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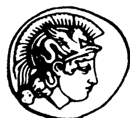

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Ontogenic changes in CD95 expression on human leukocytes: prevalence of T-cells expressing activation markers and identification of CD95$^-$CD45RO$^+$ T-cells in the fetus

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Abstract

The ontogeny of the human immune system was studied by analyzing fetal and adult tissues for the presence of various lymphocytes populations and activation/maturation markers. CD95 (fas) was expressed in hematopoietic tissues during the final stages of development of monocytes, granulocytes, NK cells and T cells, but to a much lesser extent on B cells. In the periphery, CD95 expression declined on granulocytes and NK cells. CD95 was expressed at a higher level on CD45RA$^+$ peripheral T-cells in the fetus than in the adult. Contrary to the belief that most fetal T-cells are naïve or resting, a notable number of CD45RO$^+$ T-cells were observed as well as an unique CD95$^-$CD45RO$^+$ population. Activation markers CD25, CD122, CD69 and CD80 were also present on fetal T-cells. These findings indicate that in the initial weeks following thymic maturation, a high frequency of T-cells is activated in the periphery of the fetus.

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Keywords: T cells; T-cell receptors; NK cells; B cells; Monocytes; Granulocytes; Liver; Thymus

1. Introduction

The human fetus is protected from most pathogens within the sterile environment of the uterus. Nonetheless, the cellular immune system begins its development early in gestation around the end of the first trimester. Reports suggest that T cells can be found in liver and peripheral blood as early as 7 and 9 weeks' gestation, respectively, although their overall numbers are very low [1,2]. Intrathymic CD3$^+$ T-cells can be readily identified at the 8th week of gestation [3,4] although the fetal

Abbreviations: BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; FITC, fluorescein isothiocyanate; mAb(s), monoclonal antibodie(s); PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PBS/BSA, washing buffer; PE, phycoerythrin; PerCP, peridinin chlorophyll; PI, propidium iodide; SP, single positive; TC, tricolor; TCR, T-cell receptor; UCB, umbilical cord blood.

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thymus is not anatomically completely mature until the 15th week of gestation [5]. Around this time, single positive (SP) $CD4^{+}CD8^{-}CD3^{+}$ and $CD4^{-}CD8^{+}CD3^{+}$ T cells begin to accumulate more rapidly in the periphery [4]. The purpose for the early development of lymphocytes is not known but may simply reflect the necessity for T-lymphopoiesis to keep pace with overall fetal development to assure the generation and proper selection of sufficient numbers of lymphocytes before birth. Moreover, the early development of T cells may also serve to protect the fetus from in utero infections or engraftment by maternal lymphocytes. Determining the functional capacity of fetal lymphocytes is key to defining a role for these cells in fetal immunity.

A limited number of studies have addressed the functional development of T cells from pre-term fetuses. Proliferation in response to allostimulation has been observed from fetal liver T-cells as young as 9 weeks' gestation [1,6–8]. However, cultured fetal T-cells showed some defects in cytotoxic responses which could be reversed by prestimulation with cytokine [7,9]. More on the functional capacity of fetal T-cells has been learned from analyses of T cells obtained from umbilical cord blood (UCB) at the time of birth. The T-cell repertoire appears fully formed at birth yet the repertoire appears to be composed of primarily naïve T-cells [10]. Whereas $CD45RO^{+}CD45RA^{-}$ memory T-cells are common in adult blood, UCB is primarily composed of $CD45RO^{-}CD45RA^{+}$ naive T-cells [11–15]. Other cell-surface markers important in the functioning of T cells, such as CD3 and CD28, are expressed at similar levels to adult cells, but signaling through these proteins is attenuated in UCB T-cells. Neonatal T-cells failed to increase CD25, CD154 and CD178 (Fas ligand) expression when stimulated through CD3 and CD28 [16]. Reduced proliferative responses of allostimulated UCB T-cells have also been noted [11,17–19]. Cord blood T-cells also have greatly reduced perforin expression [20]. Together with the observations of reduced CD178 expression, these findings suggest a reduced cytolytic capacity of neonatal T-cells similar to the findings on midgestation T-cells. Thus, despite T-lymphopoiesis beginning early in fetal life, some attenuation of T-cell function appears to occur in addition to the naïveté of the fetal immune system.

CD95 (fas) is an important regulator of homeostasis of the immune system. CD95 is expressed on some T cells and to varying degrees on all other leukocyte lineages. Its expression is increased with T-cell activation and CD95 expression is highest on $CD45RO^{+}$ T-cells [21]. Triggering of programmed cell death by CD95 activation, through interaction with CD178, leads to clearance of activated T-cells and thereby limits immune responses [22,23]. Neonatal T-cells express less CD95 than their adult counterparts [21,24]. Expression of CD95 is also low to negative on SP thymic cells from midgestation thymic tissues, although CD95 is expressed on immature T-cell progenitors [25]. Moreover, the expression of CD95 on T cells has been shown to increase with age up to 75 years, after which expression decreases somewhat [26]. These data suggest that CD95 expression increases with the maturation of the immune system. In this regard it is worth noting that CD95 expression can increase more rapidly in children infected by the human immunodeficiency virus [24].

In this study, it was our aim to gain a better understanding of the functional maturity of the fetal immune system by analyzing the expression of various cell surface markers on fetal T-cells as well as on other leukocyte populations. Expression of CD95 was analyzed on leukocytes harvested from peripheral blood, spleen, liver and bone marrow of midgestation fetuses and compared to CD95 expression on cells harvested from neonatal and adult tissues. Furthermore, various cell-surface markers, associated with activation, were analyzed on fetal, neonatal and adult T-cells. Our results suggest a higher level of T-cell activation in utero than would be predicted from previous studies of UCB T-cells.

2. Materials and methods

2.1. Isolation of human leukocytes from adult, neonatal and fetal tissues

Human tissues were obtained and studied under the approval of the Committee on Human Research at our institute. Male and female adult peripheral blood was

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obtained from healthy volunteers ranging in age from 24 to 61 years. Neonatal UCB and fetal hematopoietic tissues were obtained with consent of the women prior to delivery or elective abortion. Neonatal blood (birth at 33 weeks' gestation to term) and some fetal tissues were obtained at our institute. Additional fetal tissues were obtained from Advanced Bioscience Resources Inc. (Alameda, CA). Fetal tissues were harvested shortly following the abortion and were transported to the laboratory in sterile containers held on ice. Experiments on adult bone marrow were performed at Ingenex, Inc. (Menlo Park, CA) in compliance with regulations issued by the state and federal governments. Fetal tissues ranged in gestational age from 15 to 24 weeks, as determined by the foot length of the fetus. Each experiment was performed on cells obtained from an individual specimen; tissues from different specimens were not pooled for analyses.

Adult peripheral blood was obtained by venipuncture. Approximately 7 ml of blood was drawn into a vacutainer tube containing ethylene diamine tetraacetic acid (EDTA). The blood was diluted to a total volume of 50 ml in PBS/BSA washing buffer consisting of phosphate buffered saline (PBS) containing 0.3% fraction-V ethanol-extracted bovine serum albumin (BSA) (Roche Applied Science, Indianapolis, IN) and 50 μg/ml gentamicin sulfate (Life Technologies, Grand Island, NY). The cells were pelleted by centrifugation and erythrocytes were depleted by chemical lyses using ACK lyses buffer, pH 7.2–7.4, consisting of 0.15 M NH_4Cl, 1.0 mM $KHCO_3$ and 0.1 mM Na_2 EDTA (Sigma Chemical Company, St Louis, MO). The cells were pelleted by a 7-min centrifugation approximately 1 min after the addition of the ACK lyses buffer. If lysis of the erythrocytes was incomplete, the procedure was repeated. Otherwise the cells were washed once in PBS/BSA and suspended in blocking buffer consisting of PBS with 5% normal mouse serum (Gemini Bio-Products, Inc., Woodland, CA) and 0.01% NaN_3. Alternatively, peripheral blood mononuclear cells (PBMC) were prepared by centrifugation of the blood at 600 × g for 25 min on a layer of 1.077 g/ml LymphoPrep (Life Technologies). The light-density cells were harvested and washed twice before being suspended in blocking buffer for staining.

PBMC were isolated from neonatal UCB by immunomagnetic bead depletion of $CD235a^+$ erythrocytes, performed as previously described for fetal liver cells [27], and density separation using 1.077 g/ml Nycoprep (Life Technologies). The isolation of light-density neonatal blood cells was performed in an analogous fashion to the procedure described for the adult PBMC. In some cases, freshly prepared PBMC from UCB were frozen in autologous plasma with 10% dimethyl sulfoxide (Sigma Chemical Co.) and were thawed shortly before phenotypic analysis.

