Postoperative Regional Analgesia Is Effective in Preserving Perforin-Expressing Lymphocytes in Patients After Total Knee Replacement

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Background: Pain and surgical stress cause a pro-inflammatory response followed by downregulation of the immune response, which can increase the incidence of postoperative complications, such as infections or prolonged wound healing. T lymphocytes and natural killer (NK) cells have cytotoxic potential and are crucial components of cellular immunity, which is important for maintenance of immune balance. The aim of this study was to analyze the effects of 3 types of postoperative analgesia on the preservation and cytotoxic potential of T lymphocytes, NK cells, and their subpopulations, as well as NKT cells, in patients after total knee replacement (TKR) to find the most effective analgesic technique for mitigating immune suppression.

Material/Methods: Forty-eight patients scheduled for TKR were randomly allocated to Group 1 (patients received epidural analgesia), Group 2 (patients received sciatic and femoral nerve block), or Group 3 (patients received multimodal systemic analgesia). Pain intensity was assessed at rest and on movement before, immediately after, and at 24 and 72 h after surgery. Blood samples were collected at the same time points and peripheral blood mononuclear cells were isolated. The frequencies of T lymphocytes, NK cells, and NKT cells, as well as their perforin expression, were simultaneously detected and analyzed by flow cytometry.

Results: Patients in Group 1 and Group 2 experienced less severe pain than those in Group 3. The frequencies and perforin levels of T lymphocytes, their subsets, and NKT cells were significantly lower in Group 3 than in Group 1 and Group 2.

Conclusions: The present study confirmed that regional analgesia is more effective in maintaining cell-mediated immunity and perforin expression in peripheral blood lymphocytes in patients after TKR.

MeSH Keywords: Anesthesia and Analgesia • Immunity, Cellular • Nerve Block • Pain • Perforin

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**Background**

Total knee replacement (TKR) is a common orthopedic reconstructive surgical procedure that improves the mobility and the quality of life in patients with degenerative osteoarthritis [1]. It is generally associated with strong postoperative pain at the surgical site [1]. Endogenous danger-associated molecular patterns produced and released after the tissue injury are recognized by pattern recognition receptors, such as Toll-like receptors (TLRs), expressed on the surface of local antigen-presenting immune cells and non-immune cells, including primary sensory neurons, synoviocytes, fibroblasts, chondroblasts, and osteoblasts [2]. After engagement of the TLRs, the joint cells support the immune response by secreting cytokines, leading to local and systemic pro-inflammatory states [3], which cause pain [4]. Clinically manifested pain sensation is mediated by an increase in pro-inflammatory cytokines and chemokines, such as interleukin (IL)-1, IL-6, IL-8, IL-17, and tumor necrosis factor-alpha (TNF-α), in body fluids [5]. IL-6 is positively correlated with IL-17 and Western Ontario and McMaster Universities Arthritis Index [5], and IL-8 amplifies the pain mediated by IL-6 and IL-17 [6]. Periodically increased serum TNF-α in osteoarthritis patients is also correlated with pain sensation [7]. Additionally, IL-17 enhances the expression of main pro-inflammatory mediators IL-1, IL-6, IL-8, TNF-α, C-reactive protein, monocyte chemo-attractant protein 1, and matrix metalloproteinases, and supports the recruitment of neutrophils, CD4+ and CD8+ T lymphocytes, and monocytes toward local inflammatory sites [7]. Cytokines have an immunoregulatory function and are main mediators between the central nervous and immune systems [8,9]. Circulating cytokines pass directly through the blood–brain barrier by active transport or indirectly by stimulation of the vagal nerve [10]. In the brain, cytokines change the concentration of neurotransmitters [11] and play a key role in the activation of the hypothalamic-pituitary-adrenal axis as observed in stressed and depressed patients [12]. Pain increases neuroendocrine activation and catecholamine release [12–16], which potentiate the inflammatory response [12]. Catecholamines inhibit T lymphocyte immune function and decrease NK cell activity directly through β-adrenergic receptors or indirectly by inhibiting cytokines, such as IL-12 and interferon-γ, which are essential for their activity [15].

Since surgical trauma and pain suppress cellular and humoral immunity in postoperative patients, effective anesthesia and postoperative analgesia are critical. Major surgical procedures, such as TKR, are associated with neuroendocrine, metabolic, and immunological alterations that result from tissue damage, anesthesia, and pain [17,18]. If not effectively treated postoperatively, pain could additionally suppress the immune response, causing delayed wound healing, infection, and poor outcomes. Therefore, the most effective analgesia technique with minor side effects is required.

Different types of anesthesia and postoperative analgesia in patients undergoing surgery have been shown to maintain a better balance of cellular and humoral immunity [19]. Intravenous analgesia with opioids can attenuate postoperative pain, but there is strong evidence that it downregulates the immune response and leads to higher incidence of complication and prolonged recovery [20,21]. Recently, it has been shown that regional analgesia techniques can reduce the pro-inflammatory response caused by surgery and pain [12,22,23], but little is known about their effect on cell immunity and its cytokotic potential, which is important for wound healing and prevention of infections, particularly in patients undergoing TKR. T lymphocytes, natural killer (NK), and NKT cells have cytokotic potential; upon stimulation, they induce apoptosis and necrosis by secreting cytokotic mediators, such as perforin, which form pores in the target cell membrane [24]. Perforin, also called cytolsin, is a 66–70-kDa glycoprotein stored in granules in the cytotic lymphocytes and released from the granules at the immunological synapse [25].

