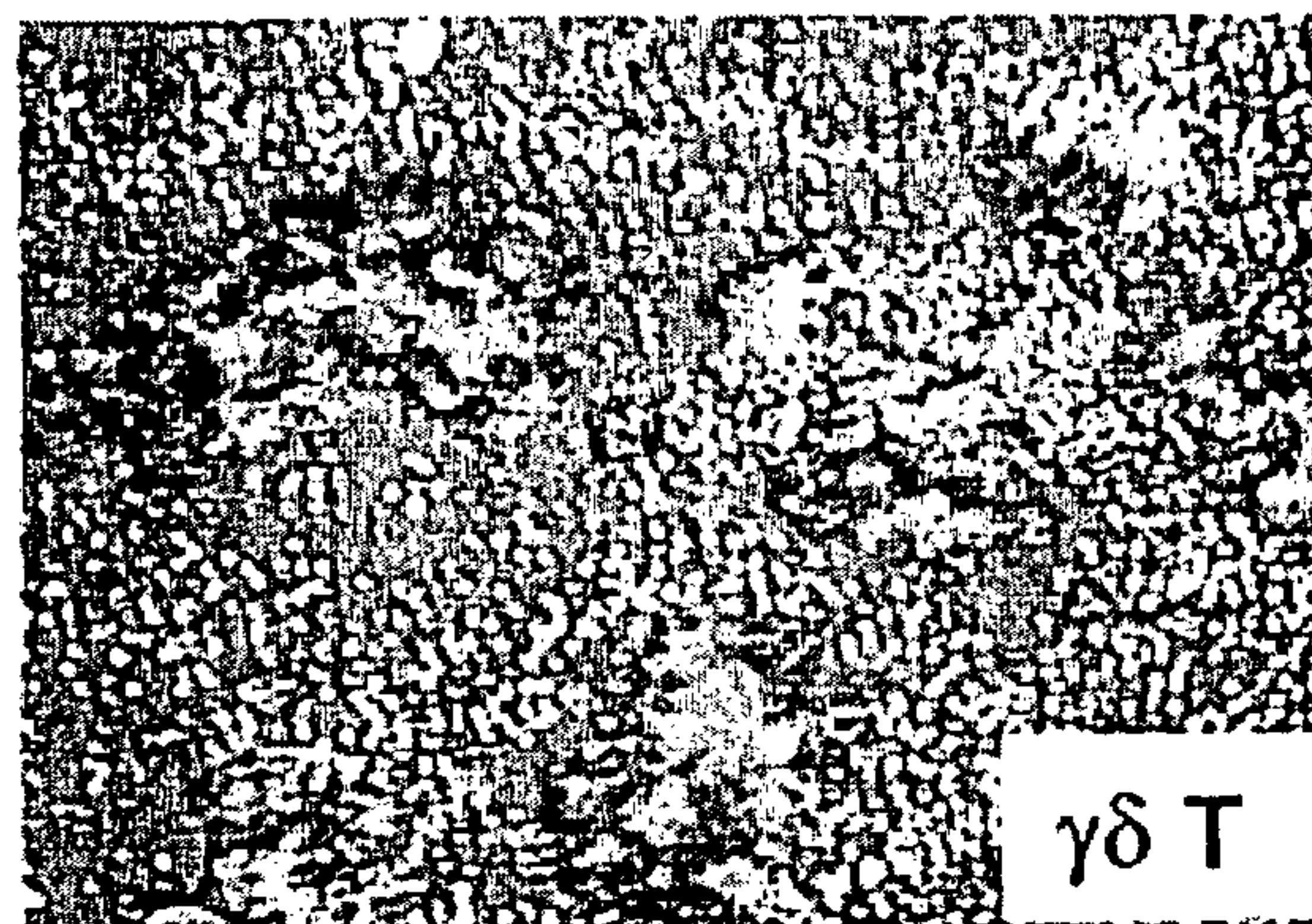
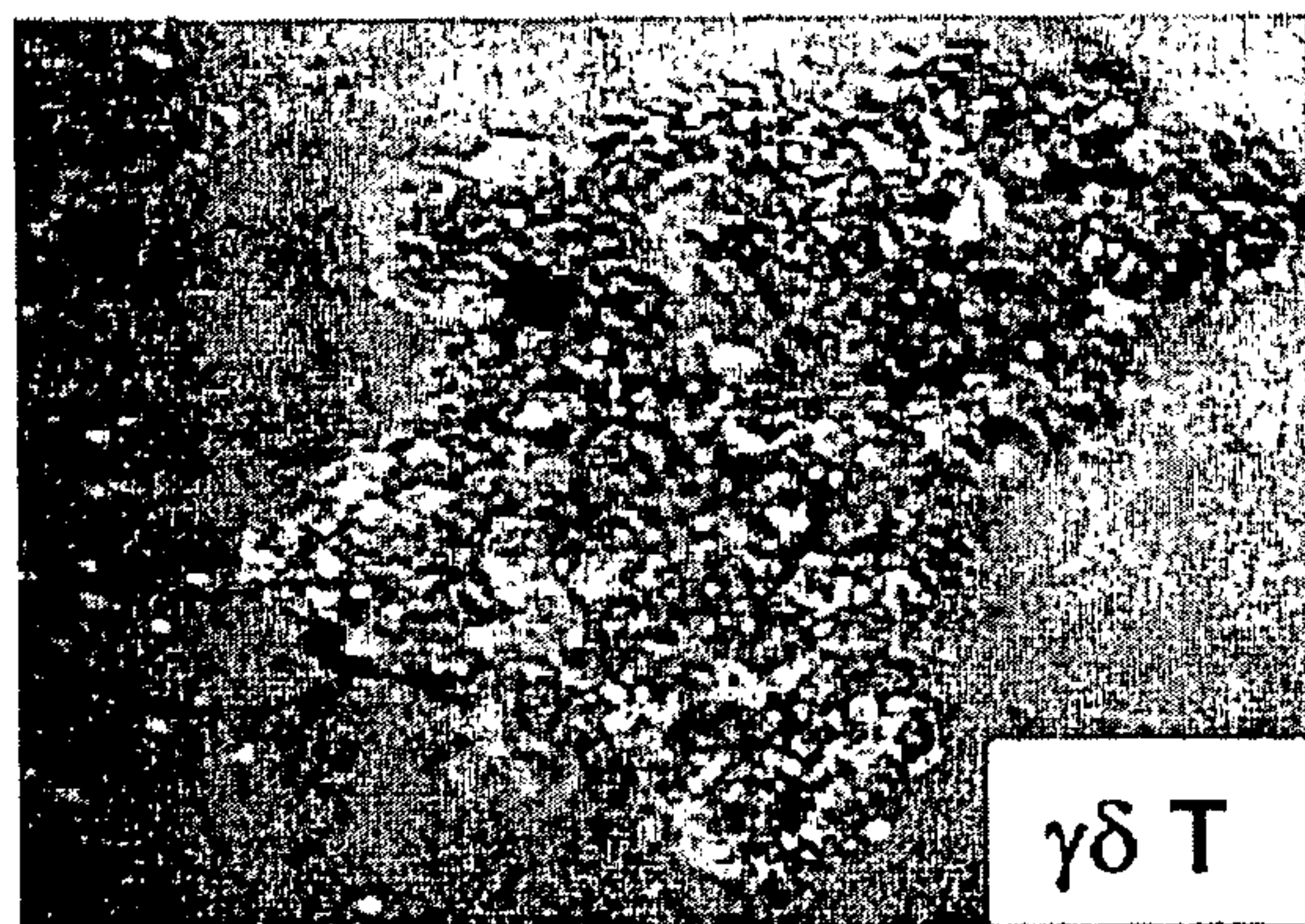
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Fig. 2