Fetal blood leukocytes were harvested from umbilical cords, placental vessels and/or hearts obtained from elective abortions. Blood was harvested from the cords after first washing the cords with PBS/BSA and then resecting (0.5 cm) the ends with scissors. The washed cords were placed in on a clean culture dish and were cut in 2 cm pieces. Fifteen to forty millilitres of PBS/BSA were injected with a 28-gage insulin syringe (Becton Dickinson & Co., Franklin Lakes, NJ) into the three cord vessels to rinse the blood out of the vessels through the fresh cut surface. In some initial experiments, fetal UCB was squeezed out through the fresh cut end using forceps. Blood samples were filtered using 70 μ nylon-mesh cell strainers (BD Biosciences, San Jose, CA) as needed to remove clots or large cellular debris. For some fetal specimens fetal blood was obtained from the placenta by direct venopuncture of surface vessels near the placenta–umbilical cord junction. Placental blood was drawn into a syringe containing heparin. Fetal hearts were collected with the pericardial sack. The surface was cleaned with PBS/BSA to remove any contaminating maternal cells. The pericardial sack was then removed and the ends of the great vessels resected. In a clean dish, the heart was cut open from the great vessels down to the apex and the blood was rinsed out of the great vessels and chambers with PBS/BSA. Erythrocytes were depleted by chemical lysis using ACK lysis buffer or immunomagnetic bead depletion of $CD235a^+$ cells [27,28]. For some analyses of lymphocytes only, fetal PBMC were prepared using LymphoPrep as described above. Blood cells were washed and suspended in blocking buffer for staining.

Splenocytes were isolated by crushing the spleen with a glass pestle through a wire mesh cell strainer (Sigma Chemical Company) and rinsed with washing buffer. The cell suspension was passed through a 70 μ

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nylon-mesh cell strainer and pelleted by centrifugation. Erythrocytes were depleted by chemical lysis using ACK lysis buffer or immunomagnetic bead depletion of CD235a$^+$ cells [27]. Alternatively, light-density splenocytes, depleted of erythrocytes and granulocytes, were isolated by centrifugation using LymphoPrep as described above. After either method, splenocytes were washed and suspended in blocking buffer for staining.

Fetal thymocytes were prepared for analysis by passage of the thymus through a cell strainer as described for the spleen samples. In order to remove erythrocytes and cellular debris, light-density thymocytes were harvested after centrifugation over a layer of 1.077 g/ml Nycoprep, as described above for the adult blood samples.

Light-density CD235a$^-$ fetal liver cells and fetal bone marrow cells were prepared by immunomagnetic-bead depletion and centrifugation over a layer of 1.077 g/ml Nycoprep as previously described [27].

2.2. Monoclonal antibodies

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll (PerCP) labeled mAbs were purchased from BD Biosciences/BD PharMingen (www.bdbiosciences.com) recognizing the following antigens: CD3-FITC (SK7), CD8-FITC (SK1), CD10-FITC (W8E7), CD14-FITC (MΦP9), CD15-FITC (MMA), CD19-FITC (4G7 or SJ25C1), CD-19-PerCP (SJ25C1), CD45RA-APC (HI100), CD45RO-APC (UCHL1), CD45RO-PE (UCHL1), CD95-PE (DX2), CD122-PE (TU27), mouse IgG_1-FITC, mouse IgG_{2a}-FITC and mouse IgM-FITC. Anti-CD56-FITC and anti-CD56-PE (C5.9) were purchased from Exalpha Corporation (Boston, MA). Labeled antibodies recognizing CD4-tricolor (TC) (S3.5), CD14-FITC (TÜK4), CD15-FITC (V1MC6), CD25-PE (CD25-3G10), CD45-PE (HI30), mouse IgG_1-FITC, mouse IgG_1-PE, mouse IgG_{2a}-PE, mouse IgG_{2b}-FITC and mouse IgM-FITC were purchased from Caltag (Burlingame, CA). Conjugated mAb were also purchased from Beckman-Coulter (Miami, FL) recognizing the following antigens: CD3-phycoerythrin-cyanine 5 (PC5) (UCHT-1), CD14-PC5 (RM052), CD16-PC5 (3G8), CD45-PC5 (J33), CD45RA-FITC (ALB11), CD45RO-FITC (UCHL1), CD56-PC5 (N901), CD69-PC5 (TP1.55.3), CD80-PE (MAB104), CD127-PE (R34.34) and mouse IgG_1-PC5. Monoclonal antibodies labeled with PE recognizing T-cell receptor (TCR) α/β (BMA031) and TCR γ/δ (5A6.E9) were obtained from Endogen (Woburn, MA). A FITC-conjugated mAb against TCR α/β was obtained from T Cell Diagnostics, Inc. (Cambridge, MA). A kit containing a panel of FITC-, PE- and a mixture of FITC- and PE-conjugated mAb recognizing different TCR Vβ chains was purchased from Beckman-Coulter and was used according to the manufacturers recommendations.

2.3. Flow cytometric analysis of cell surface markers

Approximately 2×10^5 cells suspended in up to 200 μl blocking buffer were incubated in 96-well Costar V-bottom Plate (Corning Inc., Corning, NY) with saturating amounts of mAbs on ice for at least 30 min. Cells were washed twice with 250 μl PBS/BSA containing 0.01% NaN_3 (Sigma Chemical Co.). The washed cells were suspended in PBS/BSA containing 0.01% NaN_3 and 2 μg/ml propidium iodide (PI), purchased from Sigma Chemical Co. PI was used to mark dead cells, so that they could be excluded from the analysis. PI was omitted in 3-color analyses using PC5 or PerCP labeled mAbs. Flow cytometric analysis was performed using either a FACScan or a FACSCalibur flow cytometer (BD Biosciences). Analyses of results were performed using CellQuest software (BD Biosciences).

2.4. Data presentation and statistical analysis

The median results of multiple measurements done on individual tissue samples are reported to reduce the effects of outliers. Box plots are used to present the data, which show the 10th (lower bar), 25th (box bottom), 50th (median-bar in box), 75th (box top) and 90th (upper bar) percentiles. Circles in the box plots indicate outlying data points below the 10th and above the 90th percentiles. The significance of differences observed between fetal and adult cells was determined using an unpaired Student's *t*-test. Results were considered significant when $P \leq 0.05$.

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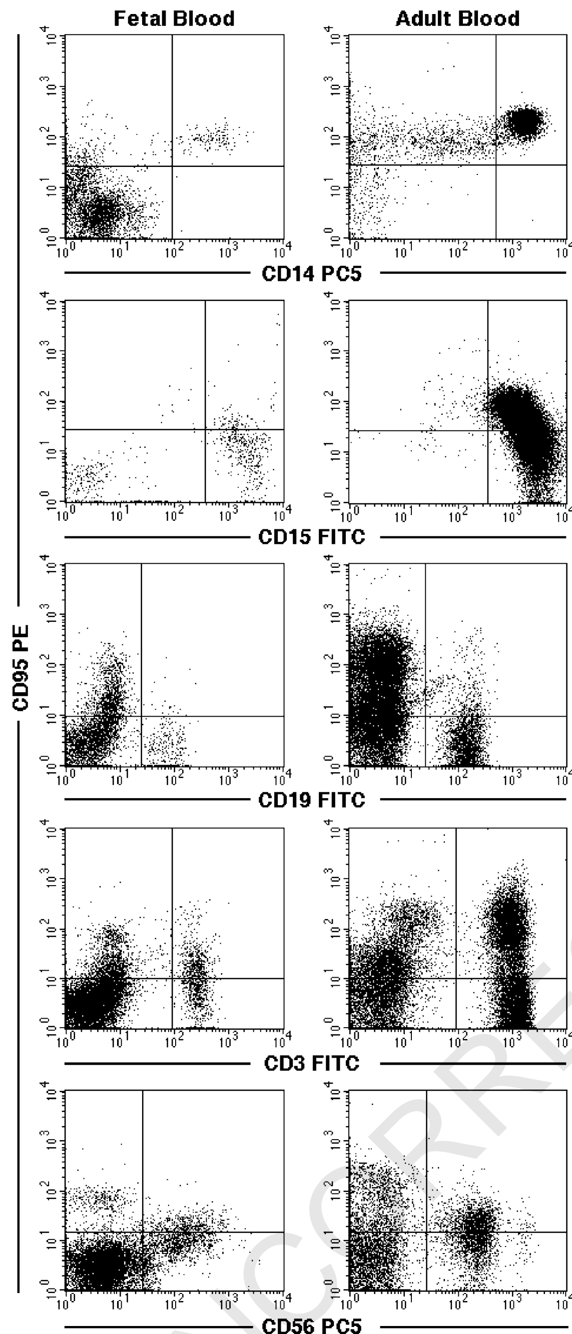

Fig. 1. Expression of CD95 by fetal and adult leukocytes. Representative data from analyses of fetal and adult peripheral-blood leukocytes are shown. The fetal leukocytes had a gestation age of 19 weeks. Adult peripheral blood was obtained from a 36 year old male. The five subsets of leukocytes were defined by the expression of their specific cell-surface antigen, as shown, as well as by their characteristic light-scatter profiles (not shown). $CD14^+$ monocytes as well as the 3 lymphocyte populations were defined as cells with a low to moderate forward-light scatter and a low side-light scatter. $CD15^+$ granulocytes were defined as cells with a high side-light scatter. Additionally, 3-color analyses further enabled the $CD19^+$ B cell population to be defined by their lack of CD14 expression (not shown), which helped to reduce non-specific background staining. $CD56^+$ NK cells were also defined by their lack of CD3 expression (not shown). Quadrants were drawn based on controls stained with mouse IgG1-PE, instead of CD95-PE, such that background staining was $\leq 2.2\%$ for the 2 myeloid populations and $\leq 0.9\%$ for the 3 lymphoid populations.

