AGE-DEPENDENT CHANGES IN RELATIVE AND ABSOLUTE SIZE OF LYMPHOCYTE SUBSETS IN THE BLOOD OF PIGS FROM BIRTH TO SLAUGHTER

MAŁGORZATA POMORSKA-MÓL AND IWONA MARKOWSKA-DANIEL

Department of Swine Diseases, National Veterinary Research Institute, 24-100 Pulawy, Poland
mpomorska@piwet.pulawy.pl

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Abstract

The aim of the presented study was to characterise the development of the cellular part of the immune system in pigs from 1 d to 20 weeks of age. Haematological examination and flow cytometry were used to establish the relative and absolute counts of various leukocyte subsets. During the first 5 months of pig life, a significant age-dependent increase in lymphocyte and granulocyte counts was noted. Moreover, a decrease in relative size of lymphocytes connected with increasing proportion of granulocytes was observed. The absolute size of CD3+ and CD21+ increased approximately two-fold during the analysed period. The absolute number of CD4+CD8−, CD4+CD8− and CD4+CD8− cells increased almost twice from birth till the 6th week of age. After this time, only CD4+CD8− subset remained stable, while the number of CD4+CD8− and CD4+CD8− subsets gradually increased 1.5- and 2.5-fold from 6 weeks to 5 months of age for CD4+CD8− and CD4+CD8−, respectively. In conclusion, changes in the absolute size of lymphocyte subsets are not always consistent with changes in their relative size. Moreover, because there are age-related differences in leukocyte subsets in porcine blood, there is a need to use appropriate age matched control groups, especially in experiments, which include immunophenotyping of lymphocytes. We suggest that immunophenotyping of lymphocytes, especially for diagnostic purposes, should be based on the absolute size rather than on the percentage of lymphocyte subpopulations.

Key words: pigs, leukocyte subpopulations, age-related changes, immunophenotyping.

The development of the cellular compartment of the porcine immune system is not complete at the time of birth (20). Differences observed in peripheral blood lymphocyte subpopulations in children compared to adults have prompted similar studies in animals (6-9, 20, 23-25). In the first weeks of life, maturation and expansion of the immune system takes place. Because of this growth process, the relative and absolute size of leukocyte subpopulations varies during neonatal, as well as later periods of life, in many animal species (3, 5-8, 12, 23, 24), as well as in human (4, 12, 17).

Up to now, little has been known about changes in leukocyte subsets in pigs, especially after the neonatal period. There were only a few reports published dealing with prenatal or early postnatal development of the immune system in piglets (1-3, 18, 26). Previous reports indicated that in pigs, contrary to other species, the large population of CD4+CD8− cells existed in peripheral blood (2, 16, 26). Thus, the changes regarding this population seem to be particularly interesting. Contrary to the perinatal period, a lot of external factors (gut microflora, environmental factors, diseases etc.) may influence the postnatal changes in the lymphocyte subsets (26). The immune, neurological, and endocrine systems are other considerable regulatory factors, which may be involved in the lymphocyte subsets changes (22, 26).

Therefore, the aim of the presented study was to summarise the data on the relative and absolute size of circulating porcine leukocytes and their subpopulations at birth and on leukocyte changes during the whole fattening period, which lasts from birth up to about 5 months of age.

Material and Methods

Animals. Fifteen clinically healthy pigs from a commercial breeding farm were used. Complete management and health data were kept for the sows and their offspring. Production was an “all in-all out” procedure with a thorough cleaning between batches. The sows and piglets were of the France hybrids FH 900. The piglets were weaned at approximately 28 d of life. The fattening period finished at about the 154th d of life. Two pigs got sick during the period of study and all data from these animals were omitted from the statistical analysis.

Blood samples were collected via vena cava cranialis venepuncture to vacuum tubes, containing
EDTA-K$_3$ as an anticoagulant (Medlab, Poland) at: 1, 7, 14, 21, 28, 42, 56, 70, 84, 98, 112, and 140 d of pigs’ age. All procedures involved in the study were approved by the Local Ethics Commission.

**Haematological examinations.** Whole blood samples were analysed for different leukocyte proportions and concentrations on a Celoscope-AutoCounter AC 920 (Swelab Instrument AB, Sweden) calibrated for porcine blood. Proportions of lymphocytes, monocytes, and granulocytes were calculated as a percentage of leukocyte concentration.

