Haematology and Blood Transfusion Vol. 29
Modern Trends in Human Leukemia VI
Edited by Neth, Gallo, Greaves, Janka

Thymic Dendritic Cells Present Blood-Borne Antigens to Medullary Thymocytes In Vivo: A Possible Role in the Generation of the T-Cell Repertoire

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A. Introduction

The thymus represents a crucial phase in the differentiation of T cells, from their earliest precursor committed to the T-cell lineage in the bone marrow, to the full array of peripheral T-effector cells. Specification of T-cell subsets, generation of receptor diversity, selection of self-MHC-restricted T-cell precursors, and induction of self-tolerance are thought to be largely or exclusively intrathymic events [24]. How such complex functional events relate to the relatively simple structure of the thymus is poorly understood. It has become apparent that the pattern of T-cell reactivity is selected by the environment [6, 26] in which T cells develop rather than being strictly genetically fixed; thus interest has focused on the definition of such selection sites. To this end direct cell–cell interactions between cells of the T-cell lineage and non-lymphoid stromal cells in the murine thymus have been characterized [25, 13, 14]. These interactions preexist in vivo, can be isolated as intact multicellular lymphostromal complexes by differential digestion of the thymus, and are thus amenable to analysis in vitro. At least three lymphostromal–cell interactions can be discerned: (1) between T cells and macrophages (MΦ), (2) between T cells and dendritic cells (DC) (both 1 and 2 referred to as thymocyte rosettes, *T-ROS*), and (3) between T cells and epithelial cells (thymic nurse cells, *TNC*). T-ROS and TNC are obtained as sequential fractions during digestion, thereby making possible a separate isolation of distinct complex types. A comparison between these interaction structures revealed the following salient points [25, 13–16, 7]. All three interactions seem to be obligatory steps for T-cell differentiation; their frequency correlates with ontogeny of T-cell maturation and is unaffected by the immune status of the animals. T-cell-stromal-cell recognition in vivo does not require syngeneity but occurs between fully allogeneic partner cells [16]. The T-cell subsets engaged in stromal-cell interactions (2%–3% of all thymocytes) are immature in surface antigen phenotype and enriched in cycling cells over unselected thymocytes. When the entry of donor bone marrow (BM)-derived Thy 1.1 pre-T cells was followed in the thymus of congenic Thy 1.2 hosts, they were found to interact first with macrophages, second with epithelial cells, and third with dendritic cells, indicating a temporal hierarchy of lymphostromal recognition during T-cell development. These kinetics do not necessarily imply a colinear maturation sequence since precursor product relationships are not known. By direct comparison of the appearance of donor T cells in lymphostromal-cell complexes after isolation in vitro, with the concomitant localization of donor T cells in situ, MΦ-ROS and TNC were located to the cortex and DC-ROS to the medulla (Kyewski, unpublished data).

Though the recognition structures governing these interactions are not known,

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Table 1.

Stromal cell type	Location	Expression of MHC antigens class		Tissue derivation	Antigen accessibility	Antigen presentation capacity	Kinetic order of T-cell interaction	Presumptive funcion
		I	II					
Macrophage	Inner cortex	Yes	No	Bone marrow	Secluded	Deficient	First	Growth and differentiation of early thymocytes
Epithelial cell	Outer cortex	Yes	Yes	Third pharyngeal pouch	Secluded	Deficient	Second	Selection according to self-MHC antigens
Dendritic cell	Medulla	Yes	Yes	Bone marrow	Accessible	Highly efficient	Third	Tolerance induction via corecognition of MHC and non-MHC self antigens

it is surmised from indirect evidence in radiation chimeras that self-MHC determinants at least in part specify these interactions [6, 26]. Given the observation that both cortical epithelial cells and medullary dendritic cells express high amounts of class II MHC-antigens constitutively in vivo (whereas cortical macrophages were found to be I-A/E negative), we tested whether non-MHC-antigens may have access to the thymus and be presented to maturing thymocytes during their maturation in vivo. This question bears particular relevance to the problem of where developing T cells expressing antigen-specific receptors are first confronted with non-MHC-self-antigens and where self-tolerance takes place. Recent evidence indicating that tolerance induction is MHC restricted would favor T-cell-accessory-cell interactions at such sites [8, 18, 19].