3. Results

3.1. Analysis of CD95 expression on fetal and adult leukocytes

Expression of CD95 was analyzed by flow cytometry on leukocyte populations found in fetal (blood, spleen, liver and bone marrow) and adult (blood and bone marrow) tissues. The gestational ages of the fetal samples ranged from 15 to 24 weeks. CD95 was expressed on at least some cells of each of the lineages analyzed in both fetal and adult tissues. Most circulating $CD14^+$ monocytes expressed CD95 at high levels (Fig. 1). In adult blood, a median 97.1% of monocytes expressed CD95. Likewise, a median 93.7% of fetal blood and 83.2% of fetal splenic monocytes showed CD95 expression (Fig. 2A). The levels of CD95 expressed on fetal and adult peripheral-blood monocytes also did not differ (Fig. 2B), although the fetal splenic monocytes had reduced levels of CD95 ($P = 0.053$). However, the reduced levels of CD95 in the spleen appeared to be the result of increased background staining, with non-specific isotype-matched control antibody, rather than reduced CD95 expression (data not shown). The expression of CD95 on the immature $CD14^+$ monocytes developing in hematopoietic tissues was also analyzed. Both in the fetal and adult bone-marrows, CD95 expression was apparent on cells expressing low and high levels of CD14 indicating that CD95 is already expressed at the time of CD14 acquisition (Fig. 3).

Few fetal $CD15^+$ granulocytes expressed CD95 (Fig. 1). In adult blood, a median 86.0% of granulocytes expressed CD95, but only 22.0% of fetal blood and 21.1% of fetal splenic granulocytes expressed CD95 (Fig. 2A). The decreased expression of CD95 in fetal tissues was significant compared to

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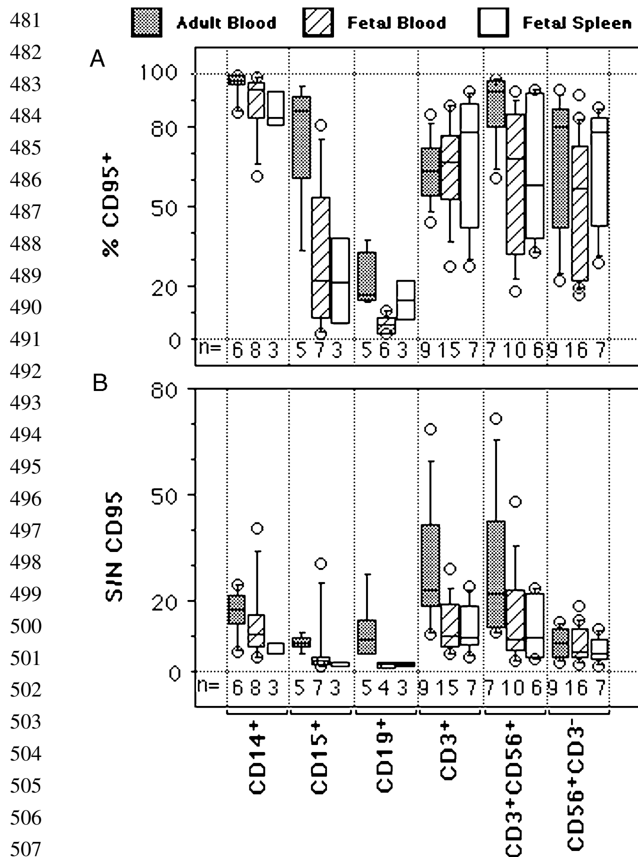

Fig. 2. Box plots of the frequency and intensity of CD95 expression on fetal and adult leukocytes. Leukocyte populations were defined by their phenotypic properties as described in Fig. 1. Box plots are shown of the percent of cells expressing CD95 (A) and the signal to noise (S/N) ratio for CD95 expression (B). The numbers (*n*) of tissue samples analyzed are indicated at the bottom of the box plots.

adult granulocytes ($P \leq 0.025$). The levels of CD95 expression on $CD15^+$ granulocytes was also significantly lower in the fetal spleen ($P = 0.006$), but did not differ between fetal and adult blood (Fig. 2B). CD95 expression appeared on those granulocytes with lower levels of CD15 expression as exemplified in Fig. 1. Furthermore, immature granulocytes in the fetal and adult bone-marrows uniformly expressed CD95 (Fig. 3). The immature $CD95^+CD15^{low}$ granulocytes were also enriched in the light-density fractions of fetal liver and neonatal UCB (data not shown), indicating that CD95 expression is a feature of young granulocytes that is decreased with maturation and increased CD15 expression.

The frequency of CD95 expression was decreased on $CD19^+$ B cells from fetal blood compared to adult blood ($P = 0.005$) (Fig. 2A). Although the level of CD95 expression was also decreased on fetal blood B-cells this difference was not significant (Fig. 2B). CD95 expression on fetal splenic B-cells did not differ significantly from adult B-cells. The overall modest levels of CD95 expressed on peripheral B-cells were also observed on B cells in the fetal and adult bone-marrows (Fig. 3). These results indicate a lack of CD95 expression on most immature B-cells and their immediate progenitors.

Both the frequency and levels of CD95 expression on $CD56^+CD3^-$ NK-cells was comparable between fetal and adult cells (Fig. 1). CD95 was expressed on a median 56.9–79.8% of fetal and adult NK cells (Fig. 2A). Immature NK cells, expressing low levels of CD56, found in the fetal liver and adult bone marrow expressed CD95 (Fig. 3). Thus, unlike the B cells, CD95 expression is a feature of maturing NK cells.

3.2. Expression of CD95 on fetal and adult T cells

A similar frequency of fetal and adult $CD3^+$ T-cells expressed CD95 (Figs. 1 and 2). However, the levels of CD95 expression on fetal blood and splenic T-cells were less than half those on adult T-cells ($P \leq 0.003$) (Fig. 2B). In general, fetal T-cells consisted of a predominant population of cells that expressed low levels of CD95 and a small subpopulation of T cells that expressed higher levels of CD95 (Fig. 1). Adult T-cells, in contrast, tended to be polarized into two subsets consisting of either $CD95^+$ or $CD95^-$ cells.

The frequency and intensity of CD95 expressed by adult $CD4^+$ T cells were previously shown by Miyawaki et al. to be higher than for $CD8^+$ T cells [21]. Our analysis of adult T-cells confirmed these findings. On the contrary, analyses of fetal blood and splenic T-cells did not indicate any significant difference in the frequency of CD95 expression by $CD4^+$ and $CD8^+$ T-cells (data not shown). However, the intensity of CD95 expressed by fetal $CD4^+$ T-cells was modestly higher than by fetal $CD8^+$ T-cells. The signal to noise (S/N) ratio for CD95 expression was 28% and 68% higher for $CD4^+$ T-cells than for $CD8^+$ T-cells from blood and spleen, respectively (data not shown). These differences did not reach significance by paired analysis ($P = 0.071$ for blood and 0.137 for

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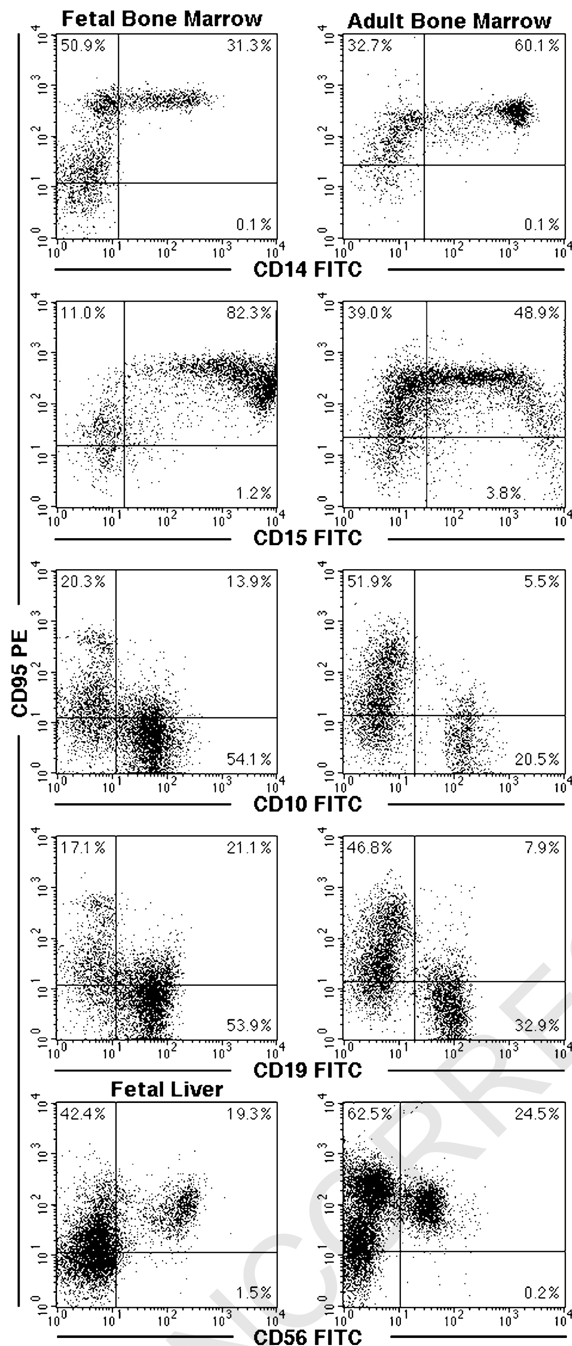

