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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 antibody(ies); 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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97 thymus is not anatomically completely mature
 98 until the 15th week of gestation [5]. Around this
 99 time, single positive (SP) CD4⁺CD8⁻CD3⁺ and
 100 CD4⁻CD8⁺CD3⁺ T cells begin to accumulate
 101 more rapidly in the periphery [4]. The purpose
 102 for the early development of lymphocytes is not
 103 known but may simply reflect the necessity for T-
 104 lymphopoiesis to keep pace with overall fetal
 105 development to assure the generation and proper
 106 selection of sufficient numbers of lymphocytes
 107 before birth. Moreover, the early development of
 108 T cells may also serve to protect the fetus from in
 109 utero infections or engraftment by maternal lym-
 110 phocytes. Determining the functional capacity of
 111 fetal lymphocytes is key to defining a role for these
 112 cells in fetal immunity.

113 A limited number of studies have addressed the
 114 functional development of T cells from pre-term
 115 fetuses. Proliferation in response to allostimulation
 116 has been observed from fetal liver T-cells as young as
 117 9 weeks' gestation [1,6–8]. However, cultured fetal
 118 T-cells showed some defects in cytotoxic responses
 119 which could be reversed by prestimulation with
 120 cytokine [7,9]. More on the functional capacity of
 121 fetal T-cells has been learned from analyses of T cells
 122 obtained from umbilical cord blood (UCB) at the
 123 time of birth. The T-cell repertoire appears fully
 124 formed at birth yet the repertoire appears to be
 125 composed of primarily naïve T-cells [10]. Whereas
 126 CD45RO⁺CD45RA⁻ memory T-cells are common
 127 in adult blood, UCB is primarily composed of
 128 CD45RO⁻CD45RA⁺ naïve T-cells [11–15]. Other
 129 cell-surface markers important in the functioning of T
 130 cells, such as CD3 and CD28, are expressed at similar
 131 levels to adult cells, but signaling through these
 132 proteins is attenuated in UCB T-cells. Neonatal T-
 133 cells failed to increase CD25, CD154 and CD178 (Fas
 134 ligand) expression when stimulated through CD3 and
 135 CD28 [16]. Reduced proliferative responses of
 136 allostimulated UCB T-cells have also been noted
 137 [11,17–19]. Cord blood T-cells also have greatly
 138 reduced perforin expression [20]. Together with the
 139 observations of reduced CD178 expression, these
 140 findings suggest a reduced cytolytic capacity of
 141 neonatal T-cells similar to the findings on midgesta-
 142 tion T-cells. Thus, despite T-lymphopoiesis beginning
 143 early in fetal life, some attenuation of T-cell function

145 appears to occur in addition to the naïveté of the fetal
 146 immune system.

147 CD95 (fas) is an important regulator of homeosta-
 148 sis of the immune system. CD95 is expressed on some
 149 T cells and to varying degrees on all other leukocyte
 150 lineages. Its expression is increased with T-cell
 151 activation and CD95 expression is highest on
 152 CD45RO⁺ T-cells [21]. Triggering of programmed
 153 cell death by CD95 activation, through interaction
 154 with CD178, leads to clearance of activated T-cells
 155 and thereby limits immune responses [22,23]. Neo-
 156 natal T-cells express less CD95 than their adult
 157 counterparts [21,24]. Expression of CD95 is also low
 158 to negative on SP thymic cells from midgestation
 159 thymic tissues, although CD95 is expressed on
 160 immature T-cell progenitors [25]. Moreover, the
 161 expression of CD95 on T cells has been shown to
 162 increase with age up to 75 years, after which
 163 expression decreases somewhat [26]. These data
 164 suggest that CD95 expression increases with the
 165 maturation of the immune system. In this regard it is
 166 worth noting that CD95 expression can increase more
 167 rapidly in children infected by the human immuno-
 168 deficiency virus [24].

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

184 2. Materials and methods

185 2.1. Isolation of human leukocytes from adult, 186 neonatal and fetal tissues

187 Human tissues were obtained and studied under the
 188 approval of the Committee on Human Research at our
 189 institute. Male and female adult peripheral blood was
 190
 191
 192

193 obtained from healthy volunteers ranging in age from
194 24 to 61 years. Neonatal UCB and fetal hematopoietic
195 tissues were obtained with consent of the women prior
196 to delivery or elective abortion. Neonatal blood (birth
197 at 33 weeks' gestation to term) and some fetal tissues
198 were obtained at our institute. Additional fetal tissues
199 were obtained from Advanced Bioscience Resources
200 Inc. (Alameda, CA). Fetal tissues were harvested
201 shortly following the abortion and were transported to
202 the laboratory in sterile containers held on ice.
203 Experiments on adult bone marrow were performed
204 at Ingenex, Inc. (Menlo Park, CA) in compliance with
205 regulations issued by the state and federal govern-
206 ments. Fetal tissues ranged in gestational age from 15
207 to 24 weeks, as determined by the foot length of the
208 fetus. Each experiment was performed on cells
209 obtained from an individual specimen; tissues from
210 different specimens were not pooled for analyses.