B. Results and Discussion

Intrathymic antigen presentation was assayed by coculture of antigen-specific I-A-restricted cloned T-helper cells with purified irradiated thymic lymphostromal-cell complexes [11, 15]. As antigens we used myoglobin, L-glutamic acid^{60}-L-alanine^{30}-L-tyrosine^{10} (GAT), and keyhole limpet hemocyanin (KLH). Proliferation of T cells was measured 72 h after culture in vitro by [^{3}H]thymidine uptake. Antigen was either injected intravascularly (i.v.) before isolation of the stimulator population or added to the culture in vitro. After injection of 0.5 mg myoglobin/g body weight i.v. into C57BL/Ka mice, T-ROS copurified with specific stimulation of I-A^b-restricted myoglobin-specific T-helper cells. This antigen-specific stimulation was a property of Thy-1.2-negative stromal cells (anti-Thy 1.2 antibody plus complement treatment did not alter the presentation capacity of *T-ROS*) and could be inhibited by more than 90% after pretreatment of the stimulator population with anti I-A^b monoclonal antibody and complement. Antigen traffic to the thymus in vivo was dose dependent within the range of 1.0–0.25 mg myoglobin/g body weight. Threshold doses for thymic and splenic antigen-presenting cells (APC) required to present antigen were similar, indicating no significant seclusion in vivo of APC enclosed in the T-ROS fraction. Similar results were obtained after injection of KLH (molecular weight 3×10^6) and GAT (molecular weight, 1×10^5) [15]. When kinetics of antigen persistence in the thymus were measured, antigen-specific stimulation of T cells was demonstrable up to 48 h after injection i.v. (Fig. 1A). The prolonged presence of antigen within the thymus argues