Fig. 3. Expression of CD95 on immature monocytes, granulocytes, B cells and NK cells. Fetal bone marrow, or fetal liver (right column) and adult bone marrow (left column) were analyzed for the expression of CD95 and the indicated leukocyte markers. All tissues were enriched for immature leukocytes by isolation of light-density CD235a^{-} cells. Additionally, the leukocyte populations of interests were enriched for display by gating on their respective characteristic light-scatter profiles as described in Fig. 1. Quadrants were drawn based on controls, such that background staining in the upper right quadrant was $\leq$2.4% for the 2 myeloid populations and $\leq$1.1% for the 3 lymphoid populations.

spleen). For comparison, the S/N ratio for CD95 expression on adult CD4^{+} T-cells was 125% higher than on CD8^{+} T-cells.

Three-color analyses of CD3, CD4 and CD8 expression were performed on fetal blood and spleen samples ranging in age from 16 to 24 weeks' gestation. Although there were no significant differences in the ratio of CD4 to CD8 SP T-cells between fetal and adult samples, the younger fetal specimens had a lower ratio than older fetal specimens (data not shown). In three fetal samples younger than 19 weeks' gestation the CD4/CD8 ratio ranged from 0.46 to 1.8. In samples between 19 and 24 weeks had ratios that ranged from 2.0 to 2.7, comparable to ratios observed from adult blood.

The tendency towards higher CD95 expression on CD4^{+} T-cells was also observed on SP T-cells in the fetal thymus (Fig. 4A). Fetal thymi of 15, 19 and 22 weeks' gestation were analyzed and CD95 was found to be on 1.9-fold more CD4 SP thymocytes than on CD8 SP thymocytes ($P = 0.057$, paired comparison). Low expression of CD95 was also observed on DP thymocytes. Double negative (DN) thymocytes were up to 83.5% CD95^{+} ($n = 3$). DN thymocytes include CD3^{-} T-cell progenitors, CD3^{+} immature T-cells and cells of various other lineages. Nonetheless, the majority of CD95 expression in the fetal thymus was on cells expressing high levels of CD3 as seen in Fig. 4B. To determine whether CD95 is expressed by T cells shortly before emigration from the thymus we analyzed CD3^{+}CD45RA^{+} and CD3^{+}CD45RO^{-} thymocytes [29–31]. There was CD95 expression on both of these overlapping subpopulations of thymocytes, indicating that CD95 is expressed at low levels on T cells that emerge from the thymus.

3.3. Expression of CD45 isoforms by fetal and adult T cells

The high frequency of CD95 expression on fetal T cells was unexpected considering published findings indicating low expression of this protein on neonatal T

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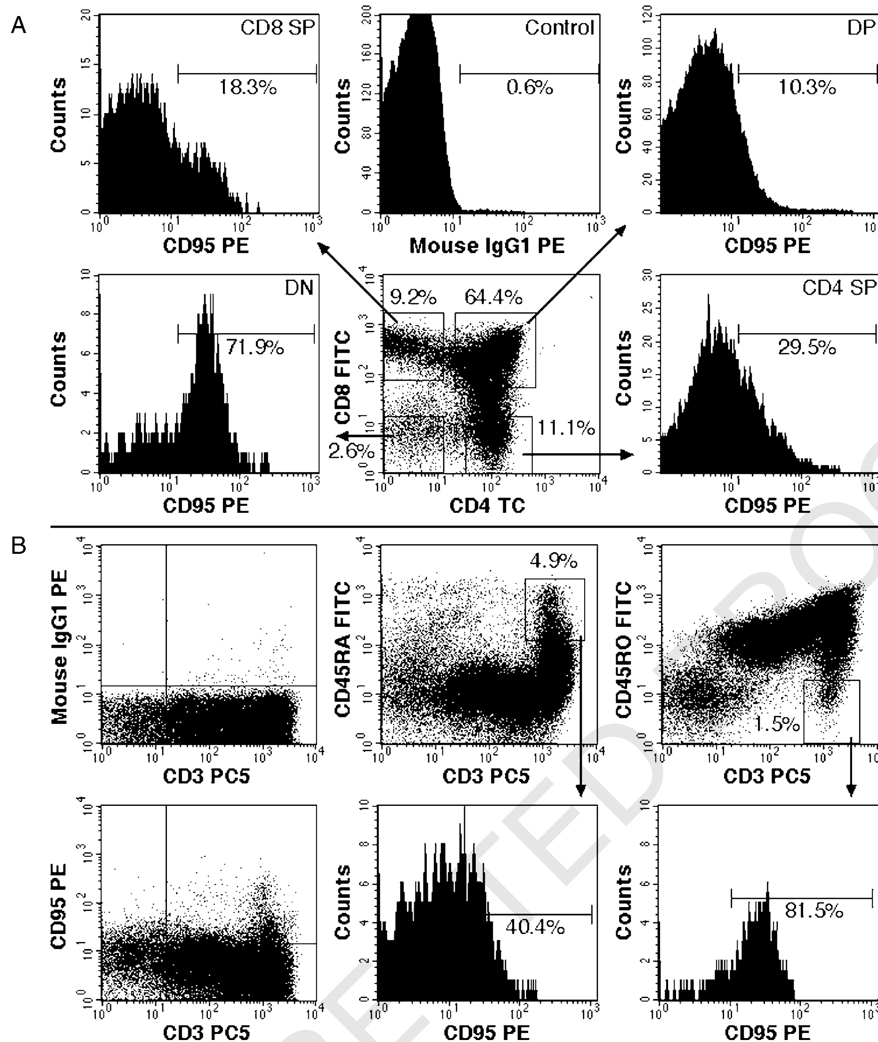

Fig. 4. Expression of CD95 during T-cell development in the fetal thymus. Expression of CD95 on DN, DP and SP thymocytes is shown in (A). Co-expression of CD3 and CD95 as well as the level of CD95 expression on $CD3^+CD45RA^+$ and $CD3^+CD45RO^-$ thymocytes is shown in (B). Representative results are shown from a 15 weeks' gestation thymus.

cells. Since CD95 expression on adult T cells is predominantly on $CD45RO^+$ T cells [21], we examined the expression of CD45RA and CD45RO on fetal, neonatal and adult T cells (Figs. 5 and 6A). Previous findings have indicated a higher frequency of $CD45RO^+CD45RA^-$ T-cells in the fetus than the in the neonate [32], thus possibly accounting for the higher rate of CD95 expression in the fetus. The $CD45RO^+CD45RA^-$ subset represented a median of 11.3% and 15.5% of T cells in the fetal blood and spleen, respectively, which was significantly higher than the median 1.5% in UCB obtained from full term newborns ($P = 0.031$ for blood and < 0.001 for spleen) (Fig. 6A). Although, the frequency of fetal $CD45RO^+CD45RA^-$ T-cells was reduced compared to the adult ($P = 0.046$ for blood and 0.072 for spleen) (Fig. 6A). Both fetal blood and spleen also had significantly reduced numbers of $CD45RO^+CD45RA^+$ T-cells compared to adults, whereas $CD45RO^-CD45RA^+$ T-cells were more prevalent in the fetus than in the adult ($P \leq 0.028$) (Fig. 6A). The majority of T cells in UCB were