211 Adult peripheral blood was obtained by venipunc-
212 ture. Approximately 7 ml of blood was drawn into a
213 vacutainer tube containing ethylene diamine tetra-
214 acetic acid (EDTA). The blood was diluted to a total
215 volume of 50 ml in PBS/BSA washing buffer
216 consisting of phosphate buffered saline (PBS) con-
217 taining 0.3% fraction-V ethanol-extracted bovine
218 serum albumin (BSA) (Roche Applied Science,
219 Indianapolis, IN) and 50 μ g/ml gentamicin sulfate
220 (Life Technologies, Grand Island, NY). The cells
221 were pelleted by centrifugation and erythrocytes were
222 depleted by chemical lyses using ACK lyses buffer,
223 pH 7.2–7.4, consisting of 0.15 M NH_4Cl , 1.0 mM
224 KHCO_3 and 0.1 mM Na_2EDTA (Sigma Chemical
225 Company, St Louis, MO). The cells were pelleted by a
226 7-min centrifugation approximately 1 min after the
227 addition of the ACK lyses buffer. If lysis of the
228 erythrocytes was incomplete, the procedure was
229 repeated. Otherwise the cells were washed once in
230 PBS/BSA and suspended in blocking buffer consisting
231 of PBS with 5% normal mouse serum (Gemini Bio-
232 Products, Inc., Woodland, CA) and 0.01% NaN_3 .
233 Alternatively, peripheral blood mononuclear cells
234 (PBMC) were prepared by centrifugation of the
235 blood at $600 \times g$ for 25 min on a layer of 1.077 g/ml
236 LymphoPrep (Life Technologies). The light-density
237 cells were harvested and washed twice before being
238 suspended in blocking buffer for staining.

239 PBMC were isolated from neonatal UCB
240 by immunomagnetic bead depletion of $\text{CD}235\text{a}^+$

erythrocytes, performed as previously described for
fetal liver cells [27], and density separation using
1.077 g/ml Nycoprep (Life Technologies). The iso-
lation 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 Chemi-
cal 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 har-
vested 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 immuno-
magnetic bead depletion of $\text{CD}235\text{a}^+$ 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

289 nylon-mesh cell strainer and pelleted by centrifugation. Erythrocytes were depleted by chemical lysis
 290 using ACK lysis buffer or immunomagnetic bead
 291 depletion of CD235a⁺ cells [27]. Alternatively, light-
 292 density splenocytes, depleted of erythrocytes and
 293 granulocytes, were isolated by centrifugation using
 294 LymphoPrep as described above. After either method,
 295 splenocytes were washed and suspended in blocking
 296 buffer for staining.
 297

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

305 Light-density CD235a⁻ fetal liver cells and fetal
 306 bone marrow cells were prepared by immunomag-
 307 netic-bead depletion and centrifugation over a layer of
 308 1.077 g/ml Nycoprep as previously described [27].
 309

310 2.2. Monoclonal antibodies

311 Fluorescein isothiocyanate (FITC), phycoerythrin
 312 (PE), allophycocyanin (APC) and peridinin chloro-
 313 phyll (PerCP) labeled mAbs were purchased from BD
 314 Biosciences/BD PharMingen (wwwbdbiosciences.com)
 315 recognizing the following antigens: CD3-FITC
 316 (SK7), CD8-FITC (SK1), CD10-FITC (W8E7),
 317 CD14-FITC (MΦP9), CD15-FITC (MMA), CD19-
 318 FITC (4G7 or SJ25C1), CD-19-PerCP (SJ25C1),
 319 CD45RA-APC (HI100), CD45RO-APC (UCHL1),
 320 CD45RO-PE (UCHL1), CD95-PE (DX2), CD122-PE
 321 (TU27), mouse IgG₁-FITC, mouse IgG_{2a}-FITC and
 322 mouse IgM-FITC. Anti-CD56-FITC and anti-CD56-
 323 PE (C5.9) were purchased from Exalpha Corporation
 324 (Boston, MA). Labeled antibodies recognizing CD4-
 325 tricolor (TC) (S3.5), CD14-FITC (TUK4), CD15-
 326 FITC (V1MC6), CD25-PE (CD25-3G10), CD45-PE
 327 (HI30), mouse IgG₁-FITC, mouse IgG₁-PE, mouse
 328 IgG_{2a}-PE, mouse IgG_{2b}-FITC and mouse IgM-FITC
 329 were purchased from Caltag (Burlingame, CA).
 330 Conjugated mAb were also purchased from Beck-
 331 man-Coulter (Miami, FL) recognizing the following
 332 antigens: CD3-phycoerythrin-cyanine 5 (PC5)
 333 (UCHT-1), CD14-PC5 (RM052), CD16-PC5 (3G8),
 334 CD45-PC5 (J33), CD45RA-FITC (ALB11),
 335 CD45RO-FITC (UCHL1), CD56-PC5 (N901),

