Intrathymic Nurse Cell Lymphocytes Can Induce a Specific Graft-versus-Host Reaction

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Summary

Single chicken thymic nurse cells (TNC) placed onto the chorionallantoic membrane (CAM), showed that intra-TNC lymphocytes (TNC-L) possess a strong graft-versus-host reactivity (GVHR) in allogeneic MHC combinations. This reaction shows the morphological, phenotypic, and functional characteristics of a classical GVH reaction (GVHR). The induction of a GVHR was significantly higher for TNC-L as compared with thymocytes or peripheral blood lymphocytes (PBL). The specificity of the GVHR was shown by serial transfer experiments onto appropriate allogeneic and syngeneic secondary embryonic hosts. In immunofluorescence analyses with monoclonal antibodies (mAb) to the chicken α/β and γ/δ T cell receptors (TCR) and the CD3, CD4, and CD8 equivalents, an enrichment of $CD3^+/CD4^+/CD8^-$ and $CD3^+/CD-4^-/CD8^+$, $TCR-\alpha/\beta^+$ and $TCR-\gamma/\delta^+$ cells was observed inside TNC as compared with extra-TNC thymocytes. A large proportion of $CD4^+$ and/or $CD8^+$ $TCR-\gamma/\delta^+$ cells were demonstrated inside TNC. A minor population among $TCR-\gamma/\delta$ extra-TNC thymocytes also expressed CD4 and/or CD8 molecules. Based on functional tests and double staining experiments, we propose that $CD4^+/CD8^+$ thymocytes enter the TNC where they may undergo positive selection for MHC restriction and further differentiation to CD4 or CD8 single-positive cells. Taken together these data support the concept that TNC contribute a specialized thymic microenvironment for T cell differentiation and maturation.

Stem cells invading the thymic anlage encounter a variety of cells and extracellular matrix structures that contribute to the acquisition of the T cell repertoire. This process involves both the maturation of T cells and the inactivation and/or deletion of potentially autoreactive cells (1). A crucial prerequisite for the specificity of the final repertoire is the process of self-recognition. During ontogeny, this recognition of self has been suggested to be dependent upon the interaction of the MHC class I and class II antigens (2–4), as well as all other cell surface and extracellular components of the thymus. In addition, nonlymphoid cellular components of the thymus, mainly the reticular epithelial cells, produce humoral factors that affect T cell differentiation (5). The way in which non-MHC antigens are recognized by T cells is not clear. MHC antigens are, however, represented on different nonlymphoid cells in the thymus and differentiating T cells come into direct and close contact with them during the sojourn in the thymus. Thymic nurse cells (TNC)[1] are of par-

ticular interest in this respect. TNC provide a specialized microenvironment (6, 7), which thymocytes enter, and there they become enclosed in individual vacuoles lined by the epithelial cell plasma membrane that displays a high density of MHC class I and II antigens. In contrast to T cells ingested by macrophages, intrathymic nurse cell lymphocytes (TNC-L) do not disintegrate and thus TNC are unlikely sites for clonal deletion (8–12).

TNC have been demonstrated in mice, rats (8, 9), humans (11, 12), and chickens (13). Whereas mammals contain numerous TNC, e.g., the mouse has over 50 TNC-L (7, 8), the chicken on average harbors only 4 T cells. The morphological criteria for the chicken TNC correspond to those of their mammalian counterparts (13). TNC are fragile, difficult to enrich in vitro, and thus elusive to functional analysis in most mammalian systems. Some studies have revealed that TNC-L exhibit T suppressor (Ts) (14), T helper (Th) (15, 16), and in the chicken system, graft-versus-host–like reactivity (GVHR) in allogeneic, and with lower frequency, in syngeneic combinations (17). The syngeneic phenomenon will be the subject of a separate paper (Penninger, J, and G. Wick, manuscript in preparation). The present paper only deals with allogeneic reactions for which we established specificity for the first time.

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[1] Abbreviations used in this paper: CAM, chorionallantoic membrane; ED, embryonic day; GVHR, graft-versus-host reaction/reactivity; PE, phycoerythrin; RSI, relative spleen weight index; SI, spleen weight index; TNC, thymic nurse cell; TNC-L, intrathymic nurse cell lymphocytes; TRITC, tetramethylrhodamine-isothiocyanate.

The experiments reported here were designed to further analyze the functional properties of TNC-L at a *single* cell level using the chorionallantoic membrane (CAM) assay (18–20), which represents a unique possibility to circumvent the technical problems for the functional study of TNC-L in mammals. The principle of the CAM assay depends on the deposition of cell suspensions from histoincompatible donor chickens containing immunocompetent T cells onto the surface of the CAM of a recipient embryo. Since MHC antigens of the recipient are expressed on the CAM, the immunocompetent T cells of the donor proliferate, which is manifested by the appearance of macroscopically visible "pocks." The number and size of pocks and the degree of splenomegaly are parameters expressed by the recipient and correlate with the degree of MHC incompatibility. One pock is induced by one donor T lymphocyte (18).