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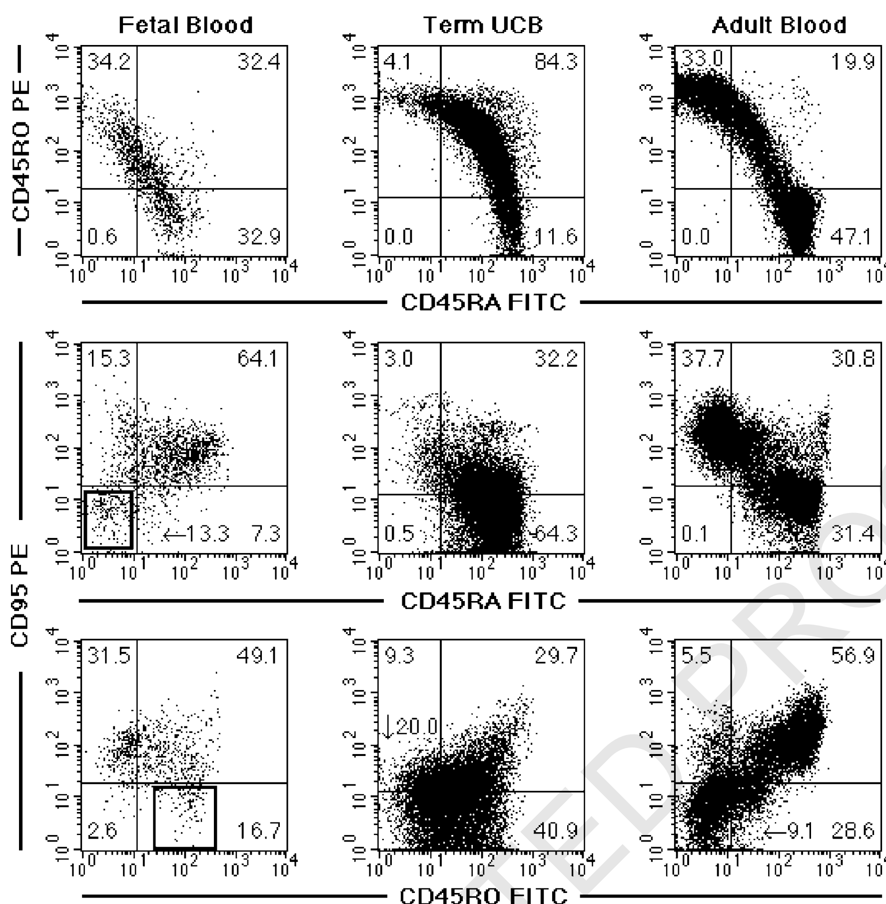

Fig. 5. Expression of CD45RA, CD45RO and CD95 by fetal, neonatal and adult T-cells. 3-color analyses were performed by staining blood cells with CD3-PC5 and the indicated mAbs. Events shown are gated on $CD3^+$ cells with low forward- and side-light scatters. The gestational ages of the fetal blood samples where 20 weeks for the top dot plot and 16 weeks for the lower two dot plots. The single UCB sample was obtained from a full-term delivery (approximately 40 weeks' gestation). Adult blood was obtained from a 29 year individual (top dot plot) and 37 year individual (middle and bottom dot plots). Numbers represent the percentage of events in the corresponding quadrants. The fetal $CD95^-CD45RO^+$/$CD95^-CD45RA^-$ T-cell population is highlighted by rectangular regions.

$CD45RO^+CD45RA^+$ ($P = 0.033$ versus adult blood), whereas the $CD45RO^-CD45RA^+$ subpopulation was similarly represented in UCB compared to adult blood. Thus, the naive $CD45RO^-CD45RA^+$ T-cell subset is enriched in the fetus, but a notable number of $CD45RO^+CD45RA^-$ T-cells are present in the fetal circulation, more so than at the time of birth.

CD95 expression was detected on both $CD45RA^+$ and $CD45RO^+$ fetal T-cells, but differences between fetal and adult T cells were apparent (Figs. 5 and 6B). The median frequency of fetal $CD45RA^+$ T-cells that expressed CD95 was 62.0% in the blood and 85.4% in the spleen (Fig. 6B). The median frequencies of CD95 expression on adult and neonatal $CD45RA^+$ T-cells were significantly lower at 45.1% and 37.1%, respectively ($P = 0.012$ for both comparisons). Most adult $CD45RO^+$ T-cells expressed CD95 (median 89.1%), as previously described [21]. However, CD95 expression was reduced on $CD45RO^+$ T-cells from fetal blood (median 59.7%), fetal spleen (median 72.8%) and UCB (median 54.9%). These differences in CD95 expression compared to adult $CD45RO^+$ T-cells were significant ($P \leq 0.009$). Moreover, examination of the pattern of CD95 expression on fetal T-cells revealed a subpopulation of $CD45RO^+CD45RA^-$ T-cells that was

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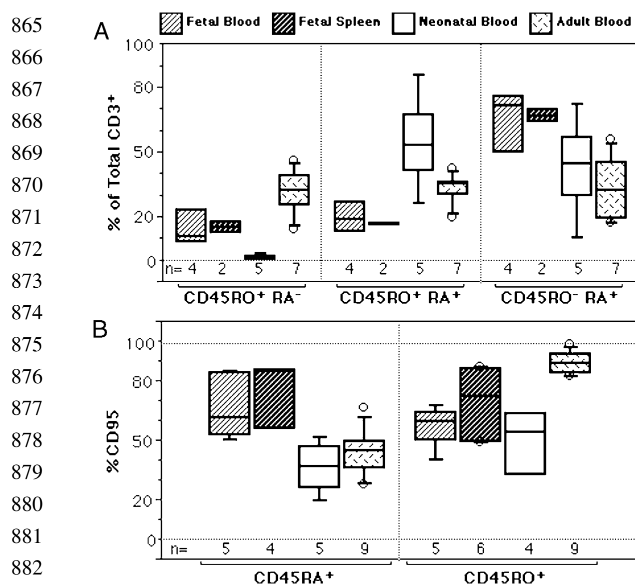

Fig. 6. Expression of CD45 subtypes and CD95 by fetal, neonatal and adult T cells. The percentages of $CD45RO^+CD45RA^-$, $CD45RO^+CD45RA^+$ and $CD45RO^-CD45RA^+$ T-cells in fetal tissues, UCB and adult blood are shown in (A). The percentages of $CD45RA^+CD3^+$ and $CD45RO^+CD3^+$ T cells that expressed CD95 are shown in (B). The numbers (*n*) of tissue samples analyzed are indicated at the bottom of the box plots.

$CD95^-$ (Fig. 5, boxed regions). These cells were best defined by their lack of CD45RA staining rather than their expression of CD45RO. This is because many $CD45RO^+$ cells can also express CD45RA, whereas all $CD45RA^-$ cells are $CD45RO^+$. The $CD95^-CD45RO^+CD45RA^-$ T-cell population was not present to any appreciable degree in either term UCB or adult peripheral blood.

3.4. Cytokine receptors and activation markers expression by fetal and adult T-cells

The expression of CD95 and CD45RO by fetal T-cells suggests the possibility that a sizable proportion of fetal T-cells have undergone activation. To support this hypothesis, we analyzed the expression of various cytokine receptors and other cell-surface markers associated with T-cell growth and activation (Table 1). Components of the IL-2 receptor complex were analyzed, which indicated that CD25 was expressed on a similar portion of fetal T-cells as on adult T-cells. In contrast, CD25 was significantly reduced on neonatal T-cells, particularly on the $CD45RO^+$ subset. CD122 was expressed on a significantly higher number of fetal T-cells than on either neonatal or adult T-cells. The α-chain subunit of the IL-7 receptor, CD127, was widely expressed on T-cells from all sources, but was significantly reduced on the $CD45RO^+$ subset of fetal T-cells. T cells in UCB expressed higher levels of CD127 compared to fetal or adult T-cells. CD132, the common γ-chain subunit of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors, was

Table 1
Expression of cytokine receptors and various activation markers on fetal, neonatal and adult $CD3^+$ T-cells

Marker	Total $CD3^+$ T-cells		Adult	$CD45RO^+CD3^+$ T-cells		Adult	$CD45RO^-CD3^+$ T-cells	Neonatal	Adult
	Fetal	Neonatal		Fetal	Neonatal		Fetal		
CD25	11.0[a]	3.4[b]	14.8	17.7	5.4[b]	22.2	3.8	2.6	3.4
CD122	14.3[a,b]	0.4	1.9	16.2[a,b]	0.7	2.2	11.5[a,b]	0.3[b]	2.0
CD127	77.8[a]	97.5[b]	84.3	67.5[a,b]	82.4	84.8	84.3[a]	100[b]	80.6
CD132	99.6	95.3[b]	100	92.5	96.3	98.4	100[a]	96.5	100
CD56	9.9	4.3	9.7	8.2	5.7	11.5	9.6	3.7	6.2
CD69	21.0[b]	12.5	10.7	ND	ND	ND	ND	ND	ND
CD80	3.7	0	0.3	5.2	0	0.6	0.6	0	0
TCR α/β	80.2[a]	97.6[b]	82.6	ND	ND	ND	ND	ND	ND
TCR γ/δ	17.3[a,b]	2.9[b]	8.2	ND	ND	ND	ND	ND	ND

Light-density cells isolated from fetal spleens and PBMC isolated from UCB and adult blood were analyzed for the expression of CD3, CD45RO and the indicated marker. T cells were defined by their expression of CD3 and by a low forward- and side-light scatter profile. Values represent the median level of expression observed on five fetal, four neonatal and six adult samples. ND = Not determined.