**A****B**

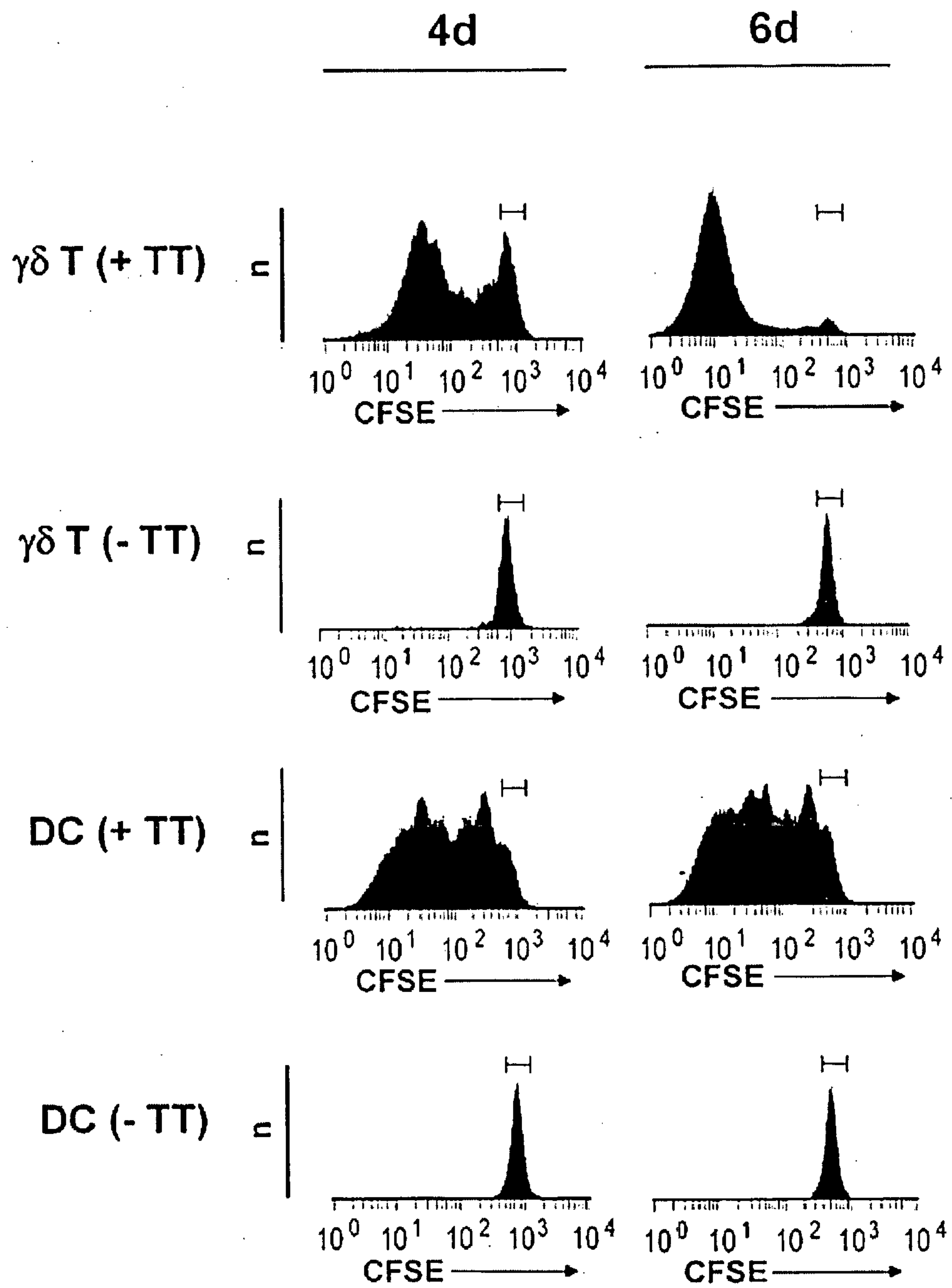
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Fig. 3



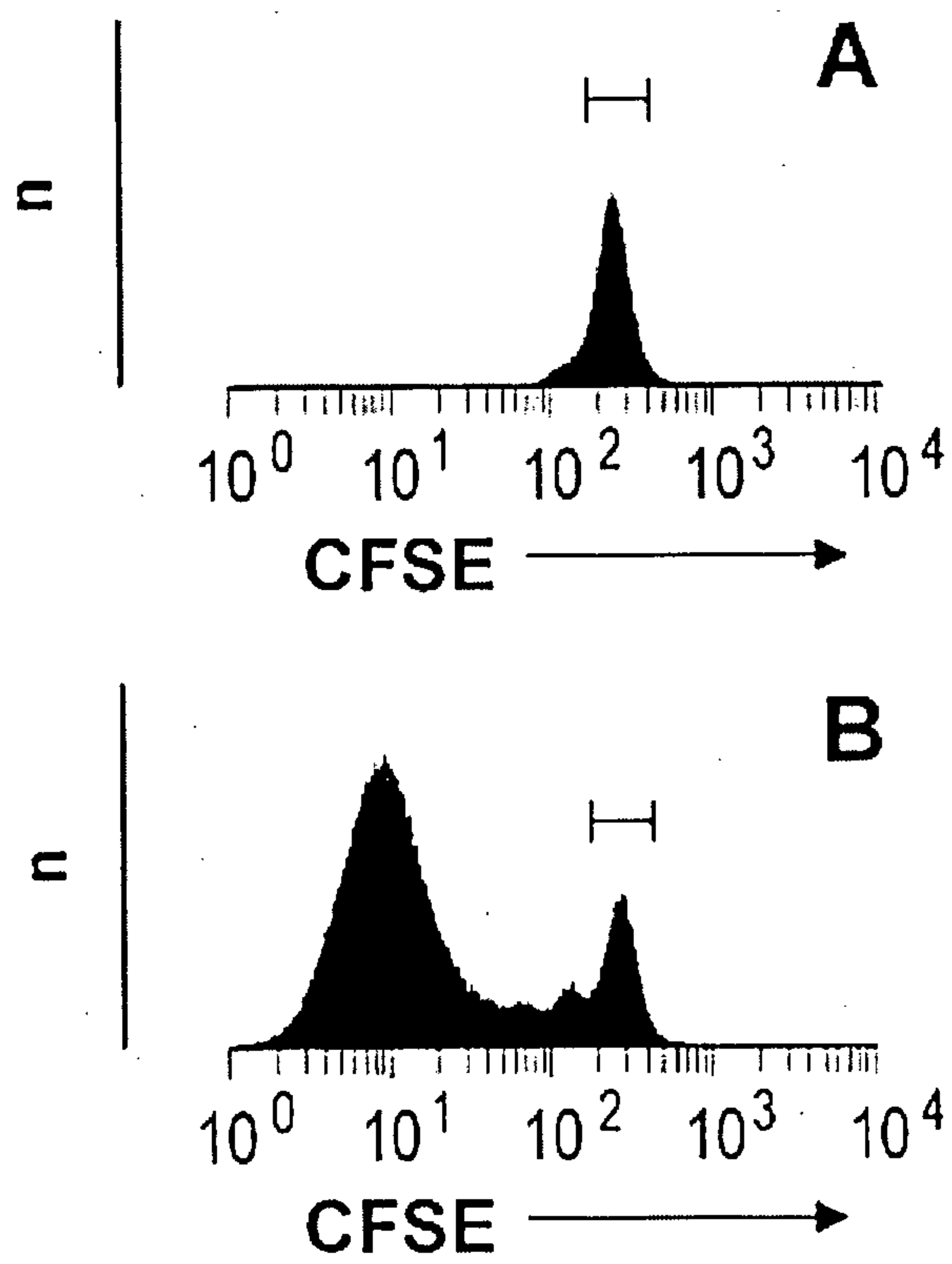
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Fig. 4



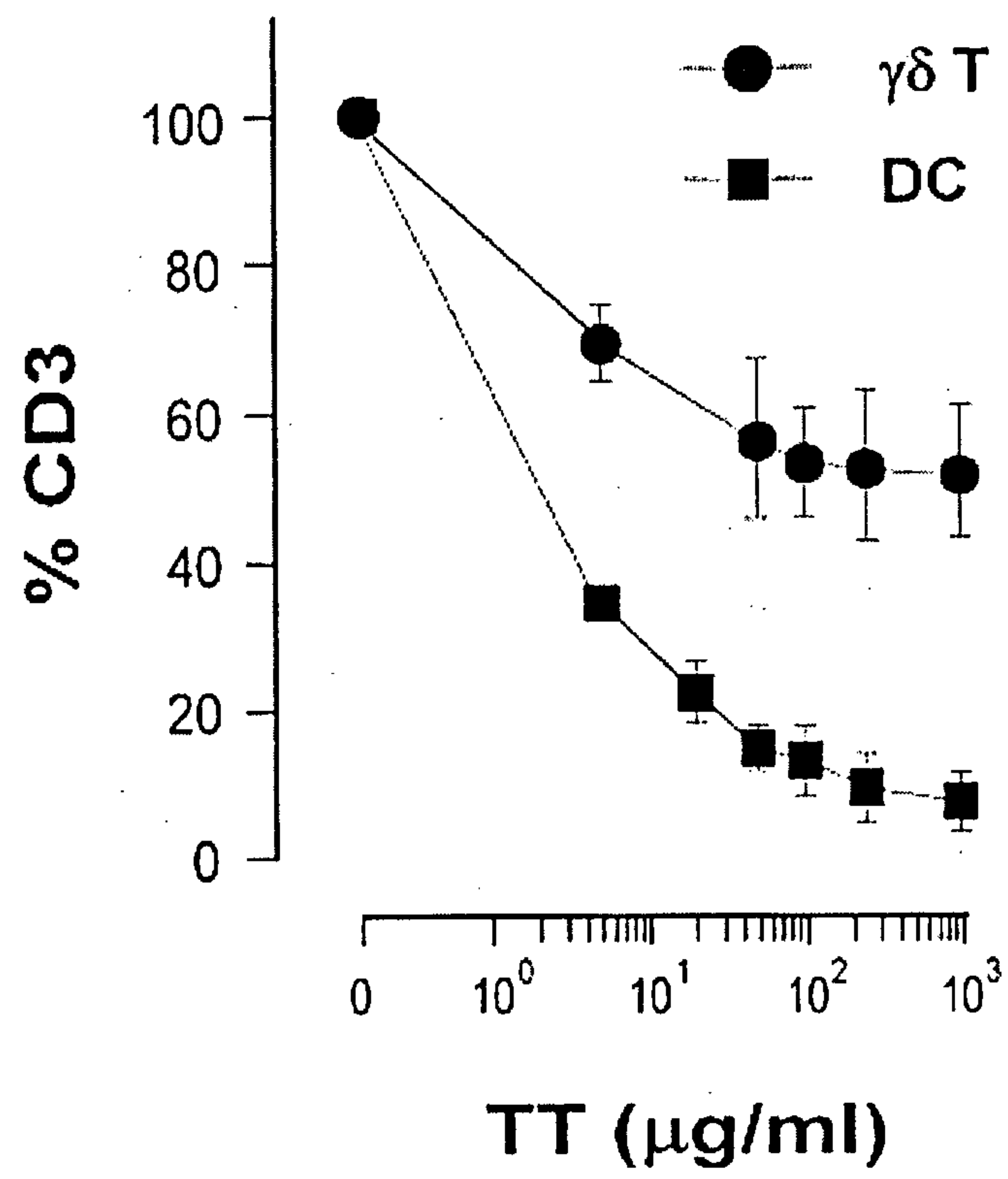
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Fig. 5