The following points were addressed in this study: (a) the surprisingly high efficiency of pock formation by a single TNC deposited onto allogeneic CAM (17); (b) the cellular composition of TNC-L induced pocks; (c) the establishing of the immunological nature of this reaction; and (d) the characterization of TNC-L with respect to their receptor expression.

Materials and Methods

Animals. The chicken MHC (B-locus) comprises three subloci, B-F (class I), B-L (class II), and B-G (class IV, only expressed on erythrocytes). In the present experiments chickens of the MHC-congeneic inbred strains CB (MHC type $B^{12}B^{12}$), B[7] (B^7B^7), and CC (B^4B^4) and the close-bred strains CS (B^4B^4) and OS($B^{15}B^{15}$) were bred and raised in the Central Animal Facility of the Medical School of the University of Innsbruck. All cell donors were 4–10 wk old. Fertilized eggs were incubated in our facilities and the embryos were used as recipients at the times indicated in Fig. 1.

Preparation of Cells. For the preparation of TNC, 4–12-wk-old donor chickens were anesthetized with Nembutal[R] (0.3 ml/kg, i.v.) and bled by heart puncture; blood-free thymi were harvested. The thymic lobes were mechanically teased into HBSS (pH 7.2; Biomed, Berlin, FRG) at room temperature to release thymocytes. The sedimented tissue was trypsinized (0.3%; Boehringer Mannheim, FRG) for 90 min at 40°C with gentle agitation. Cells released were collected and centrifuged at 250 *g* for 10 min in a discontinuous Percoll[R] gradient (Pharmacia, Uppsala, Sweden). TNC, found at the interface between the 30% (density 1.043) and 40% (density 1.056) gradient, were pooled and washed three times for 5 min and then centrifuged at 80 *g* in HBSS supplemented with 10% FCS (Biomed). In experiments where TNC-L surface molecules were studied by immunofluorescence, trypsin was replaced by 0.1% collagenase (type IV; Sigma Chemical Co., Munich, FRG).

Extra-TNC thymocytes were prepared by mechanical isolation only. PBL were isolated from heparinized peripheral blood (25 µl preservative-free heparin, 5,000 IU/ml; Immuno AG, Vienna, Austria) by slow speed centrifugation (2 × 80 *g* for 5 min). Both thymocytes and PBL were washed three times for 5 min at 250 *g* in HBSS.

Pocks were harvested from the CAM on day 16 of embryogenesis (ED16; primary hosts) or ED14 (secondary hosts), trimmed free of connective tissue under the dissection microscope, and treated with 0.1% collagenase for 1 h at 37°C. The tissue fragments of each individual pock were thoroughly mixed with a Pasteur pipette at room temperature and flushed through a 200 mesh steel sieve with HBSS. The number of mononuclear cells obtained from 1 pock amounted to 5 × 10^{5} to 1 × 10^{6}. The viability of the
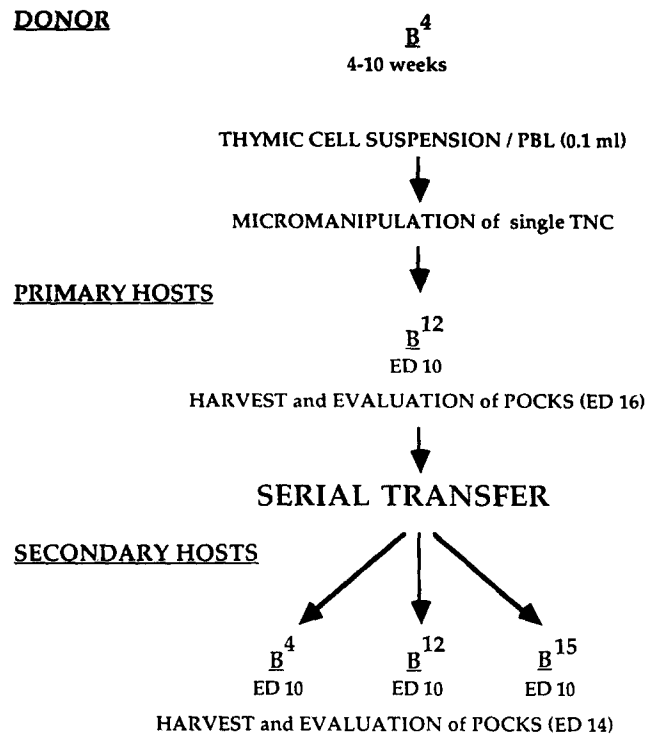
CAM-GvHR ASSAY

Figure 1. CAM-GVHR assay and serial transfer of primary pocks onto secondary recipients.

pock-derived cell suspension was assessed by trypan blue exclusion and always found to be >85%.