[a] $P \leq 0.05$ versus neonatal T-cells.

[b] $P \leq 0.05$ versus adult T-cells.

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expressed on nearly all T-cells at any stage of ontogeny. The markers CD69 and CD80 are expressed on T cells that have become activated [33–35]. The frequency of fetal T-cells expressing these markers was higher than in the adult or neonate. CD56, which is expressed by a subpopulation of cytotoxic T-cells [36], was expressed at similar levels in the fetus and adult, but was approximately half as abundant in the neonate. A significantly greater frequency of fetal T-cells were found to express γ/δ chains of the T-cell receptor.

3.5. Repertoire of TCR Vβ chain expression by fetal T-cells and thymocytes

The TCR Vβ chain repertoire expressed by fetal T-cells was studied to determine the diversity of TCR expression in the emerging immune system. Vβ chain expression was analyzed on splenic $CD3^+$ cells ranging in age from 16 to 24 weeks' gestation (Fig. 7A). A diverse repertoire was observed with the mean percent expression of each Vβ chain falling within the range of expression observed on adult specimens, as reported by the manufacturer of the test reagents. Moreover, two subsets of fetal splenic T-cells, $CD45RA^-$ ($CD45RO^+$) and $CD45RA^+$, were examined and both displayed diversity in Vβ chain expression similar to as described above, except for the following differences: The $CD45RA^-$ subset had a higher representation of Vβ11 ($P < 0.05$, $n = 3$) and Vβ5.1 ($P = 0.075$) and lower representation of Vβ14, Vβ16 and Vβ21.3 ($P < 0.05$, $n = 3$). The possibility that the $CD45RA^-$ T-cells in the spleen are thymocytes that have not gained CD45RA expression before exiting the thymus was examined by comparing the expression of Vβ chains on splenic and thymic

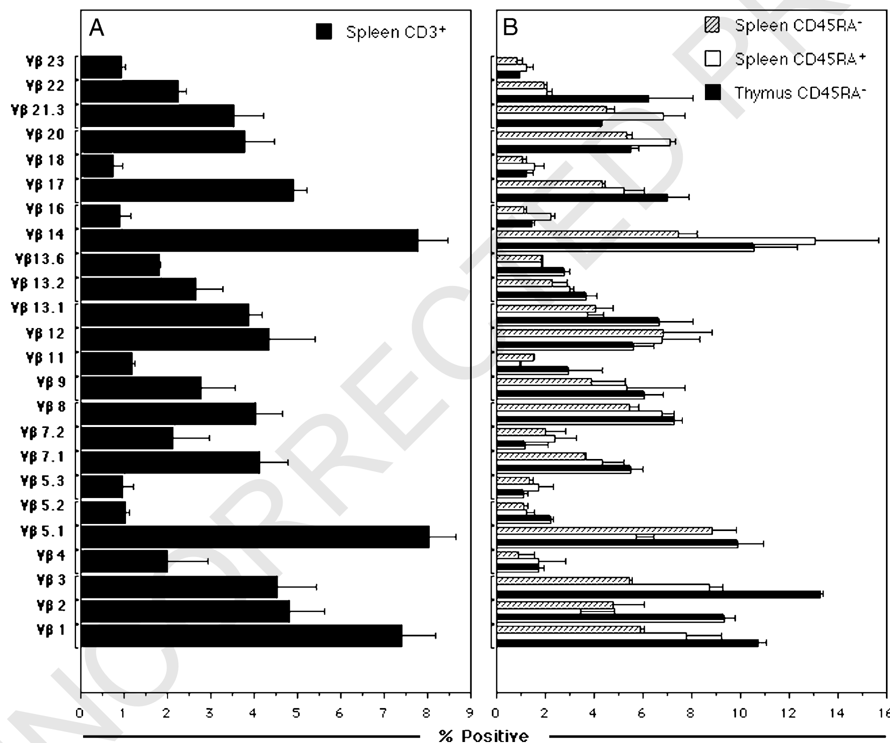

Fig. 7. Expression of TCR Vβ chains by fetal splenic T-cells and thymocytes. TCR Vβ chain expression was analyzed by 4-color flow cytometry. T cells were identified by their expression of high levels of CD3 and by their low light scatter profile. Vβ chain expression was analyzed from a cohort of 6 spleens ranging in gestation age from 16 to 24 weeks (A). Two of these spleens, of 19 and 22 weeks' gestation, were analyzed for Vβ chain expression on T-cells subdivided based on the expression of CD45RA (B). Thymocytes from these same fetal specimens were also analyzed and were gated using the same region as used to define the corresponding splenic $CD3^+CD45RA^-$ population. Results are presented as the mean ± SE.

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$CD45RA^{-}CD3^{+}$ cells (Fig. 7B). Besides wide-ranging similarity and some non-significant dissimilarities, there were notable significant differences between the thymic and splenic T-cells. Mainly, a lower representation of Vβ 1, Vβ 3, Vβ 5.2 and Vβ 13.6 ($P \leq 0.05$, $n = 2$) was observed on the splenic T-cells. These differences indicate that the splenic $CD45RA^{-}$ T-cells are not an exact match to the corresponding thymic population.

4. Discussion

The maturity of the human fetal immune system was analyzed from the perspective of CD95 expression as well as a number of additional cell-surface markers associated with T-cell activation and growth (Fig. 8). Most knowledge regarding fetal T-cells has come from the analyses of neonatal T-cells obtained from UCB. Studies have shown neonatal T-cells to be comprised of primarily $CD45RA^{+}$ naive/resting T-cells [11–15] that express low levels of the activation marker CD95 [21,24]. In contrast, our

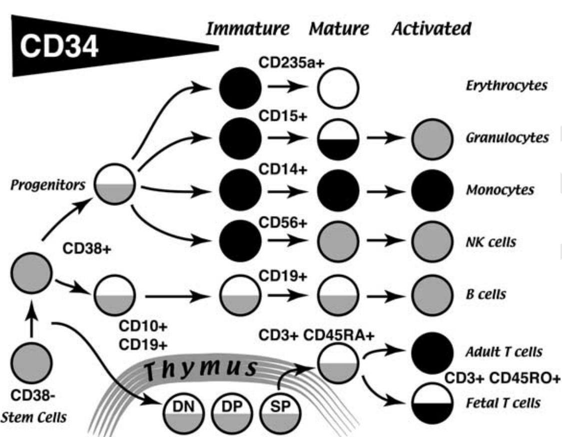

Fig. 8. Proposed expression of CD95 on human hematopoietic cells throughout ontogeny. The expression of CD95 at various stages of hematopoiesis ($CD34^{+}$ cells, black triangle), maturation and activation is indicated by shading. Black circles represent relatively high levels of CD95 expression, gray circles represent intermediate expression and open circles represent a lack of CD95 expression. Filled circles indicate that most cells of the indicated population express CD95, whereas semi-circles represent CD95 expression by a subpopulation of cells. The schema was developed from the data presented in this study as well as previously published reports [25, 27,50–53].

examination of midgestation fetal tissues indicates that the frequency of T cells that express CD95 in these tissues is comparable to that of adult T-cells. However, the levels of CD95 expression are reduced on fetal T-cells. Although fetal T-cells were predominantly $CD45RO^{-}CD45RA^{+}$ T-cells, $CD45RO^{+}CD45RA^{-}$ T-cells were present in the blood of midgestation fetuses, more so than at full term. Furthermore, a number of cell-surface markers associated with T-cell activation, were also observed on fetal T-cells at levels similar or higher than in the adult. These data indicate a, heretofore, unappreciated level of activation of peripheral T-cells circulating in the immediate weeks following thymic development in the human fetus.

Byrne et al. have reported a higher frequency of $CD45RO^{+}CD45RA^{-}$ T-cells in the midgestation fetus compared to full-term neonates [32]. Our findings confirm this observation and extend them by describing a subset of $CD45RO^{+}CD45RA^{-}$ T-cells in the fetus that lacks CD95 expression (Fig. 8). We are unaware of any previous description of a $CD95^{-}CD45RO^{+}CD45RA^{-}$ subpopulation of T cells, and we did not observe this population in post-natal blood. Nearly all adult $CD45RO^{+}CD45RA^{-}$ T-cells are known to express CD95 at high levels [21]. Indeed, the higher frequency of $CD45RO^{+}CD45RA^{-}$ T-cells in adults is a contributing factor to the higher levels of CD95 expression observed on adult versus fetal T-cells. The role of the $CD95^{-}CD45RO^{+}CD45RA^{-}$ T-cell subset in the developing immune system is presently unclear. The expression of CD45RO by these cells suggests that they may have been previously activated. Although there are some reports that suggest exposure of the fetus to external antigens can occur [37], the prevalence of the $CD45RO^{+}$ population of T cells could mean that these cells are being exposed to and subsequently responding to autologous antigens. We speculate that a developmentally early wave of activation of autoreactive T-cells may be an important step in the establishment of suppressor T-cell populations and peripheral tolerance. Indeed, the decreased expression of CD95 on these cells would be counter to the hypothetical removal of fetal autoreactive T-cells by a CD95-mediated apoptotic mechanism [32]. However, the loss of CD95 expression may still be associated with the removal

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of these cells since there is evidence that signaling through CD95 may support T-cell growth rather than apoptosis in some circumstances [38,39].