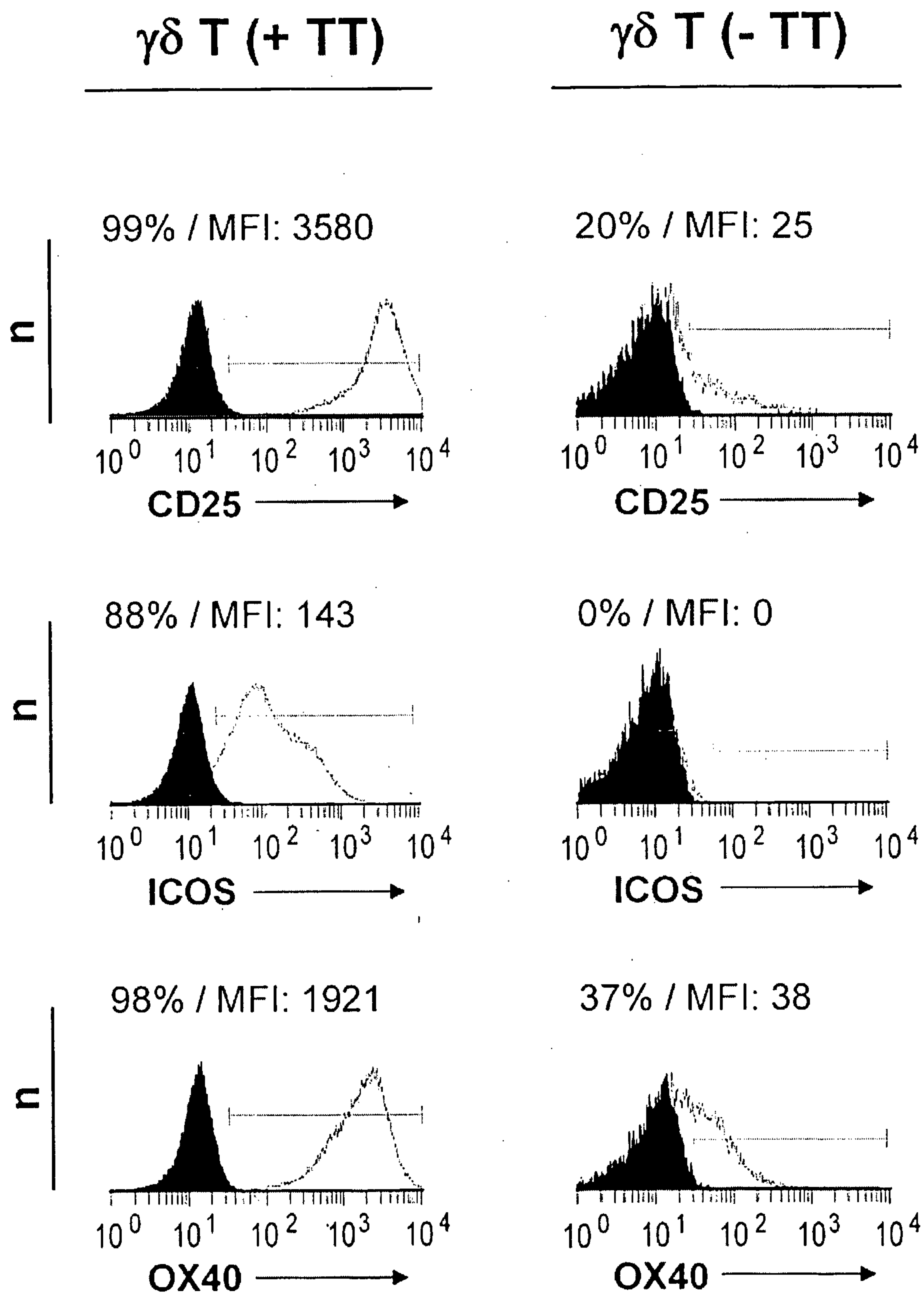
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Fig. 6



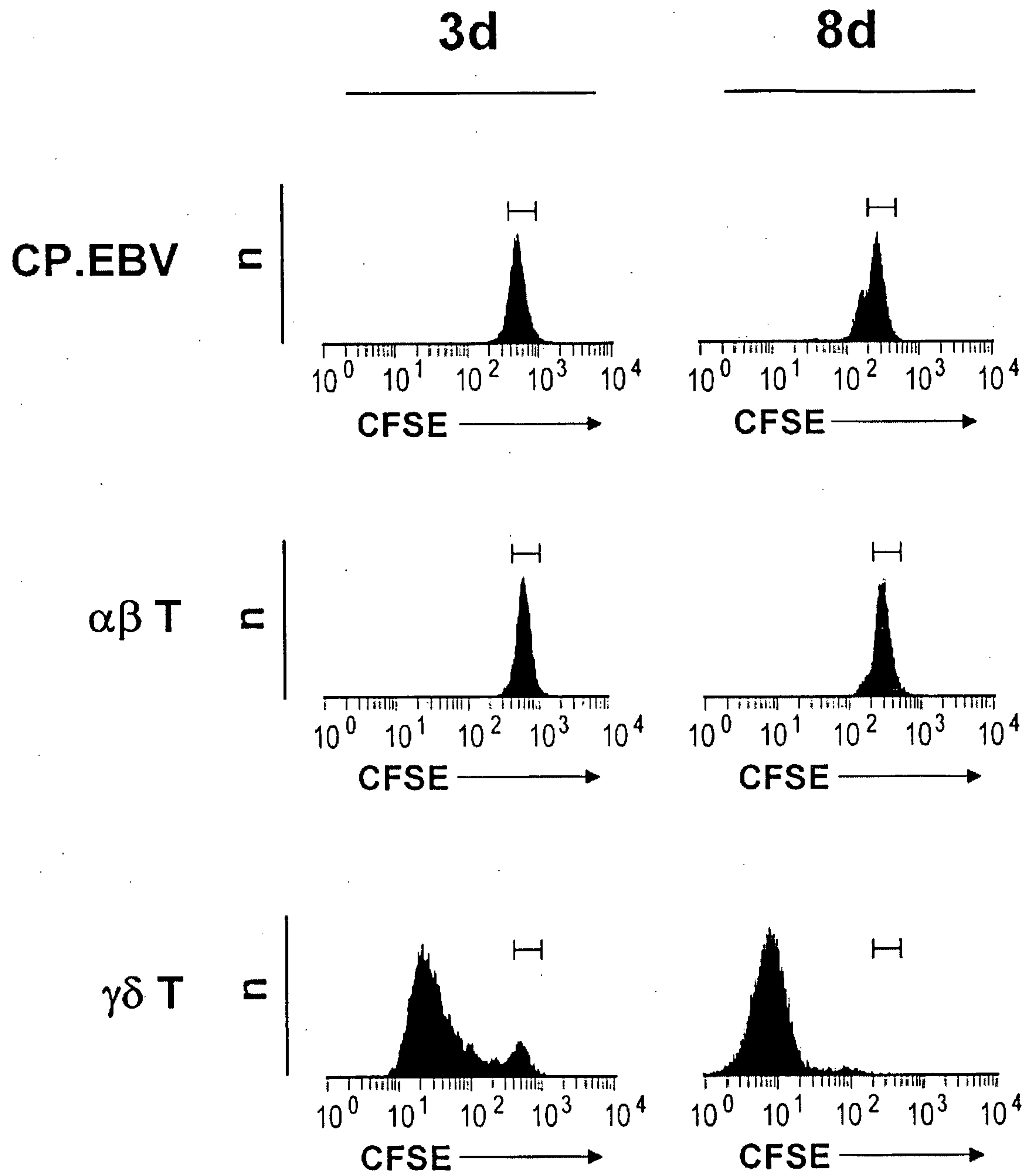
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Fig. 7