Antibodies. Murine mAbs recognizing the chicken analogues of the CD3 complex (CT3), CD4(CT4), CD8(CT8), the TCR-γ/δ heterodimer (TCR1), and the TCR-α/β heterodimer (TCR2) were kindly provided by Dr. M.D. Cooper, Birmingham, AL, and Dr. J. Cihak, Munich, FRG (21–25). The mAb to the chicken IL-2 receptor (IL-2R; code INN-LD16) and alloantibodies against chicken B-L[4] or B-L[12] (MHC Class II) antigens were prepared and characterized in our laboratory (26, 27).

Immunostaining. Alloantibodies against B-L antigens were directly conjugated either with FITC or tetramethylrhodamine-isothiocyanate (TRITC) and used in immunofluorescence analyses on 4 µm thick frozen tissue sections (17). For the determination of the contribution of donor and recipient cells, respectively, to primary and secondary pocks in a FACS III (Becton Dickinson, Sunnyvale, CA), only direct membrane immunofluorescence tests with FITC-labeled alloantibodies were performed.

For the characterization of TNC-L by double-staining immunofluorescence tests, suspensions of TNC were prepared from CB and B7 chickens and enriched as described. 25 µl of the cell suspension adjusted to 10[6] cells/ml were deposited on glass slides, air dried, and incubated with primary unconjugated mouse mAbs directed against TCR-γ/δ, TCR-α/β, or the chicken CD4. The reaction was demonstrated by indirect immunofluorescence with a TRITC-labeled rabbit anti–mouse Ig conjugate (R-270; Dako, Glostrup, Denmark). After an incubation with a 1:10 diluted normal mouse serum to block nonspecific reactivity, the preparations were

treated with biotinylated mouse mAbs against the chicken equivalents for CD3, CD4, or CD8 and visualized by a FITC-conjugated streptavidin (Seromed, Blackthorn, UK).

For FACS analysis, thymocytes from the same CB and B7 donors were prepared and analyzed in membrane immunofluorescence using different protocols: (a) Unconjugated primary mAbs against TCR-α/β, TCR-γ/δ, or CD4 visualized by a FITC-labeled rabbit anti–mouse Ig conjugate (Dako, F-261) and followed by secondary mAbs against CD3, CD4, or CD8 directly labeled with PE; or (b) primary mAbs against CD4, CD8, or CD3 directly labeled with PE, which were followed by biotinylated secondary mAbs against TCR-γ/δ visualized by FITC-conjugated streptavidin.

All reagents were diluted in PBS (pH 7.2) and used in optimal dilutions determined in pilot studies.

GvH Assay. ED10 recipient embryos were prepared under sterile conditions by drilling a 1 × 1 cm square opening into the shell at an equatorial site shown to be free of underlying larger vessels. A small hole was then produced with a needle at the blunt end of the egg and the CAM was dropped by suction with a rubber bulb. The cells to be assessed for pock formation were applied onto the surface of the CAM, which was then closed with hot paraffin. Depending on the experiment, one to five single living TNC, each of which on the average contained four engulfed thymocytes, were harvested under a micromanipulation microscope using siliconized pipettes filled with FCS to prevent adherence of TNC enriched cell suspensions. These cells were transplanted onto the CAM of ED10 recipient embryos. As controls, 10^6 PBL/100 μl HBSS, 0.1 ml peripheral blood containing 2.4 × 10^6 lymphocytes/100 μl, or purified thymocytes from the same donors were deposited in the same manner. 6 d later the CAMs were removed and the number of proliferative foci (pocks) was determined under a dissection microscope with low magnification. All pocks were documented photographically and then either subjected to routine histology, electron microscopy, or immunohistochemical analysis (Fig. 1, *top*).

For serial transfer experiments the primary pocks were harvested on ED16, trimmed free of the adhering tissue under the dissection microscope, and transplanted *in toto* onto the CAM of ED10 secondary recipients of genotypes of those of the original donor, the primary host, or an unrelated third party. On ED14 these secondary pocks were inspected microscopically, documented by photography, and processed for further analyses as described for primary pocks (Fig. 1, *bottom*).

In addition to the assessment of CAM pocks, the spleens of the secondary recipients were weighed and the spleen index (SI) and the relative spleen index (RSI) were determined. Spleens of sham-manipulated ED14 of the same MHC type as the secondary recipients were used as controls.

Spleen index was calculated as: SI = [(mg spleen weight)/(g body weight)] × 10^5. Relative spleen index was calculated as: RSI = (mean allogeneic SI)/(mean control SI). RSI >1.3 indicates a specific GVHR (28). The RSI of sham-manipulated controls has a value of 1.0.