A broader analysis of T-cell markers further supports our conclusion that the midgestation fetus contains a notable number of activated T-cells. Although a previous study failed to identify any $CD56^+$ natural cytotoxic T-cells in the fetus [40], we observed a similar number of $CD56^+$ T-cells in the fetus as in the adult. About 17% of fetal T-cells expressed the γ/δ TCR which was higher than in the neonate and adult, consistent with previous findings [41]. CD69, an activation antigen expressed early in T-cell activation [33], was expressed at nearly twice the frequency on fetal T-cells as on neonatal and adult T-cells. CD80 is a stimulatory molecule for T cells expressed by various leukocytes, which can be expressed on some T cells during the later phase of activation [34,35]. CD80 was expressed on fetal T-cells, in particular $CD45RO^+$ T cells, also at a higher frequency than in the neonate and adult. T-cell activation also results in upregulation of the α and β subunits of the IL-2 receptor, CD25 and CD122, respectively. Both components of the IL-2 receptor were found on fetal T-cells, with a notably higher number of $CD122^+$ T-cells in the fetus compared to the adult. Very little CD25 and CD122 expression was observed on neonatal T-cells. The expression of CD25 and CD122 by fetal T-cells suggests these cells may be activated, although it is possible that some of these cells represent $CD4^+$ suppressor/regulatory T-cells [42]. This regulatory subset of T cells has been described in UCB and is characterized in part by CD25 and CD122 expression [43]. IL-7 plays a critical role in the maintenance of the naïve T-cell pool through interaction with its receptor, CD127/CD132 [44]. After T-cell activation, CD127 expression is lost and, as such, is another indication that T cells have been stimulated [45]. We observed $CD127^-$ T-cells in both the fetus as well as in the adult, although most T cells in both cases express CD127. Indeed, there was a higher portion of $CD45RO^+$ T-cells that lacked CD127 expression in the fetus than in the adult. Moreover, CD127 was notably higher on neonatal T-cells compared to both fetal and adult T-cells. In total, these findings are consistent with a higher level of T-cell activation in the midgestation fetus than at term.

The abundant expression of CD95 by peripheral T-cells in the fetus prompted us to analyze the expression of CD95 on developing T-cells in the thymus (Fig. 8). CD95 expression in the fetal thymus was predominantly found on T-cells that had already begun to express high levels of CD3, although the majority of DP and SP T-cells did not express CD95. Most DN thymocytes expressed CD95. Our findings are consistent with those of Jenkins et al. who observed CD95 expression on T-cell progenitors, but found very little expression on mature $CD3^+$ SP thymocytes. These investigators also demonstrated that the thymic cells are resistant to CD95-mediated apoptosis [25]. We wished to further elucidate the degree of CD95 expression on mature thymic T-cells set to enter the circulation. Most developing T-cells in the thymus express CD45RO, which is expressed in an inverse relationship to CD45RA. Before exiting the thymus, T cells are known to down-regulate CD45RO expression and become $CD45RA^+$ [29–31]. Examination of CD95 expression on thymic $CD45RO^-$ and $CD45RA^+$ T-cells indicated that most T cells entering the periphery are $CD95^+$, consistent with the expression of CD95 observed on peripheral $CD45RA^+$ T-cells. Since adult $CD45RA^+$ T-cells, which are less likely to be recent thymic emigrants, expressed less CD95 it is likely that CD95 expression is reduced on naïve T-cells with time spent in the circulation.

There at least two additional potential explanations, besides (auto)antigen-specific activation, for the presence of T-cells in the fetal circulation with an activated phenotype. One possibility is that the markers associated with activation are expressed because of T-cell growth, associated with rapid expansion of the peripheral pool of T cells, rather than specific activation by antigen. Another possibility is that the $CD45RO^+$ T-cells in the fetal circulation are recent thymic emigrants that emerged from the thymus before changing to the $CD45RA^+$ phenotype. In attempt to distinguish these possibilities, the Vβ chain repertoire was analyzed on fetal T-cells. A diverse repertoire, comparable to that of adults, was observed as early a 16 weeks gestation. It is worth mention that methods more sensitive to minor sequence differences have shown reduced diversity within the Vβ chain families of late-gestation fetal and some neonatal blood samples [46,47], which

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presumably is true for early mid-gestation T-cells as well. Our results also demonstrated diversity within the $CD45RA^-$ ($CD45RO^+$) subset of fetal T-cells indicating that this subset is not derived from the activation and expansion of one or a few T-cell clones. Differences in Vβ chain expression between splenic and thymic $CD45RA^-$ T-cells suggest that the splenic cells are not recent emigrants from the thymus, although further experiments are required to bolster this conclusion. There were also some differences in Vβ chain expression between splenic $CD45RA^-$ and $CD45RA^+$ T-cells, which may indicate selective expansion of T cell clones. However, further study is required to distinguish the possible reasons for the expression of activation markers on fetal T-cells.

We further examined if CD95 expression can be viewed as a marker of activation or maturation for leukocyte lineages other than T-cells (Fig. 8). CD95 was expressed on immature cells of all lineages and was down-regulated with maturation, except in the case of monocytes which expressed CD95 even in the sterile fetal environment. In contrast, granulocytes reduced their expression of CD95 after entering the circulation. Likewise, CD95 expression was decreased or lost on NK cells in the circulation, possibly due to a lack of growth or activation stimulus, although increased CD95 expression is correlated with in vitro activation of NK cells [48, 49]. B cells expressed less CD95 than most other lineages during their development in the fetal or adult bone-marrow. Most $CD19^+$ and $CD10^+$ cells in hematopoietic tissues did not express CD95 and expression on circulating fetal B-cells was low and decreased compared to the adult. Miyawaki et al. first described similar results for UCB and adult peripheral blood B-cells [21]. CD95 expression on B cells correlates with increased functional differentiation and is, thus, likely low on fetal B-cells owing to their lack of stimulation and hormonal suppression. These findings show a diverse and variable expression of CD95 in the development of hematopoietic cells, indicating that CD95 expression alone is not a reliable marker of maturational status or activation.

Continued research into the development and functional status of the human fetal immune system should lead to new insights into the steps required in the development of the immune system and the establishment of peripheral tolerance towards autologous antigens. These insights may lead to better therapies for immune compromised patients and transplant patients. Efforts at fetal gene or cellular therapy would also benefit greatly from a clearer understanding of the functional capacity of the immune system at various stages of development.

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References

[1] Renda MC, Fecarotta E, Dieli F, et al. Evidence of alloreactive T lymphocytes in fetal liver: implications for fetal hematopoietic stem cell transplantation. Bone Marrow Transplant 2000;25(2):135–41.

[2] Muench MO, Rae J, Bárcena A, et al. Transplantation of a fetus with paternal Thy-1 + CD34 + cells for chronic granulomatous disease. Bone Marrow Transplant 2001; 27(4):355–64.

[3] Furley AJ, Mizutani S, Weilbaecher K, et al. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46(1):75–87.

[4] Haynes BF, Martin ME, Kay HH, Kurtzberg J. Early events in human T cell ontogeny: phenotypic characterization and immunohistological localization of T-cell precursors in early human fetal tissues. J Exp Med 1988;168(3): 1061–80.

[5] Haynes BF. Phenotypic characterization and ontogeny of the human thymic microenviroment. Clin Res 1984;32(5):500–7.

[6] Stites DP, Carr MC, Fudenberg HH. Ontogeny of cellular immunity in the human fetus: development of responses to phytohemagglutinin and to allogeneic cells. Cell Immunol 1974;11(1-3):257–71.

[7] Rayfield LS, Brent L, Rodeck CH. Development of cell-mediated lympholysis in human foetal blood lymphocytes. Clin Exp Immunol 1980;42(3):561–70.

[8] Lindton B, Markling L, Ringden O, Kjaeldgaard A, Gustafson O, Westgren M. Mixed lymphocyte culture of human fetal liver cells. Fetal Diagn Ther 2000;15(2):71–8.

[9] Wang YZ, Ning ZQ, Zhang QX. Responses of human fetal splenocytes and thymocytes to interleukin-2 and comparison with adult peripheral blood lymphocytes. Immunol Lett 1993; 37(2–3):229–33.