CD69-PC5 (TP1.55.3), CD80-PE (MAB104), 337
 CD127-PE (R34.34) and mouse IgG₁-PC5. Mono- 338
 clonal antibodies labeled with PE recognizing T-cell 339
 receptor (TCR) α/β (BMA031) and TCR γ/δ 340
 (5A6.E9) were obtained from Endogen (Woburn, 341
 MA). A FITC-conjugated mAb against TCR α/β was 342
 obtained from T Cell Diagnostics, Inc. (Cambridge, 343
 MA). A kit containing a panel of FITC-, PE- and a 344
 mixture of FITC- and PE-conjugated mAb recogniz- 345
 ing different TCR Vβ chains was purchased from 346
 Beckman-Coulter and was used according to the 347
 manufacturers recommendations. 348
 349

350 2.3. Flow cytometric analysis of cell surface markers

351
 352
 353 Approximately 2×10^5 cells suspended in up to
 354 200 μl blocking buffer were incubated in 96-well
 355 Costar V-bottom Plate (Corning Inc., Corning, NY)
 356 with saturating amounts of mAbs on ice for at least
 357 30 min. Cells were washed twice with 250 μl
 358 PBS/BSA containing 0.01% NaN₃ (Sigma Chemical
 359 Co.). The washed cells were suspended in PBS/BSA
 360 containing 0.01% NaN₃ and 2 μg/ml propidium
 361 iodide (PI), purchased from Sigma Chemical Co. PI
 362 was used to mark dead cells, so that they could be
 363 excluded from the analysis. PI was omitted in 3-color
 364 analyses using PC5 or PerCP labeled mAbs. Flow
 365 cytometric analysis was performed using either a
 366 FACScan or a FACSCalibur flow cytometer (BD
 367 Biosciences). Analyses of results were performed
 368 using CellQuest software (BD Biosciences).
 369

370 2.4. Data presentation and statistical analysis

371
 372
 373 The median results of multiple measurements done
 374 on individual tissue samples are reported to reduce the
 375 effects of outliers. Box plots are used to present the
 376 data, which show the 10th (lower bar), 25th (box
 377 bottom), 50th (median-bar in box), 75th (box top) and
 378 90th (upper bar) percentiles. Circles in the box plots
 379 indicate outlying data points below the 10th and above
 380 the 90th percentiles. The significance of differences
 381 observed between fetal and adult cells was determined
 382 using an unpaired Student's *t*-test. Results were
 383 considered significant when $P \leq 0.05$.
 384