**Flow cytometry.** Two-colour flow cytometric immunophenotyping, using the lysed whole blood method according to the procedure described below, was performed. Fifty microlitres of blood were incubated with a mAb pair directed against molecules of interest. The cells were double stained for CD3/CD21 and CD4/CD8 using mAbs as follows: mouse IgG1κ anti-pig CD3 (clone PPT), mouse IgG1κ anti-pig CD21 (BB6-11C9.6), mouse IgG2ax anti-pig CD4a (clone 74-12-4), mouse IgG2aκ anti-pig CD8a (clone 76-2-11) (SouthernBiotech, USA). FITC-conjugated mouse IgG1 anti-pig CD45 (CLONE: 1E4) (pan-leukocyte antigen) and RPE-conjugated mouse IgG2b anti-pig CD14 (clone anti-pig CD2) (monocyte antigen) mAbs were used together for gating the lymphocytes (Antigenix America INC, USA). Isotype control mAbs were used to assess nonspecific labelling.

Cells were incubated in the dark with saturating amounts of each antibody, at 4°C for 30 min. After incubation, the cells were washed twice in PBS. The red blood cells were next lysed (OptiLyse C, Immunotech, USA) and the remaining cells were incubated for the next 10 min under the same conditions. After incubation, the cells were washed twice in PBS with 2% inactivated horse serum (GIBCO, USA) and suspended in 500 μl of buffer containing PBS and 2% of formalin. Flow cytometric analyses were performed using a Coulter Epics XL 4C flow cytometer (Beckman Coulter Company, USA).

**Statistical analysis.** Data from all groups were subjected to the W. Shapiro-Wilk’s test of normality and the Levene’a test of equal variances. In the case of a lack of normality or different variances, age-dependent changes were tested with a nonparametric Kruskal-Wallis test with post hoc multiple comparisons for comparison of all pairs. In the case of normal distribution and equal variances, the one-way ANOVA with HSD Tukey’s post-test were used. Differences with $\alpha<0.05$ were considered as significant. For the analysis of the correlation between age and investigated parameters the Pearson correlation (parametric) or Spearman-Rang correlation (nonparametric) were used. All calculations were performed with the Statistica 8.0 (Statsoft, Poland) computer programme.

![Graph](image1.png)

**Fig.1.** Counts and percentage of various leukocyte subpopulations in the peripheral blood of pigs from birth up to the 140$^\circ$ d of life (mean and 0.95 confidence interval).
The results of the study demonstrated that the overall number of leukocytes significantly increased with the age of an animal. The increase was from 12.38 ±4.84 10^9/L to 25.70±3.99 10^9/L in the fifth month of life (P<0.05). The absolute numbers of leukocytes increased twofold from birth to the end of fattening period. Up to 28 d of age, the leukocyte counts were significantly lower than in older animals (≥56 d of age) (P<0.05). The absolute and relative size of the leukocytes and their subpopulation are shown in Fig.1.

A strong positive correlation was evident between the mean number of leukocytes and pig age (r=0.95, P=0.0000). The numbers of lymphocytes also increased significantly with the age of pig. The increase was from 5.11 ±10^9/L on the first day of life to 9.64 ±2.40 10^9/L in the oldest pigs (P<0.05). Up to weaning (approx. 28 d of life) the mean counts of lymphocytes were significantly lower than in weaners (over 10 weeks of life) (P<0.05). The percentage of lymphocytes was the highest in pigs between 7 and 21 d of age (but significantly only when compared to pigs over 56 d old, P<0.05). The lowest percentage of lymphocytes in the blood (33.20 ±6.77) was observed on the 84th d of life.

The absolute size of granulocytes decreased almost 1.5-fold immediately after birth. During the next 3 weeks there was no appreciable change in the numbers of granulocytes. Starting from the 4th week of life, the number of granulocytes subsequently increased ~ twice