Results

Frequency Analysis of TNC-L induced Pock Formation. In a first series of experiments two highly inbred strains CC (B^4B^4) and CB($B^{12}B^{12}$) were used as TNC donors and embryonic recipients, respectively. TNC-L displayed a very high efficiency for pock formation in this allogeneic combination, ranging from 1/14 to 1/23 in 10 experiments based on a mean count of 4 lymphocytes per TNC (Table 1). Deposition of a single TNC (Exp. 1) resulted in the formation of only

Table 1. *Frequency Analysis of TNC-L–induced Pock Formation*

Exp.	Donor cells	Host embryos	Sex	No. of CAMs inoculated	CAMs with pocks	No. of pocks/CAM one	two	three	GVHR efficiency
1	B-L^4 TNC (1) female	B-L^{12}	Male	11	2	2	—	—	1/22
			Female	5	3	3	—	—	1/7
			Total	16	5	5	—	—	1/15
2	B-L^4 TNC (2) female	B-L^{12}	Male	20	13	9	4	—	1/9
			Female	21	5	4	1	—	1/28
			Total	41	18	13	5	—	1/14
3	B-L^4 TNC (5) male	B-L^{12}	Male	29	19	14	4	1	1/23
			Female	18	13	10	2	1	1/24
			Total	47	32	24	6	2	1/23
Compilation of 10 Exp.	B-L^4 TNC (5) male and female	B-L^{12}	Male and female	153	124	82	35	7	1/18

GVHR efficiency of 4–10-wk-old B-L^4 donor TNC-L deposited on ED 10 MHC-congeneic B-L^{12} embryos. CAM-pocks were determined 6 d later. As every TNC contained 4 thymocytes on the average, GVHR efficiency was estimated as number of pocks/number of TNC × 4. In experiments 1, 2, and 3 the sex of both donors and recipients was determined.

one pock/CAM, but the transfer of two (Exp. 2) or five TNC (Exp. 3) occasionally yielded two or three pocks/CAM. In addition, no sex influence was demonstrated. The last row shows a compilation of 10 different experiments without taking the sex into account, which resulted in an average GVHR efficiency of 1/18.

For control purposes 0.1 ml of heparinized peripheral blood from the same B-L^4 donor strain that contained 2.4 × 10^6 lymphocytes was deposited on ED 10 B-L^{12}CAM, and in this instance the mean number of pocks was 30.0 ± 3.0/CAM on ED16. For 10^6 thymocytes this value was 3.8 ± 1.5/CAM. While the deposition of 0.1 ml of peripheral blood consistently caused splenomegaly, formation of the small TNC-L-derived primary pocks was not paralleled by detectable systemic manifestations (data not shown).

The histological and electron microscopic appearance of TNC-L-induced pocks corresponded to that of classical PBL-derived GVHR. They consisted of dense foci of lymphoblasts with granulocytes and macrophages in the center. The perivascular accumulation of lymphoblasts was especially evident in the periphery of the pocks. Macrophages contained phagocytozed lymphoid cells in different stages of degeneration (29).

Specificity of GVHR in Serial Transfer Experiments. To determine if the formation of TNC-L-induced pocks was immunologically specific, serial transfer experiments of primary pocks onto secondary hosts were performed according to that outlined in Fig. 1.

First, the size of primary and secondary pocks on four different hosts (B-L^4, like TNC donor; B-L^{12}, like primary recipient; B-L^{15} and B-L^7, both unrelated third-party controls) were visually and photographically recorded (data not shown). A significant enlargement occurred only in those combinations where the secondary recipient carried the same MHC haplotype as the primary host, thus showing specificity of the reaction. The next experimental series were performed to investigate if in combinations of B-L^4 → B-L^{12} → B-L^{12}, the classical determination of the SI or relative spleen weight index RSI was an appropriate correlative to analyze the specificity of pock formation (28). For control purposes primary pocks were induced by PBL, excised, and transferred *in toto* onto secondary recipients of different genetic backgrounds as described above for TNC-L pocks. As shown in Table 1 (Exps. 1 and 2), a significant increase in the RSI was only observed when the secondary recipient carried the same B-L haplotype as the primary recipient, thus proving specificity. The same results were obtained when primary TNC-L pocks (B-L^4 → B-L^{12}) were transferred to secondary B-L^{12}, B-L^4, or B-L^{15} recipients (Table 2, Exp. 3). The histological and electron microscopical appearance of secondary TNC-L-induced pocks corresponded to that of primary TNC-L or PBL-induced reactions as described above (29).