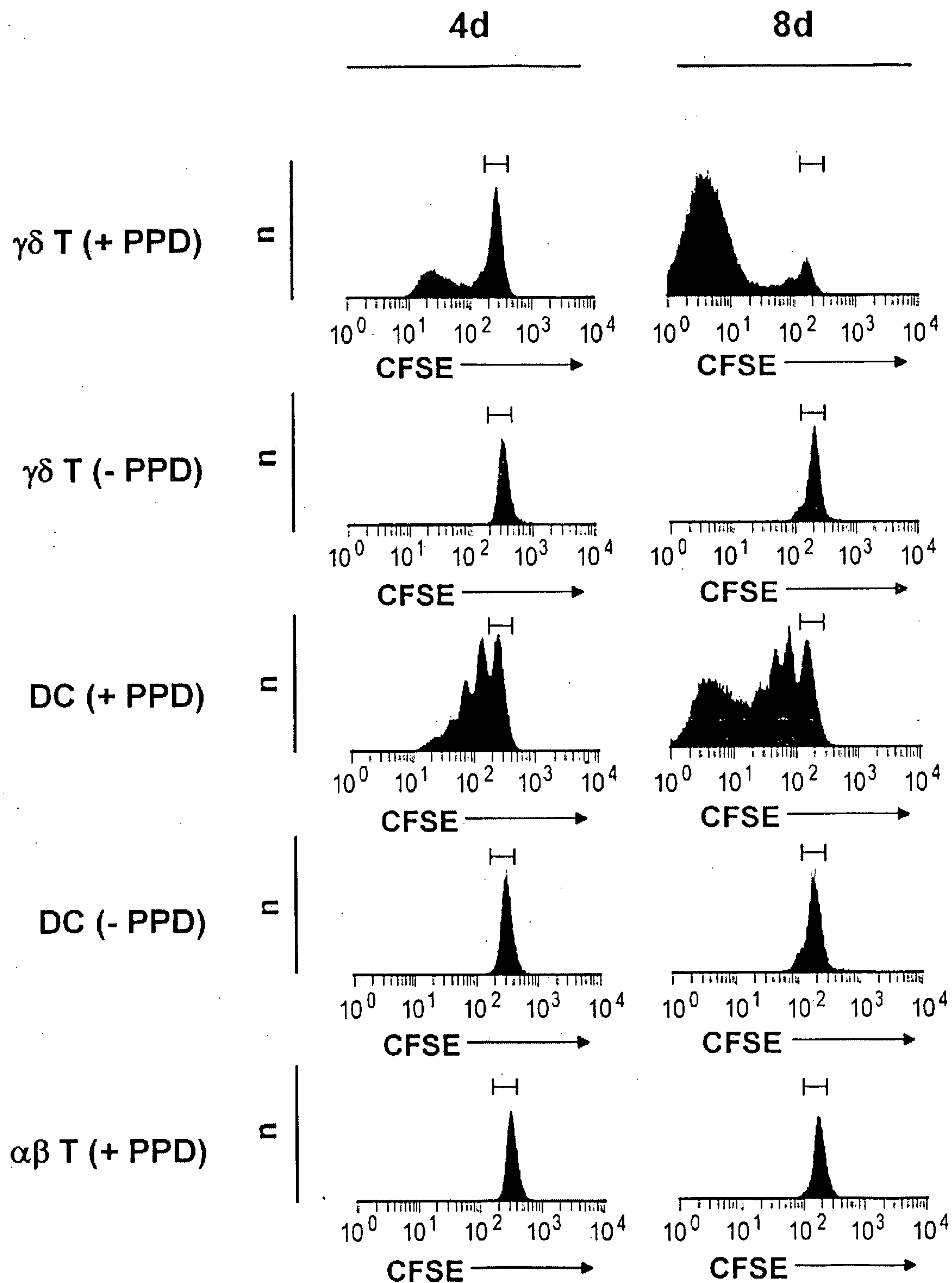
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Fig. 8



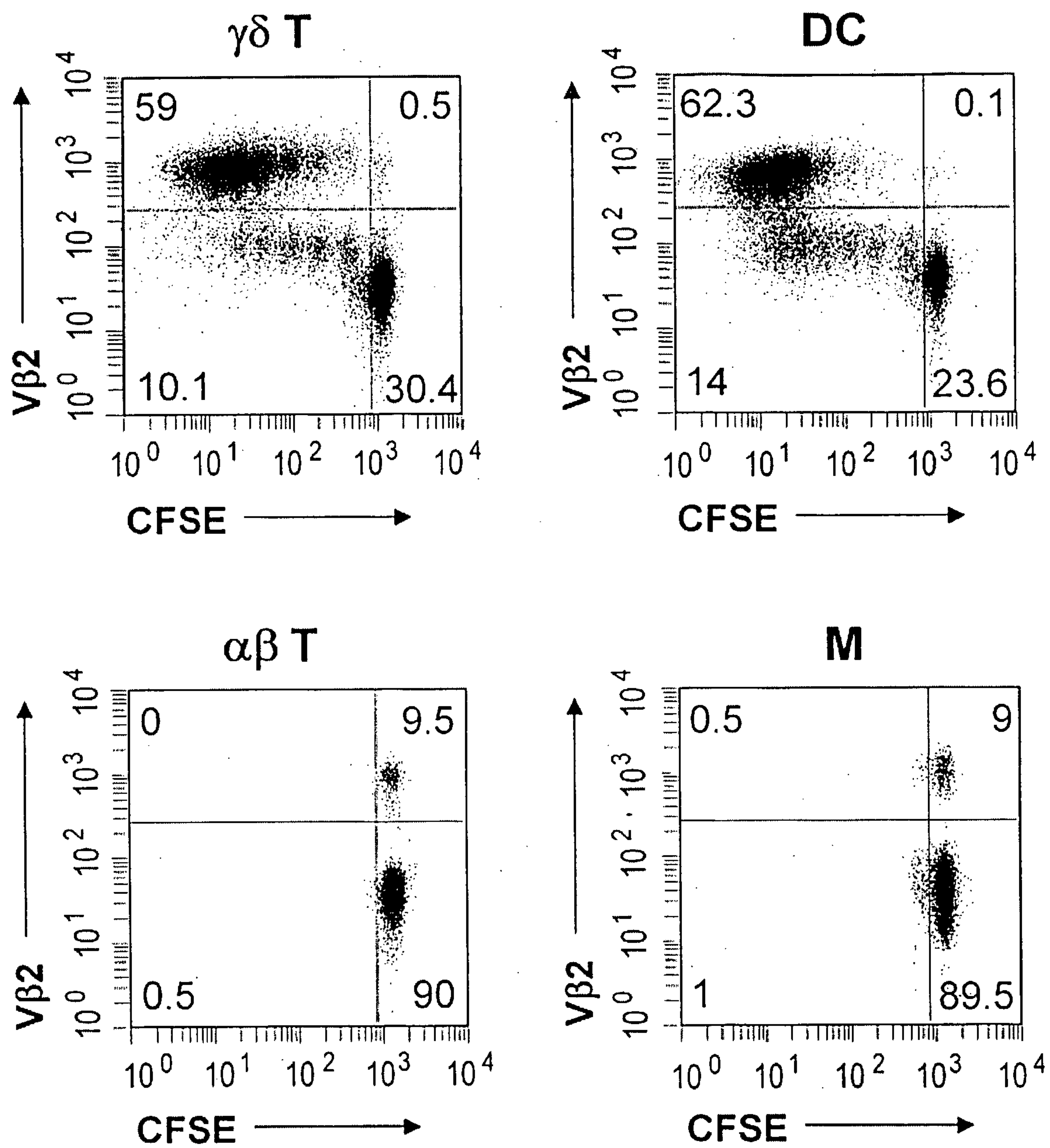
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Fig. 9



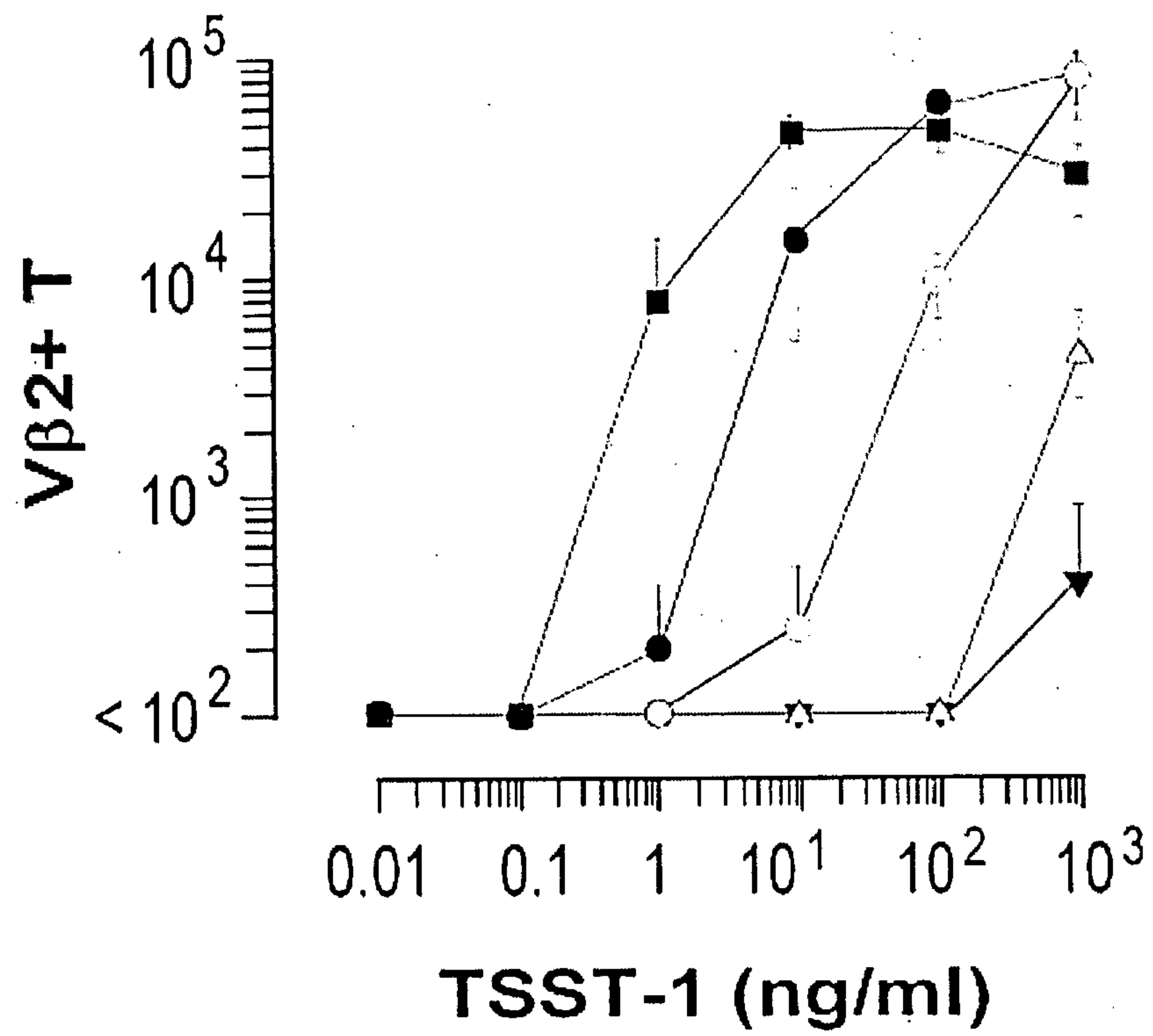
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Fig. 10