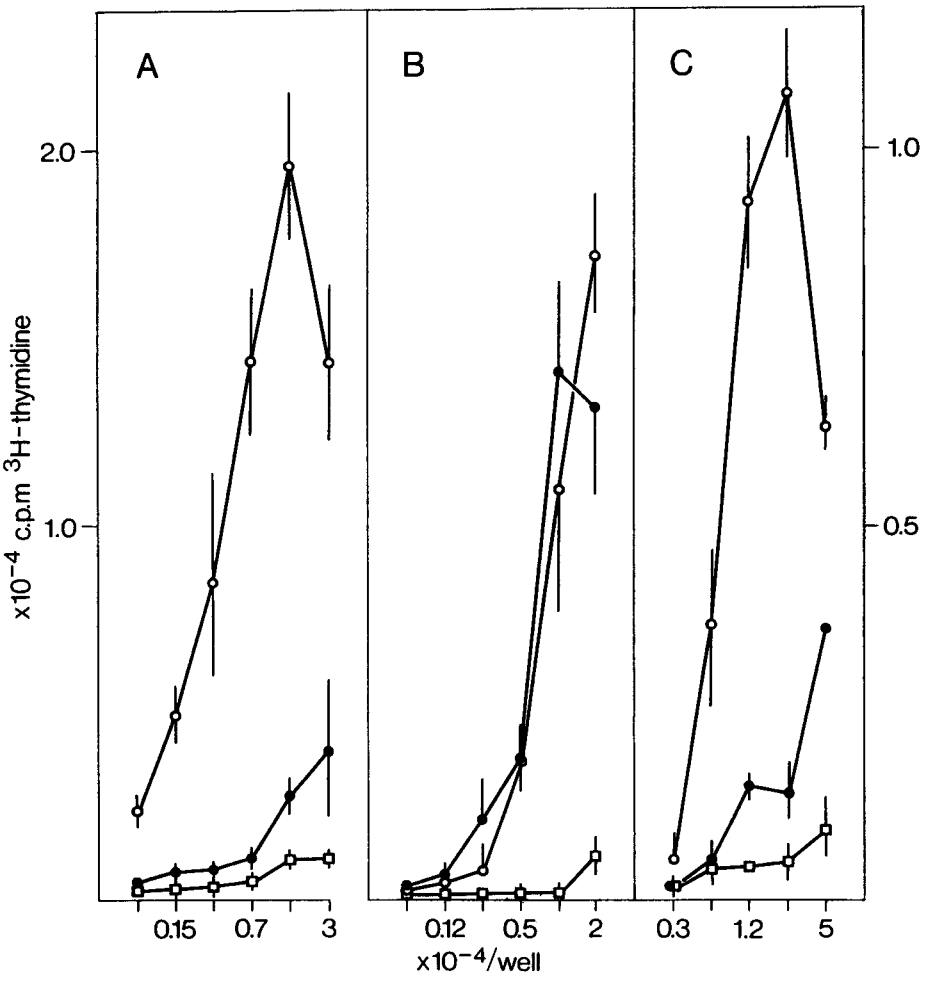

Fig. 1A–C. **A** Kinetics of antigen persistence in the thymus. C57BL/Ka mice were injected with 0.5 mg myoglobin/g body weight i.v. and 15 min (○) or 48 h (●) later nonadherent T-ROS were purified, irradiated, and cocultured with myoglobin-specific cloned T-helper cells (2×10^4/well). T-cell proliferation was measured after 72 h of coculture by [^{3}H]thymidine uptake. □ uninjected control; **B** Cellular characterization of thymic antigen-presenting cells. C57BL/Ka mice were injected with myoglobin (see **A**) and 2 h later T-ROS were isolated, fractionated, and cocultured with specific T-helper cells. ● unseparated T-ROS; ○ nonadherent T-ROS; ▣ adherent T-ROS; **C** Physiological turnover of thymic antigen-presenting cells. Nonadherent T-ROS were isolated from (C57BL/Ka × C3H/J)F_1 mice (○), (C57BL/Ka × C3H/J)F_1 BM → C3H/J newborn (●), and (Balb/c × C3H/ J) BM → C3H/J newborn (□) chimeras. Newborn mice were injected with 20×10^6 BM cells/day on days 0–4 and tested 2 weeks later. T-ROS were cocultured with cloned allo-anti I-A^b T-helper cells. *Values on abscissa* refer to numbers of stromal cells/well

against a trivial explanation of these results, namely the uptake of antigen by stromal cells (which were secluded in the intact organ), after disruption of the tissue context during the isolation procedure. Although myoglobin (molecular weight 17 000) is rapidly cleared from the circulation, the APC activity of T-ROS was unchanged when tested 15 min or 12 h after injection of antigen.

In further defining the cell type(s) responsible for uptake of antigen in vivo and presentation in vitro we separated T-ROS stromal cells into adherent and nonadherent fractions. More than 90% of adherent stromal cells are composed of I-A/E-negative, F 4/80-positive, phagocytic MΦ-like cells, whereas the nonadherent stromal cells contain 50%–80% nonphagocytic F 4/80-negative, I-A/E-positive DC-like cells [1, 14]. When separately tested for APC activity after injection of myoglobin i.v., the nonadherent fraction contained all functional APC, whereas the adherent-cell fraction even when pulsed with additional