A remote possibility that may have accounted for the splenomegaly in secondary hosts would have been the preferential migration of recruited recipient (B-L^{12}) T cells from primary pocks into the microenvironment provided by the spleen of the MHC-identical secondary host (30). To exclude the possible syngeneic homing, PBL from ED16 B-L^{12} were transferred onto the CAM of ED10 B-L^{12} and B-L^7 hosts and the spleen weight of the latter was determined on ED14. In both instances no increase in spleen weight was found (Table 3).

Table 2. *Quantitation of GvHR in Serial Transfer of Primary PBL and Primary TNC-L-induced Pocks*

Exp.	Donor cells	Primary recipient	Secondary recipient	Animals	SI*	RSI‡
				n	X ± SEM	X ± SEM
1	B-L^4 PBL	B-L^{12}	B-L^{12}	8	111.3 ± 10.2	1.49 ± 0.13
			B-L^4	13	69.5 ± 5.0	1.09 ± 0.08§
			B-L^{15}	4	78.2 ± 13.2	0.92 ± 0.15§
			B-L^7	4	65.9 ± 3.6	0.96 ± 0.05‖
2	B-L^7 PBL	B-L^4	B-L^4	3	117.6 ± 22.3	2.31 ± 0.25
			B-L^7	4	57.0 ± 0.9	0.81 ± 0.01¶
			B-L^{12}	6	83.0 ± 7.5	0.99 ± 0.09¶
3	B-L^4 TNC	B-L^{12}	B-L^{12}	12	102.1 ± 7.0	1.37 ± 0.09
			B-L^4	15	57.0 ± 6.1	0.89 ± 0.09‖
			B-L^{15}	5	72.3 ± 9.9	0.85 ± 0.11‖

Heparinized PBL (10^6/100 μl HBSS) or 5 TNC of different inbred donor strains were transplanted onto the CAM of ED 10 primary recipients, harvested on ED16, and serially transferred on ED 10 secondary hosts of genotypes comprising the original host, the primary donor, or third-party controls. Secondary pocks were evaluated on ED14. Formulas for the *spleen index (SI) and ‡relative spleen index (RSI) are indicated in Materials and Methods. RSI >1.3 indicates a specific GVHR (28). Probability, computed Student's t-test: §$p < 0.05$; ‖$p < 0.01$; ¶$p < 0.005$, significant RSI differences of secondary hosts with the same genotypes as primary hosts compared with other strain combinations.

Table 3. *Homing of Embryonic PBL*

Donor cells	Recipients	Animals	SI	RSI
		n	$X \pm SEM$	$X \pm SEM$
B-L^{12} PBL	B-L^{12}	5	66.4 ± 2.5	0.83 ± 0.03
	B-L^7	5	64.7 ± 1.9	0.82 ± 0.02

25 μl of heparinized peripheral blood that contained 6 × 10^5 lymphocytes of an ED16 B-L^{12} donor were deposited on the CAM of ED10 B-L^{12} or B-L^7 recipients. The SI and RSI were evaluated on ED14 and compared to sham-manipulated hosts of the same inbred strains.

Demonstration of Original Donor Cells in Primary and Secondary Hosts. For the assessment of the relative contribution of donor and recipient cells within each pock, frozen sections and cell suspensions were analyzed by direct immunofluorescence with antibodies against Ia-like (B-L) donor or recipient type determinants (26). On unfixed frozen sections of allogeneic pocks, B-L^4 (donor type) and B-L^{12} (recipient type) cells were demonstrated within the perivascular accumulations of mononuclear cells.

For an accurate determination of the cellular make-up of primary TNC-L-induced pocks, cell suspensions were prepared and subjected to FACS analysis. Fig. 2, *a–b*, shows that, similar to the situation in classical PBL-induced GVHR (Fig. 2,*b*), B-L^{4+} and B-L^{12+} cells were demonstrated within the TNC-induced pocks (*a*). Fig. 2, *c* and *d*, shows the results of the FACS analysis of representative secondary pocks.

In double-staining experiments, using TRITC-labeled anti-B-L alloantibodies and a mouse mAb against the chicken IL-2R visualized by a FITC-rabbit anti–mouse Ig conjugate, IL-2R$^+$ cells were not only found in the cell population derived from the immunocompetent donor, but unexpectedly, also in a considerable percentage of recruited cells from the primary and secondary embryonic hosts. In both primary and secondary TNC-L- or PBL-induced pocks the donor-derived lymphoblast population consisted of CD4$^+$ and CD8$^+$ cells in about equal proportions. The recruited lymphoid cells of the embryonic recipient did not yet reveal significant expression of these markers. In all cases studied, B lymphocytes and macrophages of the host also contributed to the pock formation (data not shown).

Surface Expression of TCR-α/β, TCR-γ/δ, CD3, CD4, and CD8 on TNC-L. Since the induction of GVHR by TNC-L would require the expression of TCR, TNC-enriched thymic cell suspensions were analyzed for TCR-γ/δ, TCR-α/β, CD3, CD4, and CD8 expression by double-staining immunofluorescence.