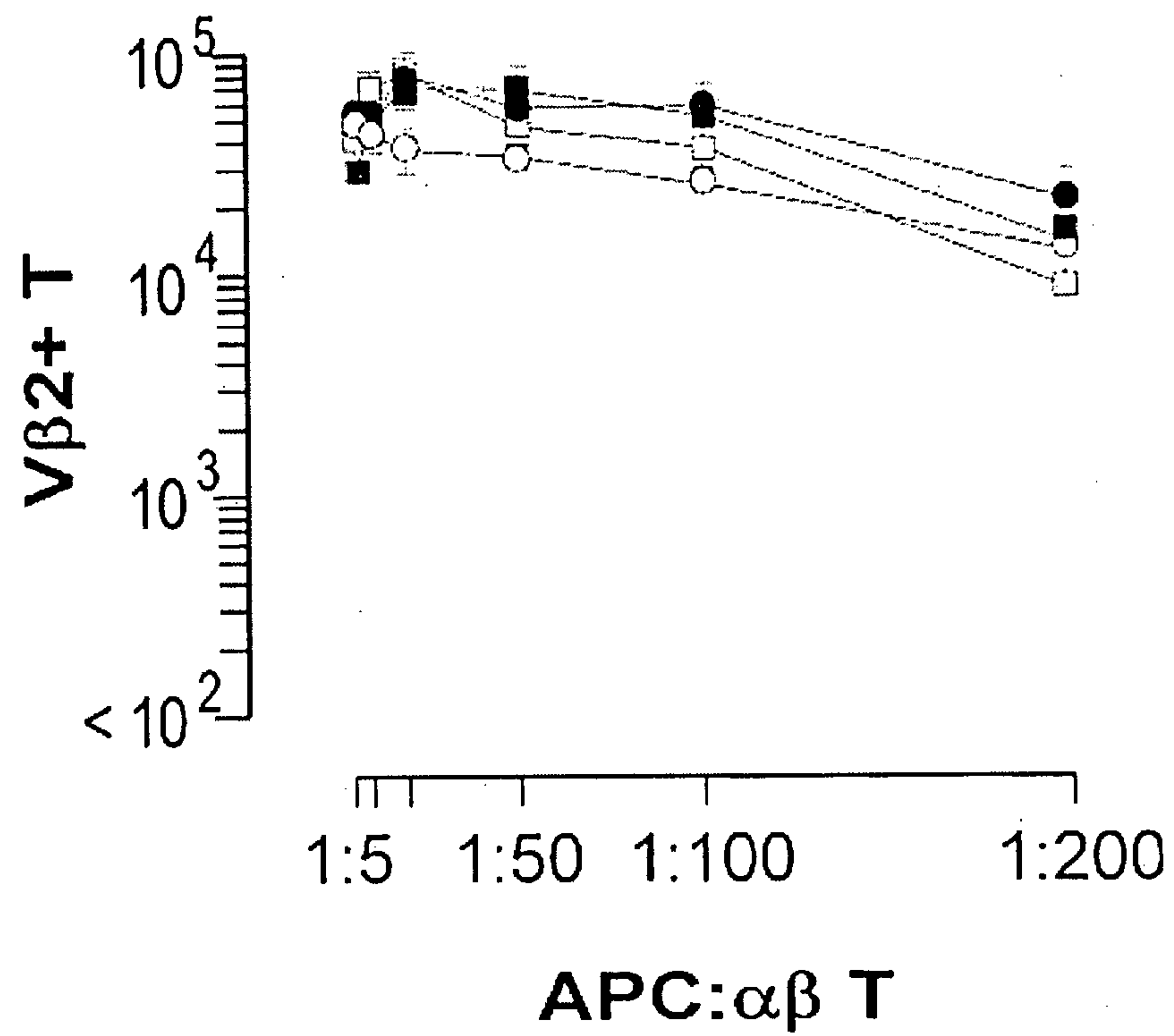
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Fig. 11



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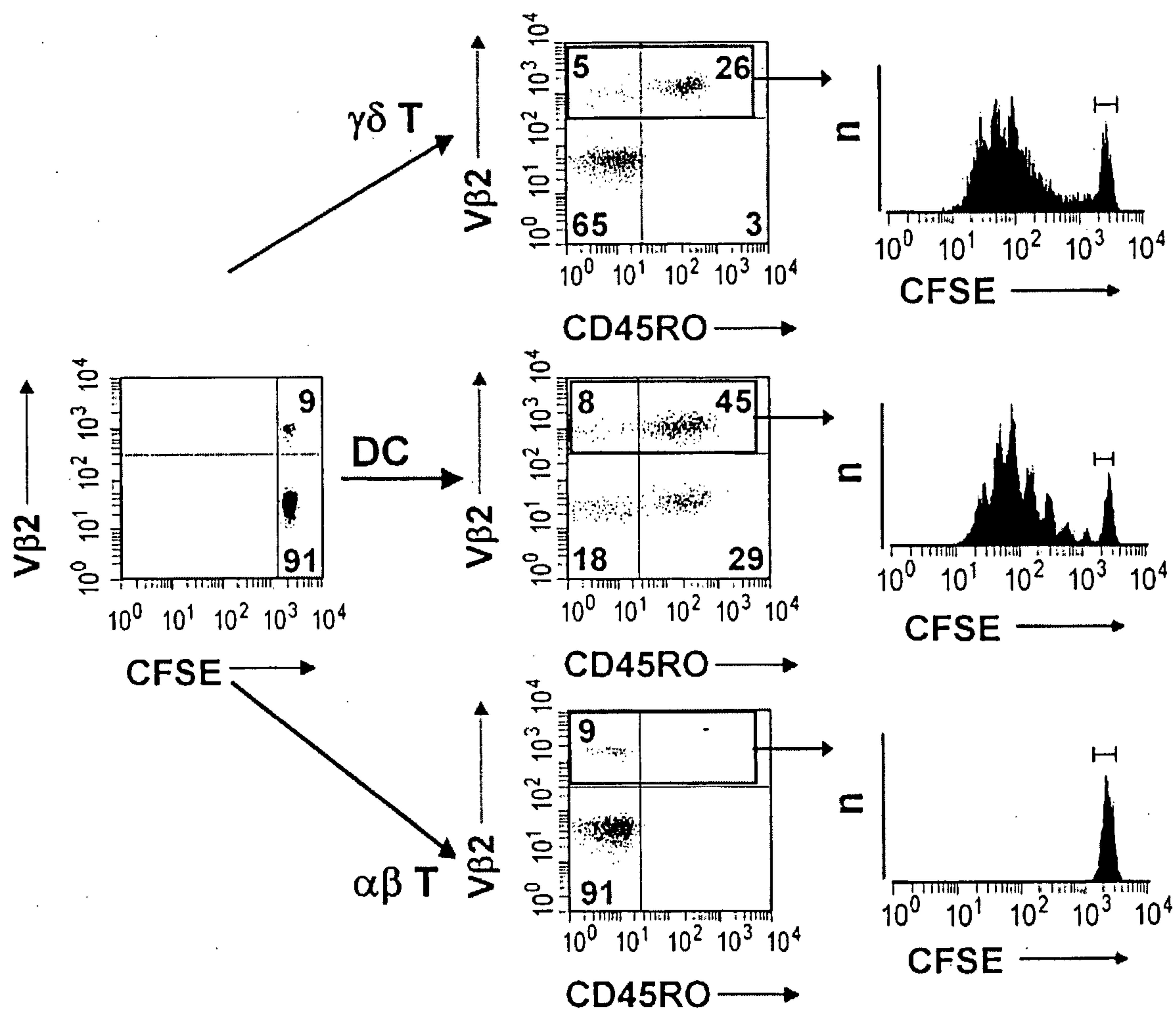
Fig. 12



- $\gamma\delta$  T, 1  $\mu$ g/ml TSST-1
- $\gamma\delta$  T, 100 ng/ml TSST-1
- DC, 1  $\mu$ g/ml TSST-1
- DC, 100 ng/ml TSST-1