This study aimed to find the most effective analgesic technique – epidural analgesia vs. peripheral nerve blockade of sciatic and femoral nerves vs. multimodal systemic analgesia – for preserving perforin-expressing T lymphocytes, NK, and NKT cells in peripheral blood after TKR.

**Material and Methods**

**Patients**

Forty-eight patients scheduled for TKR at the Clinic of Orthopedics and Traumatology Lovran, Lovran, Croatia, were included in this prospective randomized study. The patients were randomly allocated using Datlnf Ranolist software (Datlnf GmbH, Tubingen, Germany) to 1 of 3 groups (n=16 each) according to the type of postoperative analgesia received. The study included primary degenerative osteoarthritis patients who underwent TKR, were of American Society of Anesthesiologists physical statuses I to III, and provided informed consent. Patients aged <50 and >75 years, patients allergic to the analgesics and anesthetics used in this study, patients who received blood transfusion, patients with immunological and malignant disease, and those with secondary form of knee osteoarthritis and rheumatoid arthritis were excluded (Figure 1). The study was approved by the local ethics committee of the Clinic for Orthopedics and Traumatology Lovran, Lovran, Croatia and the Faculty of Medicine, University of Rijeka, Rijeka, Croatia according to the World Medical Association criteria in the declaration of Helsinki. Healthy, sex- and age-matched volunteer blood donors comprised the control group (Group 0, n=16). All subjects provided written informed consent.
Demographic data on sex, age, operative duration, anesthesia duration, and duration of hospitalization were recorded. The subjective perception of pain intensity for each patient was assessed using a 10-cm visual analogue scale (VAS) with endpoints labeled “no pain” and “the worst possible pain” before surgery (T1), immediately after surgery (T2), and 24 h (T3) and 72 h (T4) after surgery at rest and on movement.

Anesthesia procedure

For all patients, the same anesthesia procedure was applied. Twelve hours before surgery, 3800 IU of dalteparin (Fraxiparine; Aspen Pharma Trading Limited, Braine-L’Alleud, France) was administered subcutaneously, and 10 mg diazepam (Roche, Basel, Switzerland) was administered orally. The same surgeon and anesthesiologist performed all operations and anesthesia. All patients underwent regional lumbar spinal anesthesia, before which 3 mg of midazolam (Midazolam 5 mg/mL; B. Braun Melsungen AG, Germany) was administered subcutaneously, and 1 mg/kg propofol (Diprivan; AstraZeneca, Courbevoie, France) was injected. The femoral blockade was performed in an aseptic condition with 2.5 g metamizolsodium (Alkagin; Alkaloid-Int d.o.o., Ljubljana, Slovenia) administered subcutaneously, and 10 mg diazepam (Roche, Basel, Switzerland) was administered orally. The same surgeon and anesthesiologist performed all operations and anesthesia. The lumbar spinal anesthetization was performed in an aseptic condition with patients in the lateral decubitus position, using an atraumatic 25-G needle. When the subarachnoid space was reached, 3 mL of 0.5% bupivacaine (Marcaine 0.5% Spinal; AstraZeneca, Courbevoie, France) was applied. A midline approach at the L3–L4 level was used for spinal blockade.

Postoperative analgesia

Postoperative pain was managed with epidural analgesia (Group 1, n=16), blockade of sciatic and femoral nerves (Group 2, n=16), or multimodal systemic analgesia (Group 3, n=16). In Group 1, the epidural catheter was applied immediately after surgery with an 18-gauge Tuohy epidural needle (Espocan; B. Braun Melsungen, Germany) through loss-of-resistance technique. The epidural block was performed using ultrasound-guided “in plane” technique for displaying the needle on an ultrasound instrument (Sonosite EDGE Ultrasound system; Sonosite Inc., WA, USA). Sciatic blockade was performed in the lateral decubitus position with knee and hip flexure. By setting the ultrasound convex 2–5-MHz transducer (C60x/5–2MHz transducer; Sonosite, Washington, USA) in the subgluteal region and displaying the sciatic nerve in the line formed by the ischial tuberositas and posterior superior iliac spine, a 22-gauge, 100-mm-long neurostimulator needle (Stimuplex A; B. Braun Melsungen, Germany) was inserted against the nerve stimulating (Stimuplex HNS 12; B. Braun Melsungen, Germany) nerve (0.5–1 mA, 0.1 millisecond). After confirming identification of the nerve (0.5–1 mA, 0.1 millisecond), the needle was positioned near the sciatic nerve and 20 mL of 0.5% levobupivacaine was injected. The femoral blockade was performed in the supine position, using a high-frequency linear ultrasound probe (HFL50x/15-6-MHz Transducer; Sonosite Inc., WA, USA), without nerve stimulation. The transducer was placed in the inguinal region and the femoral nerve was identified. Femoral nerve block was performed with 10 mL of 0.5% levobupivacaine near the nerve. After bolus injection, the femoral catheter (Contiplex Tuohy; B. Braun Melsungen, Germany) was set up under the femoral nerve. The catheter tip was placed 3–4 cm from the needle tip. Intermittent boluses of 10 mL 0.25% levobupivacaine every 4 to 6 h were applied on the femoral catheter based on VAS score. The catheter was removed on postoperative day 3. In Group 3, multimodal systemic analgesia was used to manage postoperative pain. Initially, during surgical wound closure, paracetamol (Paracetamol Kabi, HNS 12; B. Braun Melsungen, Germany) was intravenously administered. Postoperative pain in these patients was managed with intravenously administered 1 g paracetamol every 6 h, 200 mg tramadol every 10–12 h (Tramal; Grunenthal GmbH, Aachen, Germany), and 2.5 g metamizolsodium (Alkagin; Alkaloid-Int d.o.o., Ljubljana, Slovenia).
Table 1. Demographic and clinical parameters of patients in all groups.