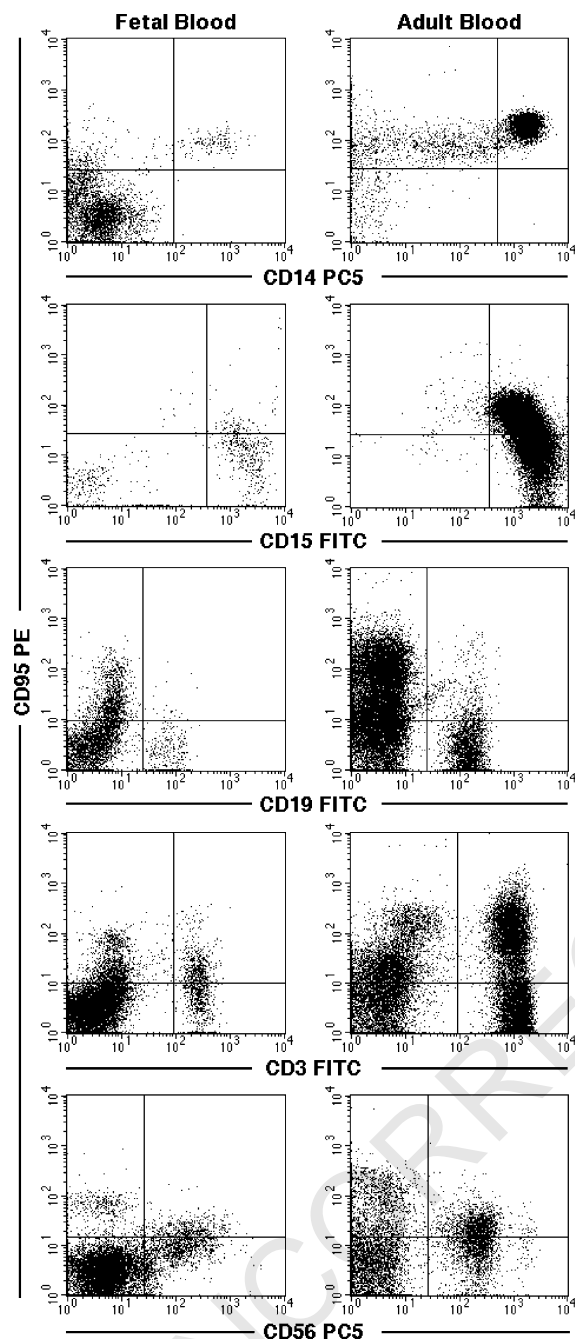


Fig. 1. Expression of CD95 by fetal and adult leukocytes. Representative data from analyses of fetal and adult peripheral-blood leukocytes are shown. The fetal leukocytes had a gestational age of 19 weeks. Adult peripheral blood was obtained from a 36 year old male. The five subsets of leukocytes were defined by the expression of their specific cell-surface antigen, as shown, as well as by their characteristic light-scatter profiles (not shown). CD14⁺

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-marrow, 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

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.

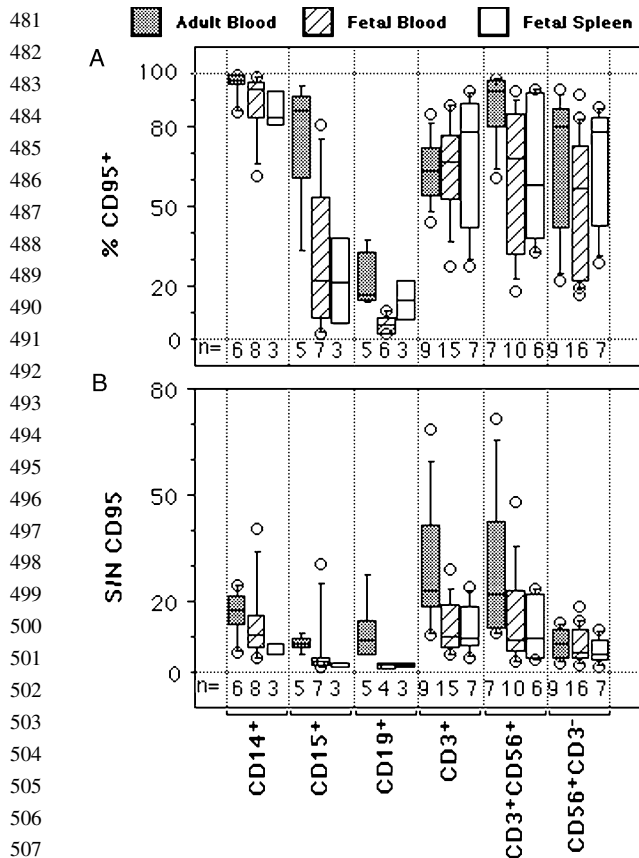


Fig. 2. Box plots of the frequency and intensity of CD95 expression on fetal and adult leukocytes. Leukocyte populations were defined by their phenotypic properties as described in Fig. 1. Box plots are shown of the percent of cells expressing CD95 (A) and the signal to noise (S/N) ratio for CD95 expression (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-marrow 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-marrow (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