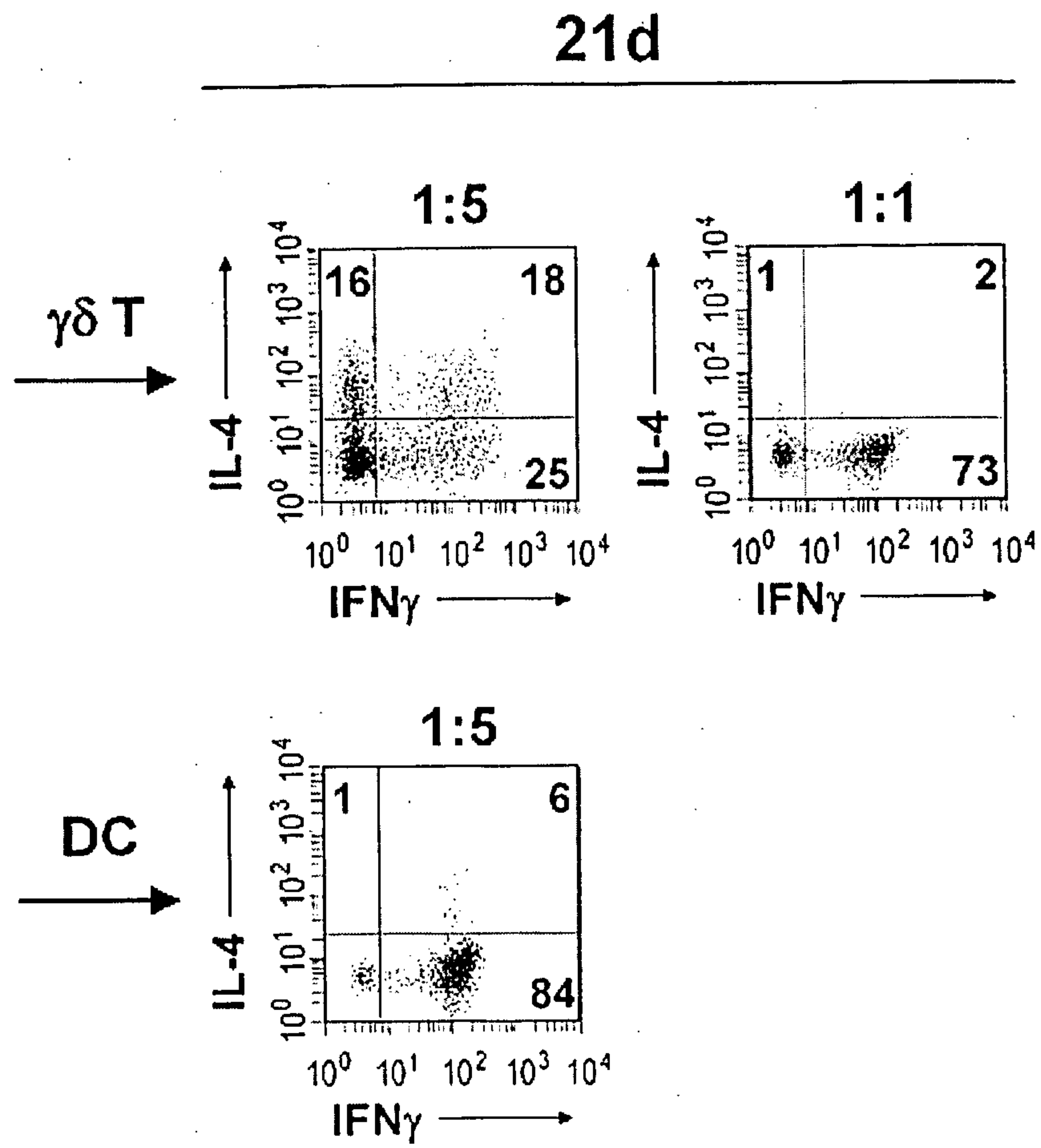
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Fig. 13A

**4d (1:5)**

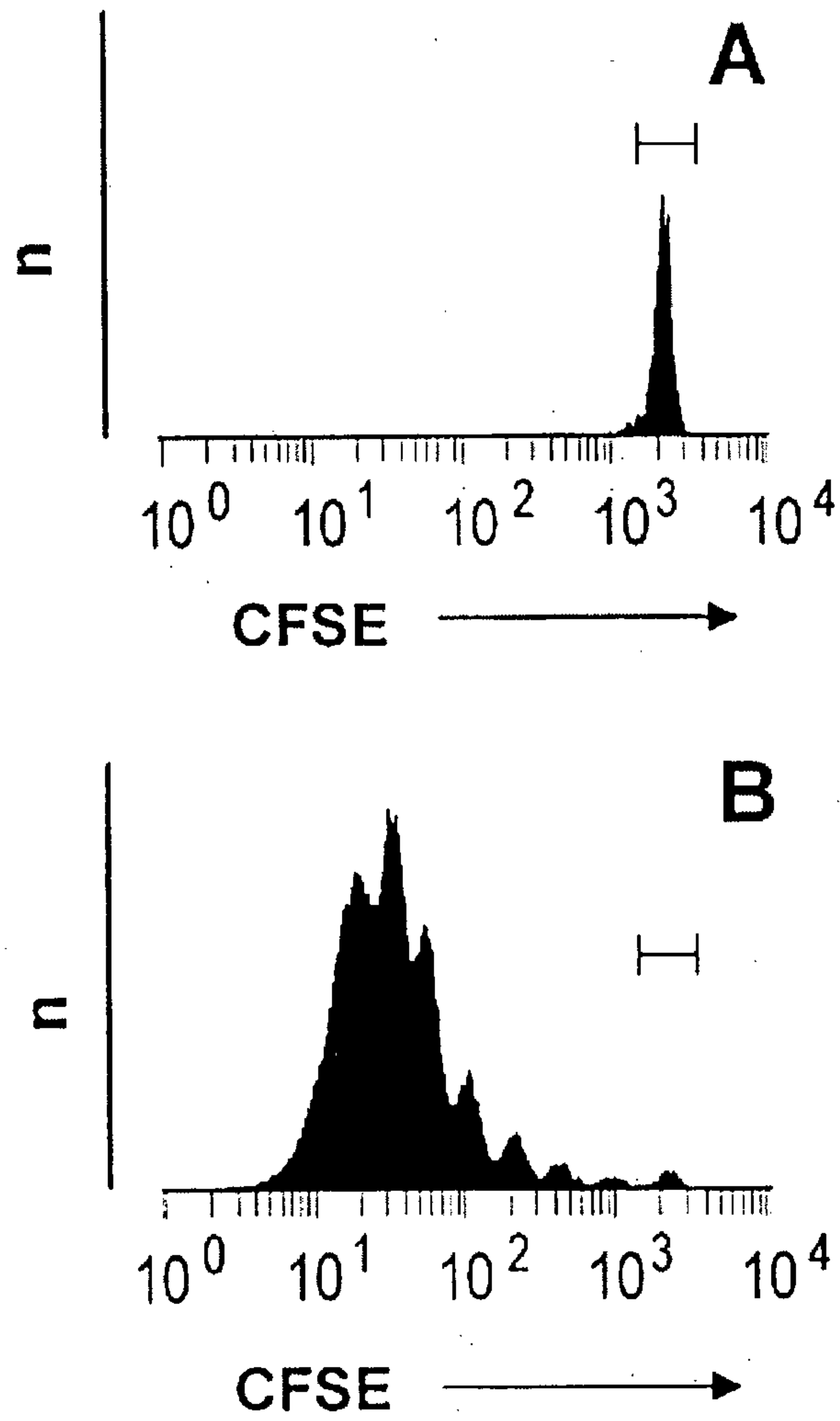
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Fig. 13B



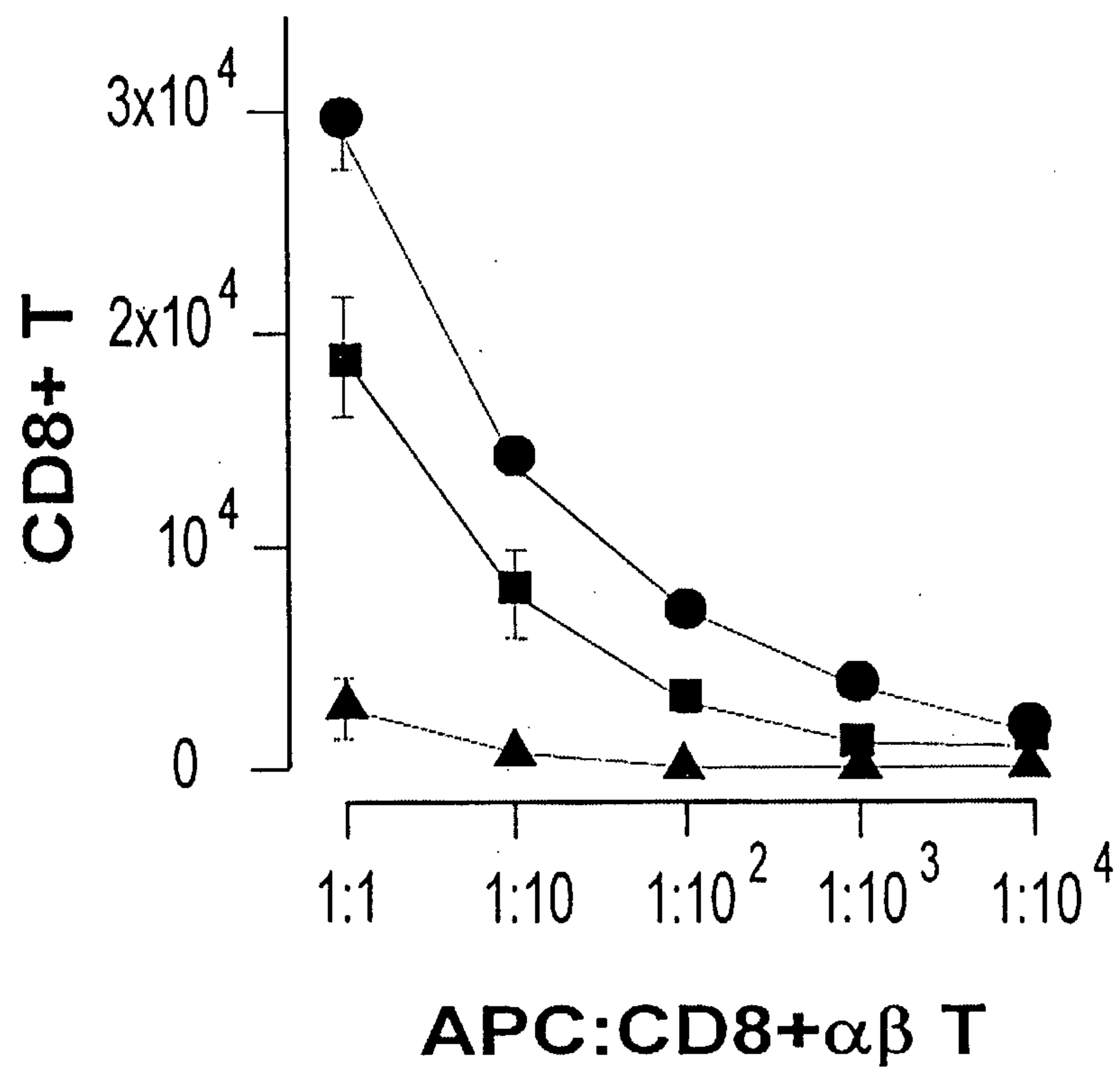
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Fig. 14