### Table 1
Absolute size of lymphocyte subpopulations in blood (number of cells x10^9/L, mean ±SD)

<table>
<thead>
<tr>
<th>Day of life</th>
<th>CD3⁺</th>
<th>CD21⁺</th>
<th>CD4⁺CD8⁻</th>
<th>CD4⁺CD8⁺</th>
<th>CD4⁺CD8⁻</th>
<th>CD4⁺CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>↑2.44±0.49</td>
<td>0.66±0.15</td>
<td>0.77±0.08</td>
<td>↑1.27±0.20</td>
<td>↑0.34±0.08</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.15±0.37</td>
<td>0.56±0.13</td>
<td>1.03±0.18</td>
<td>1.89±0.29</td>
<td>0.51±0.08</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>3.12±0.61</td>
<td>0.76±0.15</td>
<td>1.16±0.22</td>
<td>1.75±0.41</td>
<td>0.50±0.08</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>3.16±0.89</td>
<td>0.61±0.09</td>
<td>1.19±0.28</td>
<td>1.86±0.27</td>
<td>0.43±0.08</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3.36±0.37</td>
<td>0.86±0.08</td>
<td>1.12±0.09</td>
<td>1.62±0.41</td>
<td>0.47±0.07</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>4.11±0.49</td>
<td>0.94±0.19</td>
<td>1.39±0.36</td>
<td>2.15±0.31</td>
<td>0.65±0.09</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>4.51±0.49</td>
<td>0.91±0.17</td>
<td>1.58±0.29</td>
<td>2.49±0.50</td>
<td>0.72±0.17</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>4.54±0.56</td>
<td>0.87±0.19</td>
<td>1.64±0.36</td>
<td>2.91±0.58</td>
<td>1.00±0.25</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>4.94±0.82</td>
<td>1.01±0.17</td>
<td>1.39±0.36</td>
<td>2.92±0.34</td>
<td>1.04±0.18</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>4.76±0.50</td>
<td>1.21±0.26</td>
<td>1.35±0.21</td>
<td>2.96±0.15</td>
<td>1.11±0.17</td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>4.83±0.73</td>
<td>1.08±0.27</td>
<td>1.42±0.29</td>
<td>3.01±0.86</td>
<td>1.49±0.18</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>5.87±0.83</td>
<td>1.19±0.28</td>
<td>1.39±0.27</td>
<td>3.41±0.50</td>
<td>1.69±0.33</td>
<td></td>
</tr>
</tbody>
</table>

Parameters with significant age-dependent trends (P<0.05) are marked as ↑.

### Table 2
Percentage of lymphocyte subpopulations in blood

<table>
<thead>
<tr>
<th>Day of life</th>
<th>CD3⁺</th>
<th>CD21⁺</th>
<th>CD4⁺CD8⁻</th>
<th>CD4⁺CD8⁺</th>
<th>CD4⁺CD8⁻</th>
<th>CD4⁺CD8⁺</th>
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<tbody>
<tr>
<td>1</td>
<td>47.58±2.81</td>
<td>12.80±5.33</td>
<td>14.97±1.62</td>
<td>24.82±1.51</td>
<td>↑6.63±1.09</td>
<td>↓0.60±0.06</td>
</tr>
<tr>
<td>7</td>
<td>57.65±4.14</td>
<td>10.27±3.26</td>
<td>18.88±2.48</td>
<td>34.56±1.65</td>
<td>9.35±3.37</td>
<td>0.55±0.08</td>
</tr>
<tr>
<td>14</td>
<td>59.78±5.95</td>
<td>14.61±1.50</td>
<td>22.20±4.13</td>
<td>33.65±4.06</td>
<td>9.62±3.96</td>
<td>0.66±0.07</td>
</tr>
<tr>
<td>21</td>
<td>56.76±5.90</td>
<td>10.93±2.71</td>
<td>21.34±4.25</td>
<td>33.39±7.13</td>
<td>7.71±3.64</td>
<td>0.66±0.17</td>
</tr>
<tr>
<td>28</td>
<td>57.92±6.03</td>
<td>14.92±2.44</td>
<td>19.35±5.68</td>
<td>28.04±3.92</td>
<td>8.07±4.43</td>
<td>0.68±0.17</td>
</tr>
<tr>
<td>42</td>
<td>56.01±6.07</td>
<td>12.8±2.31</td>
<td>18.94±3.70</td>
<td>29.25±5.17</td>
<td>8.87±1.93</td>
<td>0.65±0.06</td>
</tr>
<tr>
<td>56</td>
<td>58.34±6.23</td>
<td>11.73±3.13</td>
<td>20.47±2.77</td>
<td>32.24±3.90</td>
<td>9.35±3.80</td>
<td>0.65±0.13</td>
</tr>
<tr>
<td>70</td>
<td>56.57±8.96</td>
<td>10.79±1.43</td>
<td>20.49±3.20</td>
<td>36.27±4.49</td>
<td>12.44±3.89</td>
<td>0.56±0.08</td>
</tr>
<tr>
<td>84</td>
<td>58.91±7.58</td>
<td>12.02±2.52</td>
<td>18.24±3.57</td>
<td>34.80±6.64</td>
<td>12.37±4.17</td>
<td>0.52±0.03</td>
</tr>
<tr>
<td>98</td>
<td>55.69±9.69</td>
<td>14.10±2.44</td>
<td>15.75±3.79</td>
<td>34.66±4.52</td>
<td>12.95±2.35</td>
<td>0.46±0.09</td>
</tr>
<tr>
<td>112</td>
<td>53.82±10.86</td>
<td>12.05±2.38</td>
<td>15.86±1.69</td>
<td>33.61±3.25</td>
<td>16.65±1.71</td>
<td>0.48±0.08</td>
</tr>
<tr>
<td>140</td>
<td>60.93±6.24</td>
<td>12.30±2.04</td>
<td>14.41±1.67</td>
<td>35.36±2.55</td>
<td>17.48±1.24</td>
<td>0.41±0.04</td>
</tr>
</tbody>
</table>