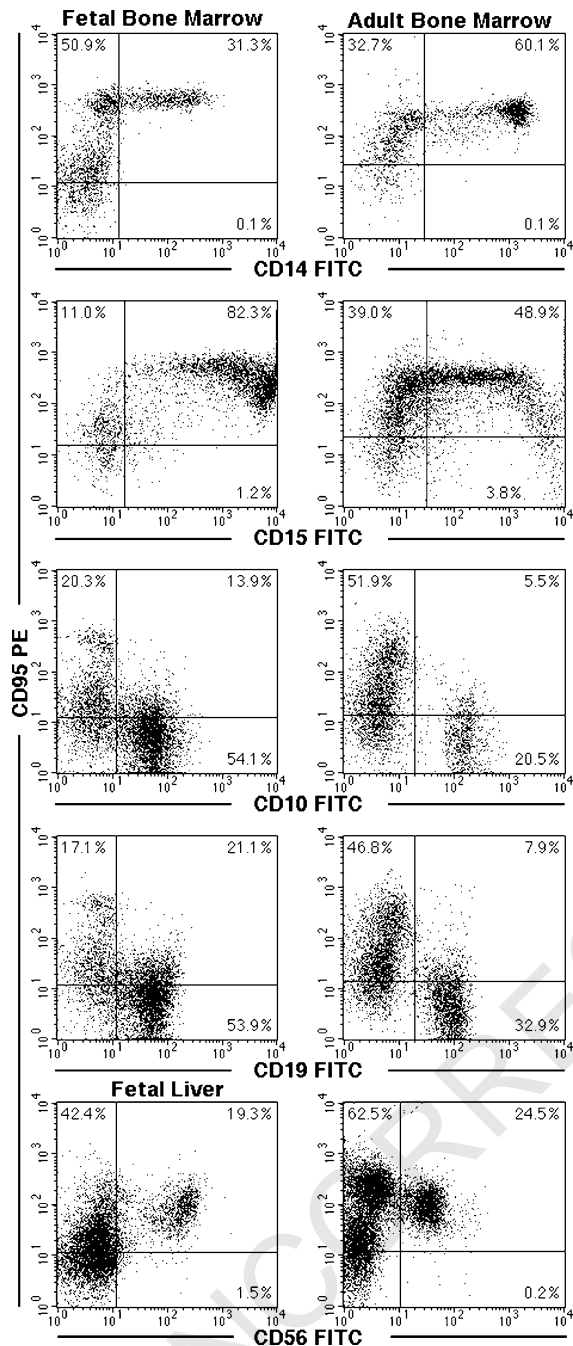


Fig. 3. Expression of CD95 on immature monocytes, granulocytes, B cells and NK cells. Fetal bone marrow, or fetal liver (right column) and adult bone marrow (left column) were analyzed for the expression of CD95 and the indicated leukocyte markers. All tissues were enriched for immature leukocytes by isolation of light-density CD235a⁻ cells. Additionally, the leukocyte populations of interests

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

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.

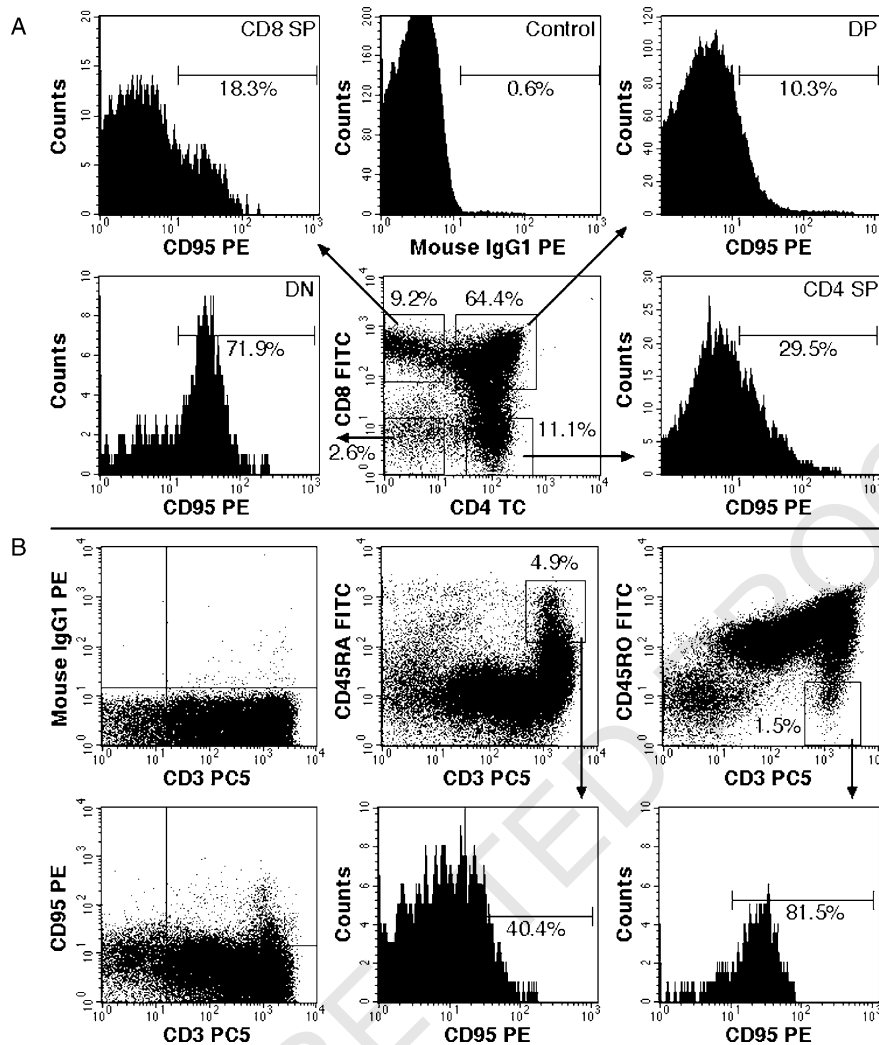


Fig. 4. Expression of CD95 during T-cell development in the fetal thymus. Expression of CD95 on DN, DP and SP thymocytes is shown in (A). Co-expression of CD3 and CD95 as well as the level of CD95 expression on CD3⁺CD45RA⁺ and CD3⁺CD45RO⁻ thymocytes is shown in (B). Representative results are shown from 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 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