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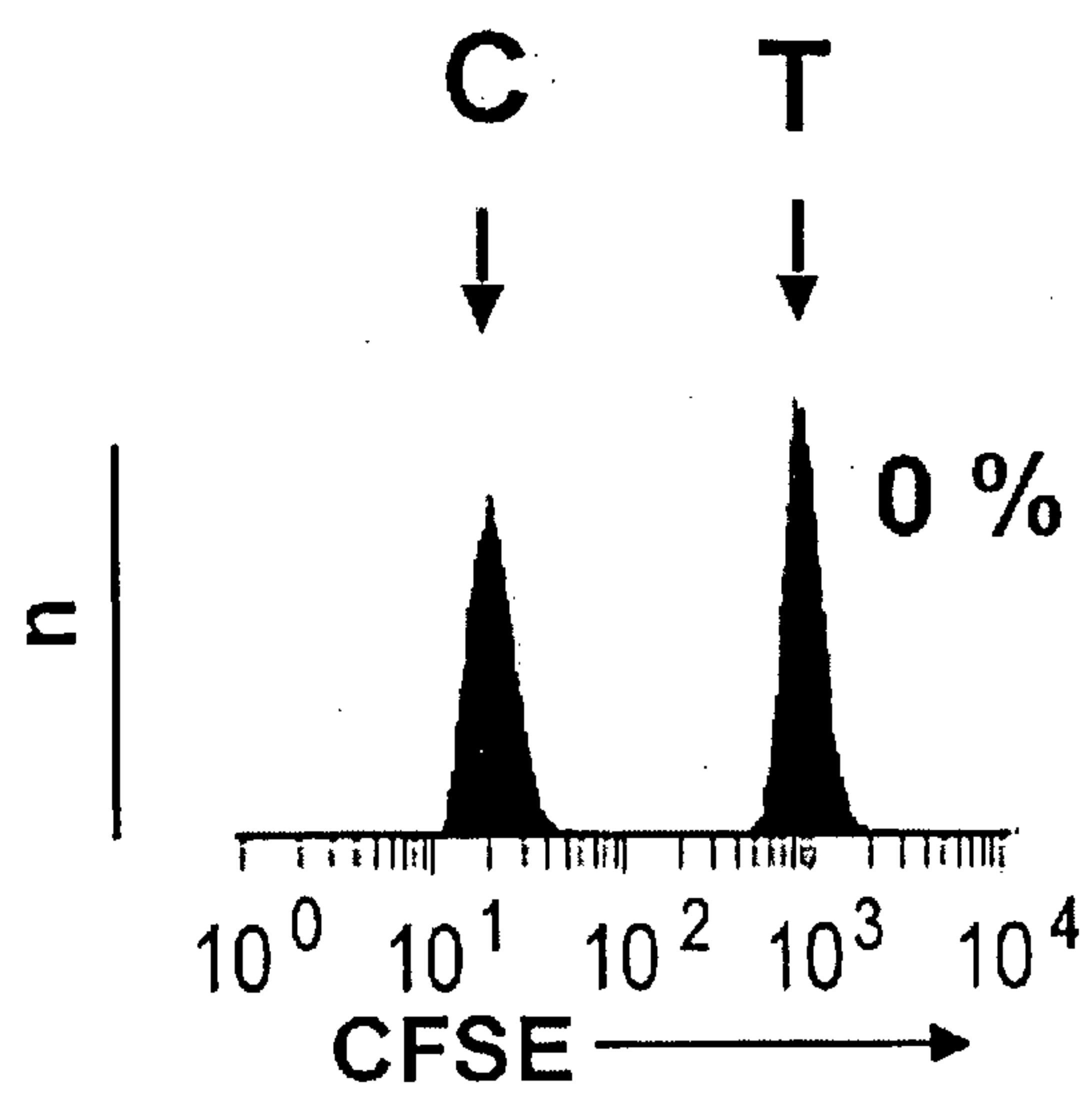
Fig. 15



—●— γδ T  
—■— DC  
—▲— αβ T

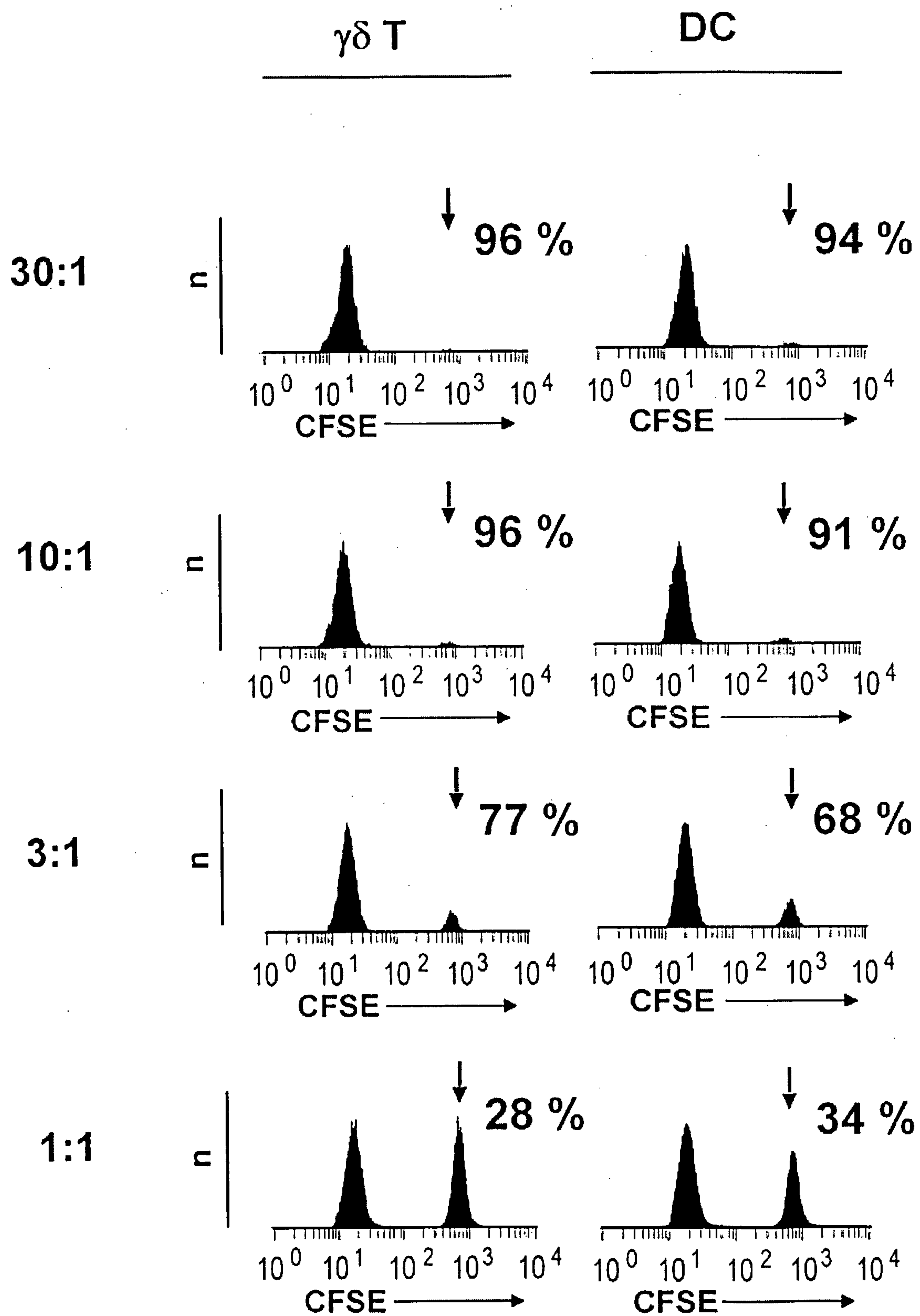
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Fig. 16A