Within the column, means with the same superscript differ significantly (P<0.05). Parameters with significant age-dependent trends (P<0.05) are marked as ↑↓.
to slaughter. The percentage content of granulocytes significantly increased from 1 d to 140 d of pigs’ age (P<0.05). Positive correlations were found, between mean number, as well as mean percentage of granulocytes, and the pigs’ age. Pearson correlations were equal respectively r=0.93, P=0.000 and r=0.77, P=0.0030. Starting from the 70th d of life, the percentage of granulocytes outnumbered about 1.5-fold the percentage of lymphocytes.

The results of flow cytometric analyses, including significant differences among neighbouring age groups, are summarised in Tables 1 and 2.

CD3+ leukocytes constituted the largest percentage; more than half of them were CD4+CD8+. No correlations were found between percentage of both, CD3 and CD21 positive cells, and the age of pigs. Immediately after birth, the percentage of CD3+ lymphocytes increased over 20% but this was not statistically significant. The percentage of CD3+ lymphocytes then remained stable until pigs were five-month-old (P<0.05). There were no significant changes between similar age groups of these cells. However, absolute numbers of CD3+ cells revealed the age-dependent trend (P<0.05). A strong positive correlation among mean numbers of CD3+ cells in pigs blood and animal age was determined (r=0.95, P<0.0000).

A slight negative correlation was found between the mean percentages of CD4+CD8+ cells and animal age (R-Spearman = -0.27, P=0.0063), contrary to the CD4+CD8+ cells, in case of which, a positive correlation was evidenced (R-Spearman = 0.34, P=0.0004). Additionally, a strong positive correlation between the mean number of CD8+CD4+ cells and animal age was observed (r=0.95, P=0.0000). Immediately after birth, a significant increase in the frequency of CD8+CD4+ cells was also noted (P<0.05). The percentages of CD4+CD8+ cells in peripheral blood were the highest between 14 and 21 d of age.

Age-dependent changes in the percentage of CD4+CD8+ double positive cells were also detected (P<0.05). A significant positive correlation between the age of pigs and the mean percentages of double positive cells in the blood, were evident (R-Spearman = 0.66, P<0.05). Additionally, the number of these cells significantly increased with a pig’s age (P<0.05), and the strong positive correlation between mean numbers of CD4+CD8+ lymphocytes and the animal’s age was observed (r = 0.97, P=0.0000). The absolute numbers of CD4+CD8+, CD4+CD8-, as well as double positive CD4+CD8+ cells, increased almost twofold from birth up to two weeks after weaning. After this time only CD4+CD8- subset remained stable until the end of study. The numbers of the remaining subpopulations gradually increased, 1.5- and 2.5-fold, respectively for CD4+CD8+ and CD4+CD8- cells.

No statistically significant differences were found between similar age groups and values of CD4/CD8 ratio. These ratios were calculated from percentages of both lymphocyte subpopulation in peripheral blood. However, there was a negative correlation between age and the CD4/CD8 ratio (R-Spearman = -0.53, P=0.0000).

Discussion

In pigs, as in studies conducted on different species of animals (5, 23), the lymphocyte/granulocyte ratio revealed changes during different periods of life. The number of granulocytes on the first day of a pig’s life was almost the same as the number of lymphocytes. Over the first three weeks of life, a decrease in granulocyte absolute size and the predominance of lymphocyte numbers were observed. Starting from the 70th d of life, the number of granulocytes outnumbered the number of lymphocytes. A similar situation was observed with respect to relative size of both mentioned leukocyte populations.

The results of our study showed that the number, as well as percentage of main lymphocyte subpopulations, changed with the age of a pig. Moreover, changes in absolute size of lymphocyte subsets were not always consistent with changes in lymphocyte subset relative size. For instance, the mean percentages of CD3+ cells remained stable at 47.58% to 60.93% from birth to 5 months of age, but at the same time the mean absolute number increased almost 2.5-fold. The increase in counts of CD3+ lymphocytes were caused mostly by rising numbers of CD4+CD8+ and double positive CD4+CD8+ cells, while the numbers of CD4+CD8- cells remained relatively stable during the whole fattening period. This demonstrates, that the relative number of lymphocyte subpopulations does not reflect their actual size and is therefore of limited value. A similar situation was observed by Comans-Bitter M. et al. (4) in humans.