Table 4 shows the percentages of TCR-α/β$^+$, CD3$^+$, CD4$^+$/CD8$^+$, CD4$^+$/CD8$^-$, and CD4$^-$/CD8$^+$ thymocytes. The overwhelming majority of TCR-α/β$^+$ thymocytes expressed both accessory molecules CD4 and CD8, and the remaining TCR-α/β population was CD4 or CD8 single-positive, i.e. showed the mature phenotype (*d* and *f*). Using
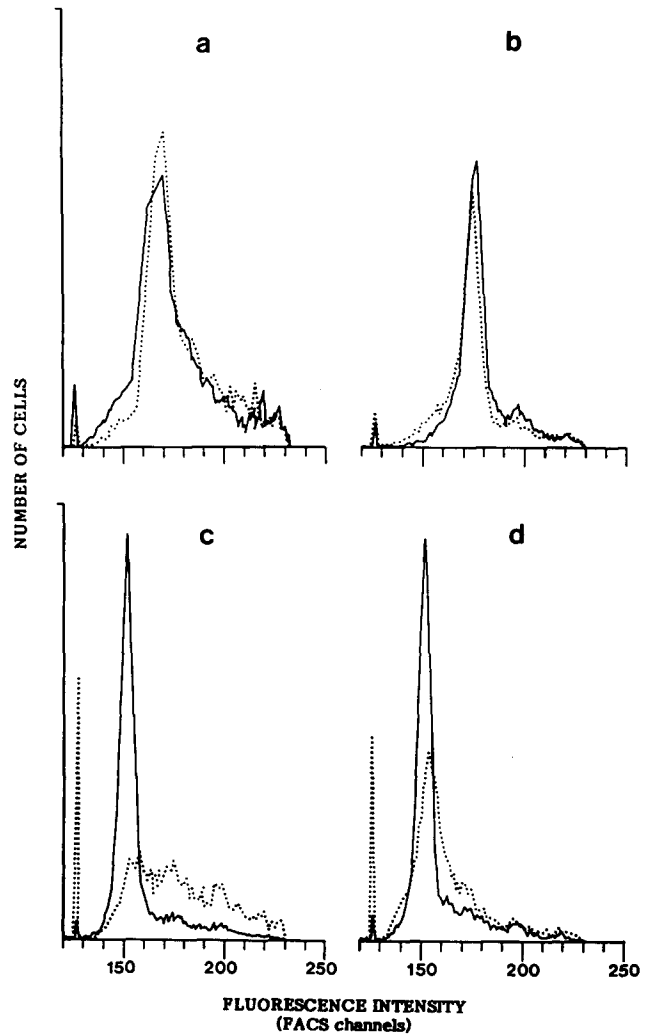

Figure 2. Immunofluorescence analysis of B-L^{4+} donor and B-L^{12+} host cells in primary and secondary pocks. In all instances solid lines represent B-L^{12+} recipient cells and dotted lines B-L^{4+} donor cells. (*a*) Primary TNC-L-induced pocks; (*b*) primary PBL-induced pock; (*c*) secondary TNC-L-induced pock; (*d*) secondary PBL-induced pock. 10^4 cells were analyzed in each instance and fluorescence intensity was displayed in relative units on a logarithmic scale. Percentages of positive cells, calculated between channel numbers 189 and 230 (*a* and *b*) or channel numbers 169 and 230 (*c* and *d*), are the following: (*a*) B-L^{4+} (donor), 14%; B-L^{12+} (recipient), 21%. (*b*) B-L^{4+}, 19%; B-L^{12+}, 18%. (*c*) B-L^{4+}, 60%; B-L^{12+}, 17%. (*d*) B-L^{4+}, 28%; B-L^{12+}, 15%. Reciprocal crossreactivity of the anti-B-L^4 and anti-B-L^{12} conjugates with PBL of the nonidentical haplotype was excluded by FACS analysis. Furthermore, an unrelated FITC-labeled conjugate (swine anti–rabbit Ig) stained <2% of each cell suspension, i.e., similar to autofluorescence (curves not shown).

various staining protocols, we also detected a high percentage of TCR-γ/δ$^+$ cells that expressed CD4 or CD8 molecules (*e* and *g*). While CD4$^+$/CD8$^+$ cells were more numerous among thymocytes outside TNC, CD-3$^+$ and single-positive (CD4$^+$/CD8$^-$ and CD4$^-$/CD8$^+$) cells were enriched among TNC-L(*a*). TCR-α/β$^+$/CD3$^+$ TNC-L clearly prevailed over TCR-γ/δ$^+$/CD3$^+$ TNC-L (*b* and *c*). The majority of