antigen in vitro remained nonstimulatory (Fig. 1B). Though this separation method needs further confirmation by enrichment protocols using strictly lineage-specific surface markers, it was reproducibly found that depletion of strongly adherent MΦ did not affect the ability of the T-ROS fraction to present antigen. The lack of class II MHC-antigen expression on cortical macrophages forming T-ROS is compatible with this result. In order to test DCs and TNCs separately for their accessibility and capacity to present antigen, we turned our attention to their different embryonic origins. DCs are strictly bone marrow derived whereas epithelial cells are derived from the third pharyngeal pouch. Thus, $P_1 \rightarrow (P_1 \times P_2)$ F_1 radiation chimeras were analyzed in which DCs were completely replaced by P_1-type cells and epithelial cells remained of the F_1 type. When antigen presentation by cells isolated from such animals was tested 10 weeks after reconstitution with T-helper cells restricted to P_2-type I-A antigens, no T-cell proliferation was measured using either purified T-ROS (that is bone-marrow-derived stromal cells) or epithelial cells as stimulators. The latter, however, expressed class II-MHC-determinants of P_2-type abundantly, as determined by fluorescence microscopy. This lack of antigen presentation by thymic epithelial cells after injection of myoglobin i.v. could not be overcome by providing optimal doses of antigen in vitro [15]. This result indicates an intrinsic deficiency of epithelial cells in stimulation of T-helper cells, rather than a seclusion from antigen in vivo. It is not clear to date whether the epithelial cells lack the ability to process antigen and/or fail to produce obligatory costimulation factors (e.g., interleukin-l). Interestingly, thymic epithelial cells have been successfully grafted across allogeneic barriers without being rejected [21]. Given the assumption that DCs are responsible for antigen presentation in the thymus, we further assessed the physiological turnover of thymic DCs (equivalent here to nonadherent T-ROS). To this end we used nonradiation chimeras. Newborn P_1 mice were given multiple injections with F_1 bone marrow cells at daily intervals. Such animals establish a stable bone marrow chimerism which is proportional to the dose of donor cells injected (Kyewski, unpublished data). In such "normal" hosts, without prior ablation of bone marrow-derived hemopoietic lineages, F_1-BM-derived cells establish stem-cell chimerism and replace host cells during physiological turnover. When nonadherent T-ROS from such $F_1 \rightarrow P_1$ newborn chimeras were cocultured with cloned T-helper cells restricted to P_2-type I-A antigens, specific proliferation was detected (Fig. 1C). This proliferative response amounted to about 10%–20% of the magnitude induced by normal F_1 mice-derived T-ROS, indicating a significant replacement in the thymus of host-type DCs by cells of donor origin. The result indicates that medullary DCs, in contrast to cortical epithelial cells, undergo a constant physiological turnover and replacement by extrathymic DCs. Thus, in addition to the direct entry of blood-borne antigens into the thymus, circulating antigen-laden-DCs may contribute to the spectrum of intrathymically presented antigens.

The described results, in concert with earlier studies on these cell interactions [25, 13–16, 7], indicate a strict compartmentalization of thymic stromal cells with regard to their accessibility to circulating antigens and their intrinsic capacity to present these antigens to T cells. Macrophages and epithelial cells (here isolated by virtue of their interactions with thymocytes in vivo) seem to be highly inefficient in presentation of soluble protein antigens and are presumably secluded from blood-borne antigens by a vascular blood-cortex barrier. In contrast, presenting DCs are strictly confined to the medulla, which in turn displays a vascular architecture permissive to the passage of macromolecules [20]. Thus, an important aspect of cortex/medulla dichotomy with regard to T-cell recognition resides in either the prevention or facilitation of T-cell encounters with non-MHC antigens in conjunction with self MHC-antigens.

In the following we speculate on the possible roles of the three recognition steps in the context of the development of the T-cell repertoire. Pre-T cells probably enter the thymus at the cortical side of the cortical-medullary junction and first interact with