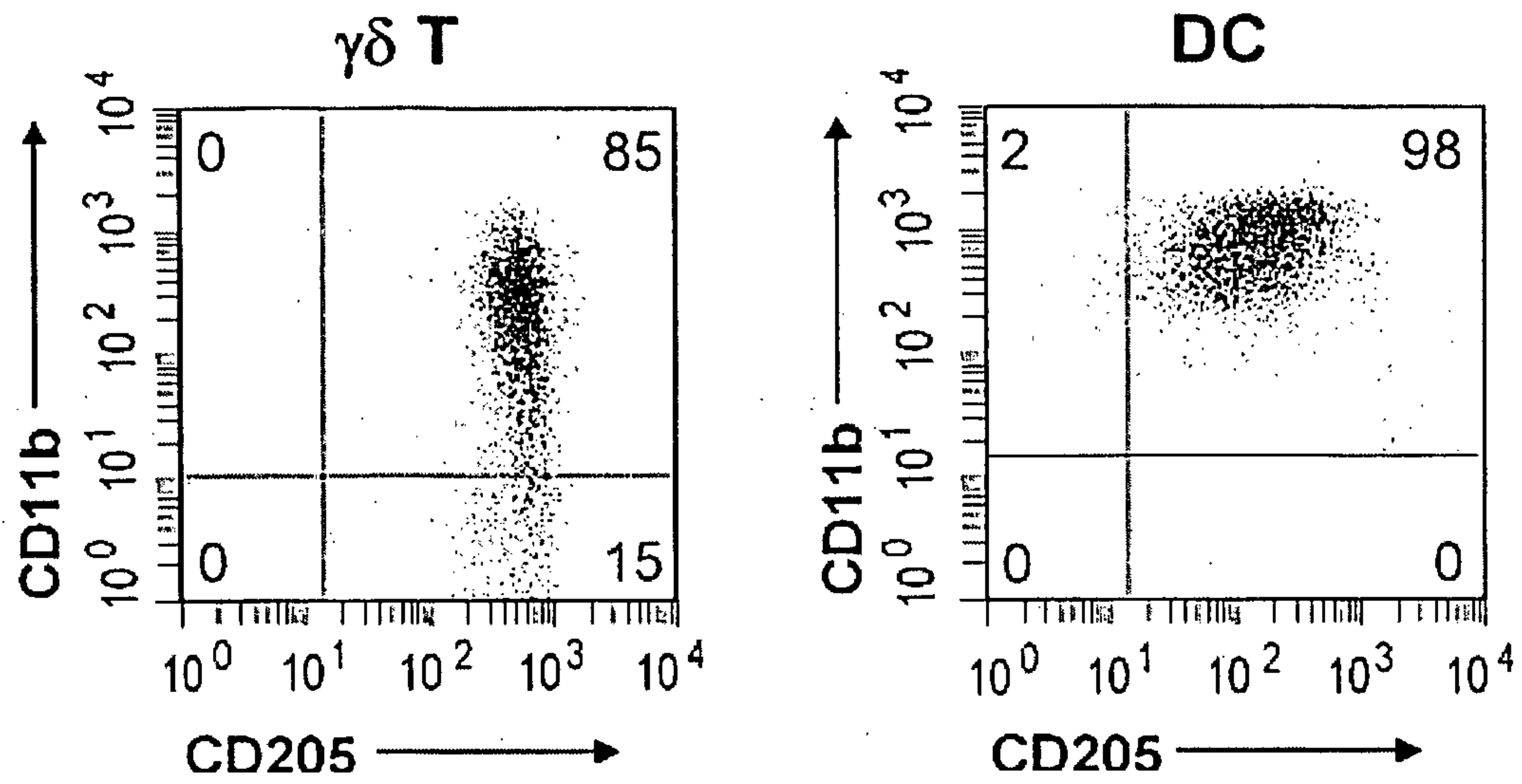
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Fig. 16B

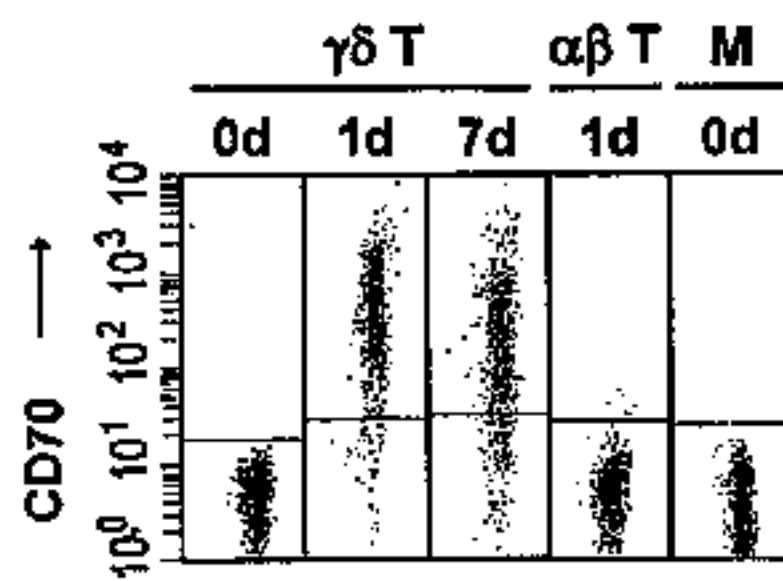
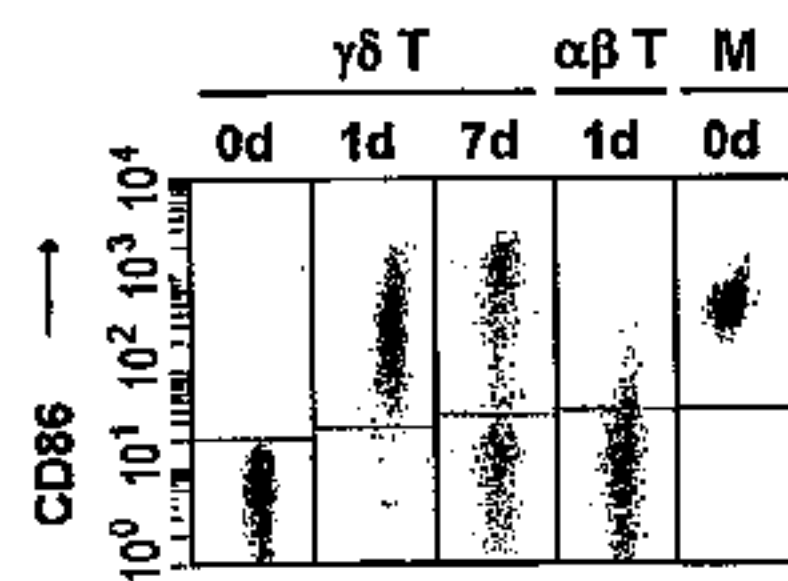
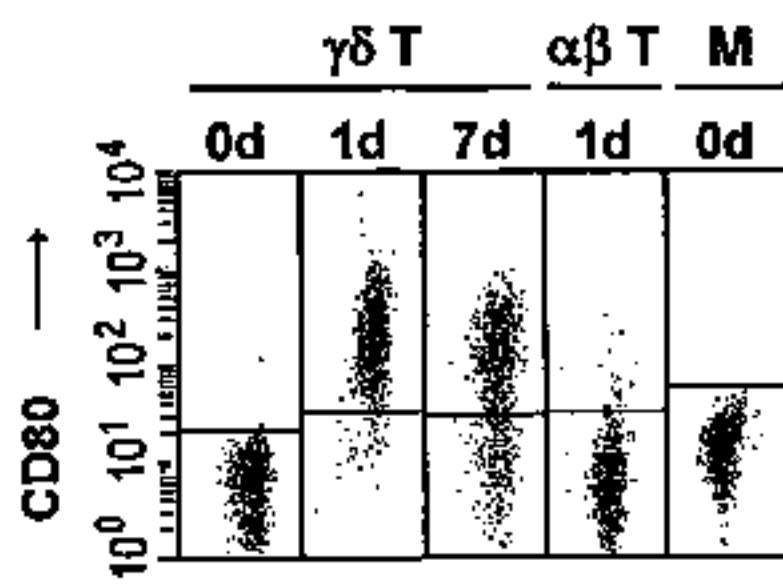
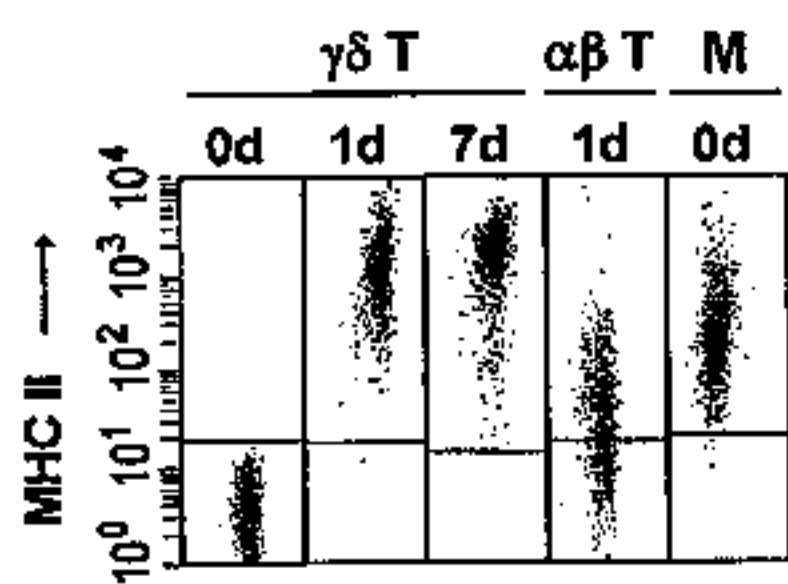


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Fig. 17



A



B