Table 4. *Double-staining of TNC-L and thymocytes*

Cell phenotype		Experiments	Percentage positive cells ± SEM		
			Thymocytes*	Experiments	TNC-L‡
		n		*n*	
a	CD4 + CD8 +	7	60.3 ± 4.4	4	46.4 ± 4.5
	CD4 + CD8 −	7	5.8 ± 1.6	4	24.3 ± 4.1
	CD4 − CD8 +	7	10.2 ± 2.0	4	27.3 ± 5.5
b	TCRα/β + CD3 +	4	38.5 ± 7.5	2	72.2 ± 1.3
	TCRα/β + CD3 −	4	0	2	0
	TCRα/β − CD3 +	4	12.0 ± 5.6	2	18.1 ± 0.04
c	TCRγ/δ + CD3 +	3	7.0 ± 0.9	2	17.5 ± 0.5
	TCRγ/δ + CD3 −	3	0	2	0
	TCRγ/δ − CD3 +	3	31.0 ± 4.0	2	73.0 ± 2.9
d	TCRα/β + CD4 +	2	34.6 ± 6.4	5	44.1 ± 1.5
	TCRα/β + CD4 −	2	4.3 ± 0.6	4	24.7 ± 1.9
	TCRα/β − CD4 +	2	39.8 ± 0.8	4	24.5 ± 3.1
e	TCRγ/δ + CD4 +	6	1.9 ± 0.2	4	10.1 ± 1.4
	TCRγ/δ + CD4 −	6	6.1 ± 0.9	4	6.6 ± 0.4
	TCRγ/δ − CD4 +	6	67.6 ± 4.3	4	61.7 ± 4.0
f	TCRα/β + CD8 +	2	36.3 ± 6.3	5	40.6 ± 4.7
	TCRα/β + CD8 −	2	2.1 ± 0.1	5	18.9 ± 4.2
	TCRα/β − CD8 +	2	39.1 ± 2.8	5	24.5 ± 5.9
g	TCRγ/δ + CD8 +	6	2.6 ± 0.3	6	8.9 ± 0.8
	TCRγ/δ + CD8 −	6	5.6 ± 0.5	6	10.5 ± 1.5
	TCRγ/δ − CD8 +	6	57.5 ± 7.1	6	58.7 ± 2.8

* In each experiment 10,000 thymocytes were analyzed in a FACS III (Becton Dickinson, Sunnyvale, CA) using two different protocols (see Materials and Methods) and the percentages of positive cells were calculated by gated analysis. Visual evaluation of thymocytes that showed the same results as the FACS analysis are not indicated.

‡ In each experiment at least 100 TNC/slide were visually evaluated.

$CD4^+$ and $CD8^+$ TNC-L carried the α/β TCR (*d* and *f*), a smaller fraction of both subpopulations was also γ/δ TCR-γ/δ^+ (*e* and *g*). We further showed that TNC-L were composed of both a larger proportion of TCR-α/β and a minor proportion of TCR-γ/δ^+ cells (*b* and *c*). The ratio of TCR-α/β^+ to TCR-γ/δ^+ cells was similar to that in the general thymocyte population, but the proportion of each subpopulation was significantly increased.

Discussion

Based on experimental data obtained so far, various schemes of T cell differentiation and selection in the thymus have been proposed that are not mutually exclusive (31–33): First, $CD4^-/CD8^-$ stem cells enter the thymic anlage and become $CD4^+/CD8^+$ (double-positive) T cell precursors.

These double-positive T cells interact with thymic stroma cells and undergo positive selection for self-MHC (33–35). T cells reacting with self MHC and non-MHC with high affinity are eliminated and/or inactivated by negative selection (36, 37). Experiments using transgenic mouse models suggest that the specific interaction of the TCR on thymocytes with thymic MHC antigens determines the differentiation of $CD4^+/CD8^+$ cells into either $CD4^+/CD8^-$ or $CD4^-/CD8^+$ mature T cells (38).

The actual *sites* within the thymus where self-recognition and T cell differentiation occur in vivo has been elusive. The specific experimental possibilities in the chicken with its extramaternal embryonic development combined with the micromanipulation of single cells made it possible to investigate the phenotype and functional potential of developing thymocytes engulfed within TNC. Using congeneic inbred

strains differing only in the MHC we found a surprisingly high alloreactivity by TNC-L far exceeding the GVHR of mature PBL and extra-TNC thymocytes (17). Since the influence of the male-specific antigen was excluded the observed GVHR of TNC-L solely depended on the recognition of foreign MHC.

Serial transfer experiments proved that the GVHR of TNC-L is a specific immunological reaction. Because it is difficult to quantitate pock size, the spleen weight of recipients was used as a parameter for GVHR (28). Syngeneic homing was excluded as a cause of the increase in spleen weight by appropriate controls.