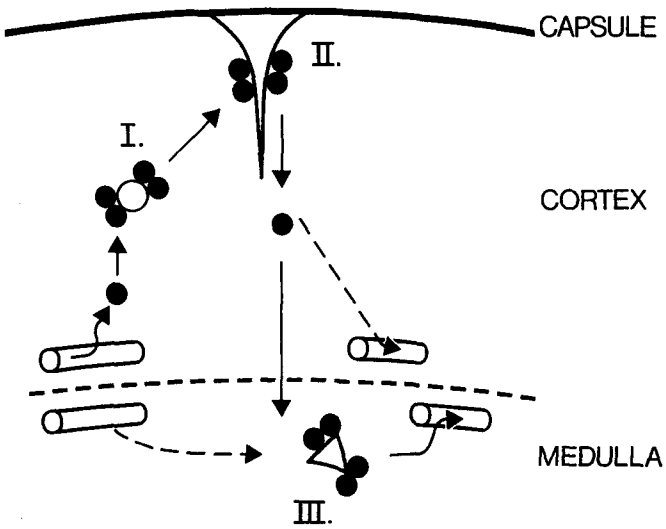

Fig. 2. *Solid arrows* indicate conjectural routes of intrathymic T-cell migration intercalated by lymphostromal recognition and selection steps between thymocytes and macrophages (*I*), epithelial cells (*II*), and dendritic cells (*III*). *Dashed arrows* indicate equally possible traffic routes

and proliferate around macrophages in the inner cortex (Fig. 2). The role of these macrophages may resemble those in hemopoietic islands in the bone marrow, in which they support the growth and differentiation of erythropoietic and granulopoietic cell lineages by direct cell–cell contact [2, 5]. This early stage of thymocyte–accessory-cell interaction may not yet involve the T-cell receptor for antigen recognition but may induce its expression. After this initial MΦ-induced growth and differentiation phase a second interaction with epithelial cells follows in the outer cortex. As proposed previously, this interaction may represent the recognition of self-MHC determinants expressed on epithelial cells by a minor selective set of clonally expressed T-cell receptors, thus determining the self-MHC restriction of cytotoxic and helper T cells [6, 26]. If correct, one would predict that all T cells associated with epithelial cells should have productively rearranged T-cell-receptor genes and express membrane-bound T-cell-receptors [12]. In the absence of non-MHC antigens this recognition step has to be different from the obligatory corecognition of MHC antigens and nominal antigens exhibited by mature T cells [23, 3]. A third type of intercellular recognition, now in the medulla between thymocytes and dendritic cells, displays the characteristic MHC-restricted recognition of non-MHC antigens, which enter this compartment and are presented here to maturing T cells. By conjecture this site may constitute a possible microenvironment where self-tolerance is induced. This proposition receives indirect support from the recent observation that thymic epithelial cells when grafted across allogeneic barriers do not induce T-cell tolerance to their own class II MHC antigens [21]. DC-thymocyte interactions would conform to the prediction that tolerance induction is MHC restricted [8, 18, 19]. In addition, the ontogeny of thymocyte-DC interactions parallels the induction of self-tolerance [17] (Kyewski, unpublished data). These considerations, however, leave the cellular and molecular mechanisms of how self-tolerance is induced and maintained completely unexplained. According to this model, recognition structures on stromal cells select for complementary receptors on T cells expressed at the respective stage of their interaction. With the advent of monoclonal antibodies and molecular probes specific for the T-cell-receptor certain predictions of this model may be tested [9, 10]. In the absence of direct evidence for a precursor–product relationship between the various interactions, and the unknown fate of the selected thymocytes, the developmental pathways of T cells still remain conjectural [22] (Fig. 2).

The outlined results and speculations depict the complexity of intrathymic T-cell maturation with regard to cell-to-cell communication, compartmentalization, and directed lymphocyte traffic. The possibly critical involvement of stromal cells in growth control of hematopoietic cell lineages has recently been reemphasized [4]. In view of the notion that some forms of acute T-cell leukemias may represent an arrest in differentiation rather than an irreversible transformation step, the analysis of the inductive signals responsible for T-cell growth and differentiation may aid our understanding of the mechanism of T-cell leukemogenesis. T-cell transformation, at least in the murine model, has been shown to be strictly dependent on an intact thymic microenvironment.

Acknowledgment. This work has been conducted under the stimulating guidance of the late Henry S. Kaplan, Cancer Biology Research Laboratory, Dept. of Radiology in collaboration with Dr. C. G. Fathman, Div. of Immunology, Dept. of Medicine, Stanford University Medical School. The author was supported by a postdoctoral fellowship of the DFG.

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