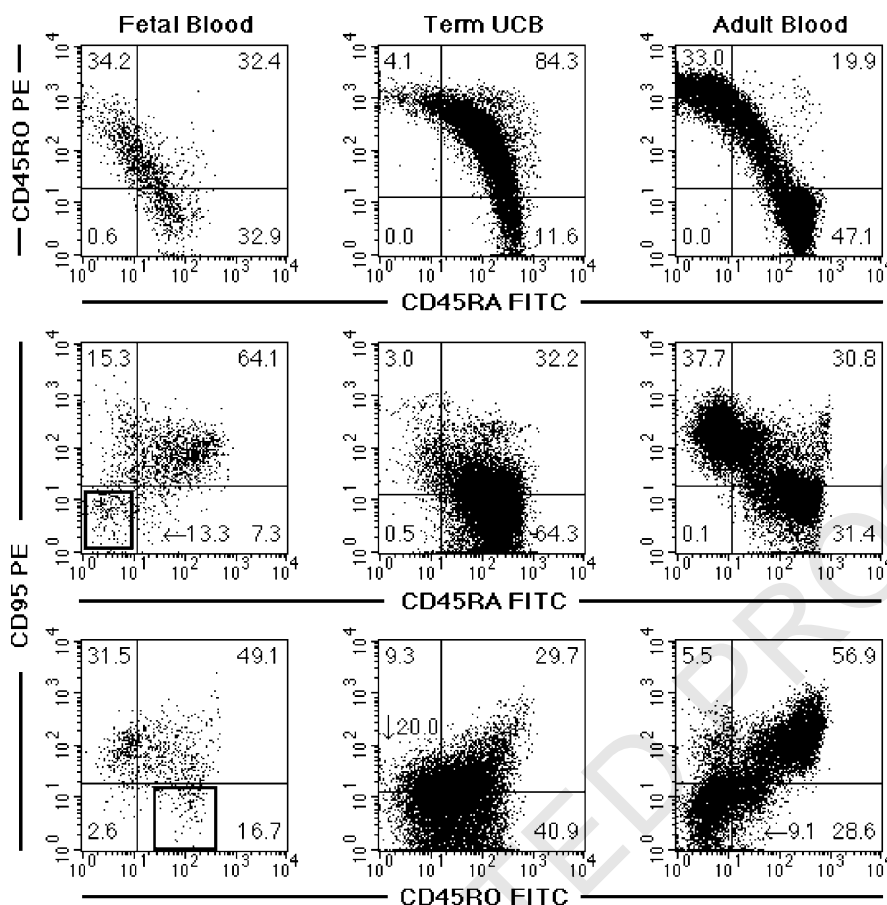


Fig. 5. Expression of CD45RA, CD45RO and CD95 by fetal, neonatal and adult T-cells. 3-color analyses were performed by staining blood cells with CD3-PC5 and the indicated mAbs. Events shown are gated on CD3⁺ cells with low forward- and side-light scatters. The gestational ages of the fetal blood samples were 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

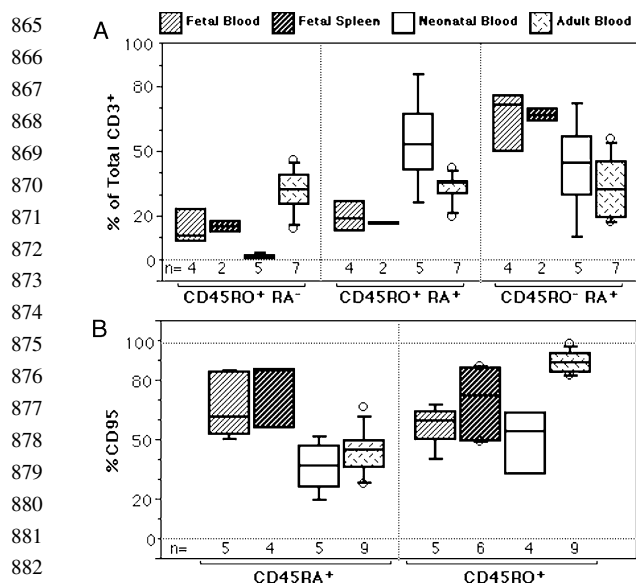


Fig. 6. Expression of CD45 subtypes and CD95 by fetal, neonatal and adult T cells. The percentages of CD45RO⁺CD45RA⁻, CD45RO⁺CD45RA⁺ and CD45RO⁻CD45RA⁺ T-cells in fetal tissues, UCB and adult blood are shown in (A). The percentages of CD45RA⁺CD3⁺ and CD45RO⁺CD3⁺ T cells that expressed CD95 are shown 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 CD3 ⁺ T-cells		Adult CD3 ⁺ T-cells	
	Fetal	Neonatal	Fetal	Neonatal	Fetal	Neonatal	Fetal	Neonatal	Fetal	Neonatal
CD25	11.0 ^a	3.4 ^b	14.8	17.7	5.4 ^b	22.2	3.8	2.6	3.4	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	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	80.6
CD132	99.6	95.3 ^b	100	92.5	96.3	98.4	100 ^a	96.5	100	100
CD56	9.9	4.3	9.7	8.2	5.7	11.5	9.6	3.7	6.2	6.2
CD69	21.0 ^b	12.5	10.7	ND	ND	ND	ND	ND	ND	ND
CD80	3.7	0	0.3	5.2	0	0.6	0.6	0	0	0
TCR α/β	80.2 ^a	97.6 ^b	82.6	ND	ND	ND	ND	ND	ND	ND
TCR γ/δ	17.3 ^{a,b}	2.9 ^b	8.2	ND	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 ≤ 0.05 versus neonatal T-cells.