There are at least two critical periods in pig’s life. Both periods are connected with the huge changes that take place in the pig’s surroundings, and the exposure to many „new” antigens immediately after birth and then again after weaning. The results of the presented study showed that just after birth, the number of CD4+CD8+ cells, which contain activated and memory T cells (11, 12, 14, 15), increased almost twofold. Directly after weaning, the number of these cells increased again by about 40%. It might be a result of enlarged proliferation after contact with many external antigens, as well as the maturation process (4). These data are in agreement with the results obtained by Borghetti et al. (2), who investigated the age related changes in leukocyte counts of pigs till the 41st d of life. Up to weaning, the absolute sizes of lymphocytes in piglets were significantly lower than in weaners (P<0.05).
The relationship between CD4⁺ and CD8⁺ cells in pigs’ blood has been investigated in variety of studies (1, 2, 9, 10, 19). As it has been shown, the number of CD8⁺ cells prevailed over CD4⁺ cells, and the CD4⁺/CD8⁺ ratio was lower than 1.0 (1, 3, 10, 18). Our results revealed that CD4⁺/CD8⁺ ratios decreased with the age, which is in accordance with results obtained by other authors (2, 3, 9, 12, 19). In the presented study, the lower CD4⁺/CD8⁺ ratio (calculated from the relative size of lymphocyte subsets) in the 5th month of age, was mainly the result of the reduction of CD4⁺CD8⁻ cell percentage with a relatively stable proportion of CD4⁺CD8⁺ cells. The values of CD4⁺CD8⁻ ratios calculated from absolute numbers of CD4⁺CD8⁻ and CD4⁺CD8⁺ cells also decreased with pig age. This effect was caused not only by declining amounts of CD4⁺CD8⁻ cells, but it was also the consequence of increasing numbers of CD4⁺CD8⁺ lymphocytes.

As it was mentioned, beside single positive CD4 and CD8 cells, the population of double positive lymphocytes existed in pigs. The proportions of these lymphocytes range between 4% and 60% (12, 13). It is evident that the amount of CD4⁺CD8⁺ lymphocytes in peripheral blood of pigs increases with the age of pig (2, 9, 13, 26). A similar tendency was observed also in the presented study. In our study the percentage of this subset increased from 6.63% on the first day of life to 17.48% in 20-week-old pigs, while the absolute number of these cells increased almost 5-fold in the same period. Stepanova et al. (12), in contrast to our results, have shown that double positive CD4⁺CD8⁺ cells were very rare during the first month of life (up to 0.5%) and that the numbers of CD8⁻ cells did not predominate over CD4⁺ lymphocytes in piglets, as well as in adult pigs (6 months of age). It is likely that these differences were influenced by the conditions in which the animals were kept. In the above mentioned study, the animals were housed under experimental conditions in which the external antigenic stimulation was lower than at conventional farms.

The age-dependent increase in the amount of double positive lymphocytes seems to be the result of antigen-dependent maturation of naïve CD4⁺ T helper lymphocytes to antigen-specific memory T helper cells (2, 12, 16). As detected previously in humans (11) and partly in pigs (15, 21), the CD4⁺CD8⁻ lymphocytes are involved in the antiviral immune response. It is possible that this subset of lymphocytes is involved also in the immune response against others pathogens.

In conclusion, during the fattening period there was a significant age-dependent increase in total leukocyte number together with numbers of lymphocytes and granulocytes. However, a decrease in the percentage of lymphocytes, connected with an increasing proportion of granulocytes, was observed. The changes related to the number of leukocyte subsets were not always correlated with the changes in the percentage of respective subpopulations. Furthermore, we conclude that postnatal changes related to the size of the main subpopulations of lymphocytes were characterised by an increasing number of all analysed subsets. There was a strong positive correlation between mean numbers of particular lymphocyte subpopulations and pigs’ age (r value at least 0.66, P<0.05).

On the basis of this study, we suggest that immunophenotyping of lymphocytes, especially for diagnostic purposes, should be based on the absolute size, rather than on the percentage of lymphocyte subpopulations, according to appropriate age-matched reference values. Moreover, because there are age-related differences in lymphocyte subsets distributed in the peripheral blood of pigs, there is a need to use appropriate age matched control groups. The ages should be matched as closely as possible especially in those experiments which include immunophenotyping of blood lymphocytes.

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**References**

pigs from 1 to 40 weeks of age. Vet Immunol Immunopathol 1994, 40, 105-117.