Since MHC class II–positive donor cells were found in primary and secondary pocks induced by TNC-L or PBL, the donor cells reflected activated T lymphocytes. The presence of mature T cells of host origin at early stages of embryonic development (ED16 and ED14) that expressed class II molecules and IL-2R in primary and secondary pocks is not surprising. Accelerated maturation of the immune system by the induction of GVHR has been described (28, 39).

While CD4$^+$/CD8$^+$ thymocytes were more numerous outside TNC, within the TNC-L, CD3$^+$ and CD4 or CD8 single-positive TNC-L were enriched. TNC-L, therefore, comprised a heterogenous population with a high number of mature thymocytes of both Th or Ts/c phenotype. This confirms previously observed functional differences for TNC-L (14–16). Since CD4 or CD8 single-positive thymocytes are generally found in the thymic medulla, we interpret our results as an indication that the engulfed thymocytes differentiate into mature CD4$^+$ or CD8$^+$ T cells within the subcapsular TNC and subsequently migrate to the medulla. Whether this step of differentiation is consistent with positive selection events is yet to be clarified (31, 33).

We also showed that TNC-L were composed of both a larger proportion of TCR-α/β and a minor proportion of TCR-γ/δ^+ cells. The ratio of TCR-α/β^+ to TCR-γ/δ^+ cells was similar to that in the normal chicken thymocyte population (23), but the number of each subpopulation was significantly increased. All TCR-α/β^+ and TCR-γ/δ^+ cells co-expressed the signal transducing molecule CD3 (40). A considerably larger percentage of CD4$^+$ cells expressed the TCR-α/β than the TCR-γ/δ, but both of these subpopulations were enriched within TNC. Similar observations were made for TCR-α/β^+/CD8$^+$ and TCR-γ/δ^+/CD8$^+$ TNC-L. Although our observation that CD4$^+$ or CD8$^+$ thymocytes expressed TCR-γ/δ is contradictory to other studies in the chicken that found only CD4$^-$ and CD8$^-$ TCR-γ/δ thymocytes (21, 25), it is consistent with the recent description of CD4$^+$ and/or CD8$^+$ thymocytes of the γ/δ phenotype in C57BL/6 mice (41). In accordance with

these data we also found a minor population of CD4$^+$ TCR-γ/δ^+ cells in the periphery and within the spleen (data not shown). CD8$^+$ TCR-γ/δ^+ peripheral T cells have been shown previously (21, 23, 25). Nonspecific staining was excluded by two different immunofluorescence protocols (see Materials and Methods) revealing the same results.

A possible explanation of the discrepancy within the chicken system using the same mAb may be due to differences in the animal strains used. Whereas the mAb were produced and originally studied in outbred strains, we examined the highly inbred CB and B^7 strains which possibly express TCR-γ/δ molecules of a selected clonotype. The high number of CD4$^+$ or CD8$^+$ TCR-γ/δ^+ cells among TNC-L led to the assumption that TNC also provide a special microenvironment for the maturation of TCR-γ/δ^+ cells. Whether TCR-γ/δ^+ thymocytes can be CD4/CD8 double-positive remains to be determined.

These data support the concept that TNC provide a particular thymic microenvironment for T cell differentiation and maturation. Our functional data also demonstrate that TNC-L are an accumulation of especially allo-aggressive T cells, by far exceeding the GVHR potential of extra-TNC thymocytes and mature T cells in the peripheral blood. Since no T cell death was found within TNC, we postulate that they are sites for positive selection for MHC class I and class II antigen recognition and that deletion of potentially autoreactive cells occurs later on their way to the medulla, most probably when non-MHC self antigens are presented to them in the context of MHC self-antigens by dendritic cells and macrophages (42). An interesting possibility for the transport of non-MHC self-antigens into the thymic cortex via the transcapsular route bypassing the blood-thymic barrier has been reported (43). This model would also be in line with the observation that dead T cells can be found in large numbers around and within thymic macrophages. In contrast to macrophages and dendritic cells, TNC cannot activate thymocytes by engaging the TCR with non-MHC self-molecules (44, 45). The threshold for deletion of an autoreactive cell could be its affinity: those with high affinity would be targets for deletion, those with low affinity surviving the process, such as autoreactive T suppressor cells (31, 33). From a practical viewpoint, with regard to the possible role of TNC as a site for the process of self-recognition, it is noteworthy that we have previously reported a profound deficit of TNC in Obese strain (OS) chickens, which are afflicted with spontaneous autoimmune thyroiditis (SAT) (13, 46). Since this deficiency was observed well before the beginning of thyroid infiltration and even in embryos, it was suggested that this phenomenon may be significant in the pathogenesis of this autoimmune disease.

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