^b P ≤ 0.05 versus adult T-cells.

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 V β chain expression by fetal T-cells and thymocytes

The TCR V β chain repertoire expressed by fetal T-cells was studied to determine the diversity of TCR expression in the emerging immune system. V β 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 V β 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 V β chain expression similar to as described above, except for the following differences: The CD45RA⁻ subset had a higher representation of V β 11 ($P < 0.05$, $n = 3$) and V β 5.1 ($P = 0.075$) and lower representation of V β 14, V β 16 and V β 21.3 ($P < 0.05$, $n = 3$). 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 V β chains on splenic and thymic

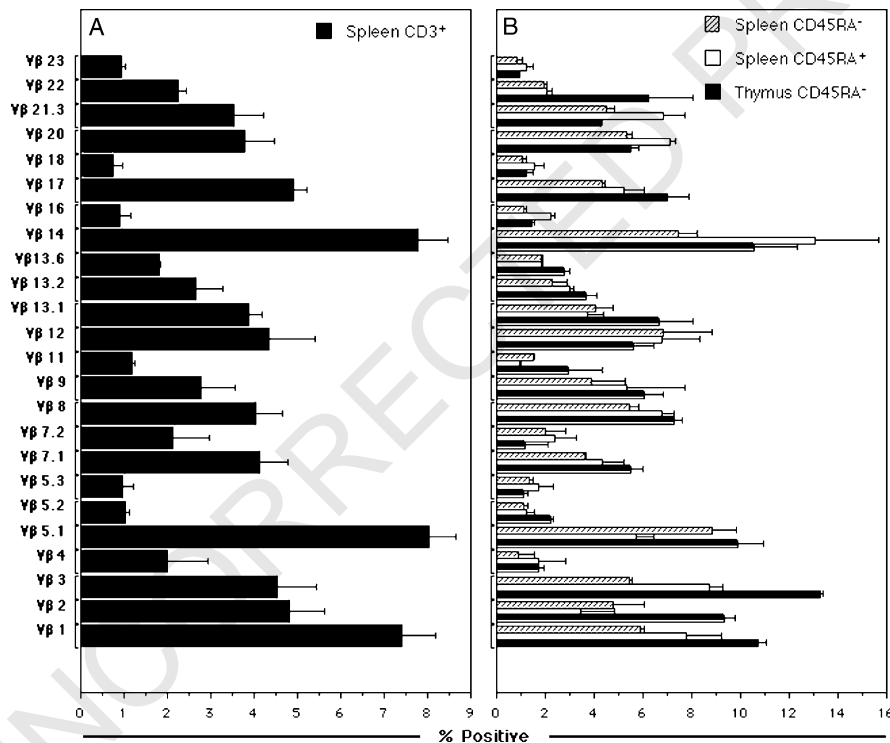


Fig. 7. Expression of TCR V β chains by fetal splenic T-cells and thymocytes. TCR V β chain expression was analyzed by 4-color flow cytometry. T cells were identified by their expression of high levels of CD3 and by their low light scatter profile. V β chain expression was analyzed from a cohort of 6 spleens ranging in gestation age from 16 to 24 weeks (A). Two of these spleens, of 19 and 22 weeks' gestation, were analyzed for V β 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 \pm SE.

1057 CD45RA⁻CD3⁺ cells (Fig. 7B). Besides wide-
 1058 ranging similarity and some non-significant dissim-
 1059 ilarities, there were notable significant differences
 1060 between the thymic and splenic T-cells. Mainly, a
 1061 lower representation of V β 1, V β 3, V β 5.2 and V β
 1062 13.6 ($P \leq 0.05$, $n = 2$) was observed on the splenic T-
 1063 cells. These differences indicate that the splenic
 1064 CD45RA⁻ T-cells are not an exact match to the
 1065 corresponding thymic population.