UNCORRECTED PROOF

ARTICLE IN PRESS

[10] Garderet L, Dulphy N, Douay C, et al. The umbilical cord blood alphabeta T-cell repertoire: characteristics of a polyclonal and naive but completely formed repertoire. Blood 1998;91(1):340–6.

[11] Harris DT, Schumacher MJ, Locascio J, et al. Phenotypic and functional immaturity of human umbilical cord blood T lymphocytes. Proc Natl Acad Sci USA 1992;89(21): 10006–10.

[12] Lai R, Visser L, Poppema S. Postnatal changes of CD45 expression in peripheral blood T and B cells. Br J Haematol 1994;87(2):251–7.

[13] Roncarolo MG, Bigler M, Ciuti E, Martino S, Tovo PA. Immune responses by cord blood cells. Blood Cells 1994; 20(2-3):573–85.

[14] Bofill M, Akbar AN, Salmon M, Robinson M, Burford G, Janossy G. Immature CD45RA(low)RO(low) T cells in the human cord blood. I. Antecedents of CD45RA + unprimed T cells. J Immunol 1994;152(12):5613–23.

[15] Keever CA, Abu-Hajir M, Graf W, et al. Characterization of the alloreactivity and anti-leukemia reactivity of cord blood mononuclear cells. Bone Marrow Transplant 1995;15(3): 407–19.

[16] Sato K, Nagayama H, Takahashi TA. Aberrant CD3- and CD28-mediated signaling events in cord blood T cells are associated with dysfunctional regulation of Fas ligand-mediated cytotoxicity. J Immunol 1999;162(8):4464–71.

[17] Harris DT, LoCascio J, Besencon FJ. Analysis of the alloreactive capacity of human umbilical cord blood: implications for graft-versus-host disease. Bone Marrow Transplant 1994;14(4):545–53.

[18] Risdon G, Gaddy J, Horie M, Broxmeyer HE. Alloantigen priming induces a state of unresponsiveness in human umbilical cord blood T cells. Proc Natl Acad Sci USA 1995; 92(6):2413–7.

[19] Porcu P, Gaddy J, Broxmeyer HE. Alloantigen-induced unresponsiveness in cord blood T lymphocytes is associated with defective activation of Ras. Proc Natl Acad Sci USA 1998;95(8):4538–43.

[20] Berthou C, Legros-Maida S, Soulie A, et al. Cord blood T lymphocytes lack constitutive perforin expression in contrast to adult peripheral blood T lymphocytes. Blood 1995;85(6): 1540–6.

[21] Miyawaki T, Uehara T, Nibu R, et al. Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. J Immunol 1992;149(11): 3753–8.

[22] Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. Nat Immunol 2000;1(6):469–74.

[23] Krammer PH. CD95's deadly mission in the immune system. Nature 2000;407(6805):789–95.

[24] McCloskey TW, Oyaizu N, Bakshi S, Kowalski R, Kohn N, Pahwa S. CD95 expression and apoptosis during pediatric HIV infection: early upregulation of CD95 expression. Clin Immunol Immunopathol 1998;87(1): 33–41.

[25] Jenkins M, Keir M, McCune JM. Fas is expressed early in human thymocyte development but does not transmit an apoptotic signal. J Immunol 1999;163(3):1195–204.

[26] Potestio M, Pawelec G, Di Lorenzo G, et al. Age-related changes in the expression of CD95 (APO1/FAS) on blood lymphocytes. Exp Gerontol 1999;34(5):659–73.

[27] Bárcena A, Muench MO, Song KS, Ohkubo T, Harrison MR. Role of CD95/Fas and its ligand in the regulation of the growth of human CD34++CD38 − fetal liver cells. Exp Hematol 1999;27(9):1428–39.

[28] Bárcena A, Muench MO, Roncarolo MG, Spits H. Tracing the expression of CD7 and other antigens during T- and myeloid-cell differentiation in the human fetal liver and thymus. Leuk Lymph 1995;17(1-2):1–11.

[29] Pilarski LM, Gillitzer R, Zola H, Shortman K, Scollay R. Definition of the thymic generative lineage by selective expression of high molecular weight isoforms of CD45 (T200). Eur J Immunol 1989;19(4):589–97.

[30] Egerton M, Pruski E, Pilarski LM. Cell generation within human thymic subsets defined by selective expression of CD45 (T200) isoforms. Hum Immunol 1990;27(4):333–47.

[31] Fujii Y, Okumura M, Inada K, Nakahara K, Matsuda H. CD45 isoform expression during T cell development in the thymus. Eur J Immunol 1992;22(7):1843–50.

[32] Byrne JA, Stankovic AK, Cooper MD. A novel subpopulation of primed T cells in the human fetus. J Immunol 1994;152(6): 3098–106.

[33] Testi R, Phillips JH, Lanier LL. T cell activation via Leu-23 (CD69). J Immunol 1989;143(4):1123–8.

[34] Azuma M, Yssel H, Phillips JH, Spits H, Lanier LL. Functional expression of B7/BB1 on activated T lymphocytes. J Exp Med 1993;177(3):845–50.

[35] Wyss-Coray T, Mauri-Hellweg D, Baumann K, Bettens F, Grunow R, Pichler WJ. The B7 adhesion molecule is expressed on activated human T cells: functional involvement in T–T cell interactions. Eur J Immunol 1993;23(9):2175–80.

[36] Lanier LL, Chang C, Azuma M, Ruitenberg JJ, Hemperly JJ, Phillips JH. Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). J Immunol 1991;146(12):4421–6.

[37] Devereux G, Seaton A, Barker RN. In utero priming of allergen-specific helper T cells. Clin Exp Allergy 2001;31(11): 1686–95.

[38] Alderson MR, Armitage RJ, Maraskovsky E, et al. Fas transduces activation signals in normal human T lymphocytes. J Exp Med 1993;178(6):2231–5.

[39] Kabra NH, Kang C, Hsing LC, Zhang J, Winoto A. T cell-specific FADD-deficient mice: FADD is required for early T cell development. Proc Natl Acad Sci USA 2001;98(11): 6307–12.

[40] Thilaganathan B, Mansur CA, Morgan G, Nicolaides KH. Fetal T-lymphocyte subpopulations in normal pregnancies. Fetal Diagn Ther 1992;7(2):53–61.

[41] Krangel MS, Yssel H, Brocklehurst C, Spits H. A distinct wave of human T cell receptor gamma/delta lymphocytes in the early fetal thymus: evidence for controlled gene rearrange-

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ment and cytokine production. J Exp Med 1990;172(3): 847–59.

[42] Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002;2(6): 389–400.

[43] Wing K, Ekmark A, Karlsson H, Rudin A, Suri-Payer E. Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood. Immunology 2002;106(2): 190–9.

[44] Webb LM, Foxwell BM, Feldmann M. Putative role for interleukin-7 in the maintenance of the recirculating naive CD4+ T-cell pool. Immunology 1999;98(3):400–5.

[45] Webb LM, Foxwell BM, Feldmann M. Interleukin-7 activates human naive CD4+ cells and primes for interleukin-4 production. Eur J Immunol 1997;27(3):633–40.

[46] Schelonka RL, Raaphorst FM, Infante D, Kraig E, Teale JM, Infante AJ. T cell receptor repertoire diversity and clonal expansion in human neonates. Pediatr Res 1998;43(3): 396–402.

[47] Alfani E, Migliaccio AR, Sanchez M, Passarelli AM, Migliaccio G. Characterization of the T cell receptor repertoire of neonatal T cells by RT-PCR and single strand conformation polymorphism analysis. Bone Marrow Transplant 2000;26(1):83–9.

[48] Medvedev AE, Johnsen AC, Haux J, et al. Regulation of Fas and Fas-ligand expression in NK cells by cytokines and the involvement of Fas-ligand in NK/LAK cell-mediated cytotoxicity. Cytokine 1997;9(6):394–404.

[49] Haux J, Johnsen AC, Steinkjer B, Egeberg K, Sundan A, Espevik T. The role of interleukin-2 in regulating the sensitivity of natural killer cells for Fas-mediated apoptosis. Cancer Immunol Immunother 1999;48(2–3):139–46.

[50] De Maria R, Testa U, Luchetti L, et al. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 1999;93(3):796–803.

[51] Cleary AM, Fortune SM, Yellin MJ, Chess L, Lederman S. Opposing roles of CD95 (Fas/APO-1) and CD40 in the death and rescue of human low density tonsillar B cells. J Immunol 1995;155(7):3329–37.

[52] Bárcena A, Park SW, Banapour B, Muench MO, Mechetner E. Expression of Fas/CD95 and Bcl-2 by primitive hematopoietic progenitors freshly isolated from human fetal liver. Blood 1996;88(6):2013–25.

[53] Josefsen D, Myklebust JH, Lynch DH, Stokke T, Blomhoff HK, Smeland EB. Fas ligand promotes cell survival of immature human bone marrow CD34+CD38− hematopoietic progenitor cells by suppressing apoptosis. Exp Hematol 1999;27(9):1451–9.

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