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1068 4. Discussion

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1070 The maturity of the human fetal immune system
 1071 was analyzed from the perspective of CD95
 1072 expression as well as a number of additional cell-
 1073 surface markers associated with T-cell activation and
 1074 growth (Fig. 8). Most knowledge regarding fetal T-
 1075 cells has come from the analyses of neonatal T-cells
 1076 obtained from UCB. Studies have shown neonatal T-
 1077 cells to be comprised of primarily CD45RA⁺ naive/
 1078 resting T-cells [11–15] that express low levels of the
 1079 activation marker CD95 [21,24]. In contrast, our
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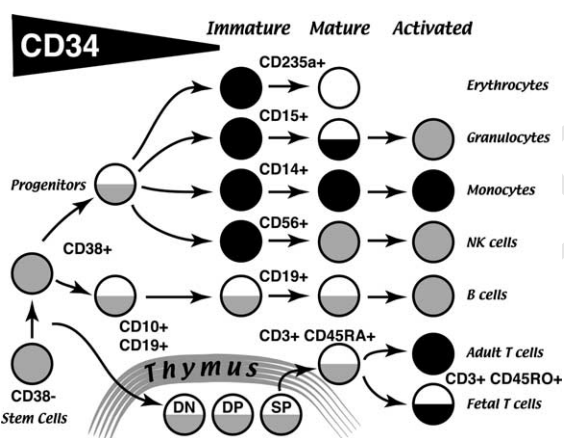
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1105 examination of midgestation fetal tissues indicates
 1106 that the frequency of T cells that express CD95 in
 1107 these tissues is comparable to that of adult T-cells.
 1108 However, the levels of CD95 expression are reduced
 1109 on fetal T-cells. Although fetal T-cells were pre-
 1110 dominantly CD45RO⁻CD45RA⁺ T-cells,
 1111 CD45RO⁺CD45RA⁻ T-cells were present in the
 1112 blood of midgestation fetuses, more so than at full
 1113 term. Furthermore, a number of cell-surface markers
 1114 associated with T-cell activation, were also observed
 1115 on fetal T-cells at levels similar or higher than in the
 1116 adult. These data indicate a, heretofore, unappreciated
 1117 level of activation of peripheral T-cells circulating in
 1118 the immediate weeks following thymic development
 1119 in the human fetus.

1120 Byrne et al. have reported a higher frequency
 1121 of CD45RO⁺CD45RA⁻ T-cells in the midgestation
 1122 fetus compared to full-term neonates [32]. Our findings
 1123 confirm this observation and extend them by
 1124 describing a subset of CD45RO⁺CD45RA⁻ T-cells
 1125 in the fetus that lacks CD95 expression (Fig. 8).
 1126 We are unaware of any previous description of a
 1127 CD95⁻CD45RO⁺CD45RA⁻ subpopulation of T
 1128 cells, and we did not observe this population in post-
 1129 natal blood. Nearly all adult CD45RO⁺CD45RA⁻ T-
 1130 cells are known to express CD95 at high levels [21].
 1131 Indeed, the higher frequency of CD45RO⁺CD45RA⁻
 1132 T-cells in adults is a contributing factor to
 1133 the higher levels of CD95 expression
 1134 observed on adult versus fetal T-cells. The role of the
 1135 CD95⁻CD45RO⁺CD45RA⁻ T-cell subset in
 1136 the developing immune system is presently unclear.
 1137 The expression of CD45RO by these cells suggests that
 1138 they may have been previously activated. Although
 1139 there are some reports that suggest exposure of the
 1140 fetus to external antigens can occur [37], the
 1141 prevalence of the CD45RO⁺ population of T cells
 1142 could mean that these cells are being exposed to and
 1143 subsequently responding to autologous antigens. We
 1144 speculate that a developmentally early wave of
 1145 activation of autoreactive T-cells may be an important
 1146 step in the establishment of suppressor T-cell
 1147 populations and peripheral tolerance. Indeed, the
 1148 decreased expression of CD95 on these cells would
 1149 be counter to the hypothetical removal of fetal
 1150 autoreactive T-cells by a CD95-mediated apoptotic
 1151 mechanism [32]. However, the loss of CD95
 1152 expression may still be associated with the removal



1096 Fig. 8. Proposed expression of CD95 on human hematopoietic cells
 1097 throughout ontogeny. The expression of CD95 at various stages of
 1098 hematopoiesis (CD34⁺ cells, black triangle), maturation and
 1099 activation is indicated by shading. Black circles represent relatively
 1100 high levels of CD95 expression, gray circles represent intermediate
 1101 expression and open circles represent a lack of CD95 expression.
 1102 Filled circles indicate that most cells of the indicated population
 1103 express CD95, whereas semi-circles represent CD95 expression by
 1104 a subpopulation of cells. The schema was developed from the data
 1105 presented in this study as well as previously published reports [25,
 1106 27,50–53].

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 V β chain repertoire was analyzed on fetal T-cells. A diverse repertoire, comparable to that of adults, was observed as early as 16 weeks gestation. It is worth mention that methods more sensitive to minor sequence differences have shown reduced diversity within the V β chain families of late-gestation fetal and some neonatal blood samples [46,47], which

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 V β 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 V β 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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