| Group                  | Age (years)* | Gender (M/F) | Duration of surgery (min) | Duration of anaesthesia (min) | P value |
|------------------------|--------------|--------------|---------------------------|-------------------------------|---------|
| Group 1                | 68.5 (64–72) | 6/10         | 72.5 (65–88.5)            | 316 (277.5–407.5)             | Ns      |
| Group 2                | 67 (62–70)   | 6/10         | 67.5 (64.5–77.5)          | 339 (299–369.5)               | Ns      |
| Group 3                | 70.5 (64–74.5)| 7/9         | 65.5 (60–73)              | 318 (294–359.5)               | Ns      |

Group 1: patients received epidural analgesia; Group 2: patients received sciatic and femoral nerve block; Group 3: patients received multimodal systemic analgesia. Data are presented as median (25th–75th percentile). Differences between groups were not significant (Ns; p>0.05).

Isolation of peripheral blood mononuclear cells (PBMCs)

We drew 20-ml blood samples from each patient. Blood was overlaid onto Lymphoprep (Lymphoprep; Fresenius Kabi, Oslo, Norway). PBMCs were isolated through a centrifugation procedure.

Cell surface and intracellular labeling of PBMCs

The isolated PBMCs (2×10⁶/sample) were incubated for 20 min at room temperature (RT) with 10% heat-inactivated human AB serum to block non-specific Fc receptor binding. Then, the cells were washed in fluorescence-activated cell sorting (FACS) buffer containing NaCl (140 mM), KH₂PO₄ (1.9 mM), Na₂HPO₄ (16.5 mM), KCl (3.75 mM) (all from Kemika, Zagreb, Croatia), Na₂-EDTA (0.96 mM) (Fluka, Buchs, Switzerland), and NaN₃ (1.5 mM) (Difco, Detroit, MI, USA) and were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at RT. Thereafter, the cells were washed twice in FACS buffer and permeabilized for 20 min at RT with saponin buffer (0.1% saponin; Sigma, Poole, Dorset, USA), 2% goat serum in phosphate-buffered saline (PBS; NaCl 8 g, KCl 0.2 g, Na₂PO₄·12H₂O 2.87 g, and KH₂PO₄ 0.2 g (all from Kemika, Zagreb, Croatia) dissolved in 1 L of distilled water). To appropriate cell samples (3 μg diluted in 200 μL of saponin buffer), mouse dG9 IgG2b anti-perforin monoclonal antibody or isotype-matched control (mouse MA-21, IgG2b; BD Biosciences, Erembodegem, Belgium) conjugated with fluorescein isothiocyanate (FITC) was added and incubated at +4°C for 30 min. Then, the samples were washed twice with saponin buffer and resuspended in 1 mL of FACS buffer. A combination of CyChrome Phycoerythrin-5 (Cy-PE5) conjugated anti-CD3 (mouse UCHT1, IgG1) and PE-conjugated anti-CD4 mAb (mouse RPA-T4, IgG1), Cy-PE5 anti-CD3 and PE anti-CD8 (mouse RPA-T8, IgG1), or Cy-PE5 anti-CD3 and PE anti-CD56 (mouse B159, IgG1) were used for surface labeling. As controls, we used PE-, FITC-, or CY-PE5-conjugated mouse isotype-matched antibodies (all from ebScience, San Diego, CA, USA). After fixing the labeled samples in 400 μL of 2% paraformaldehyde, they were analyzed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Statistical analysis

Statistical analysis was performed using Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The Kolmogorov-Smirnov test was used to test for normal distribution of the data. Non-parametric tests were used because the data did not demonstrate normal distribution. Friedman’s test and the post hoc Wilcoxon rank sum test were used to compare data points within groups. Kruskal-Wallis test was used to analyze differences among groups, followed by Mann-Whitney U-test for post hoc analysis. Bonferroni adjustments were applied when multiple comparisons were made.

We calculated the minimum required sample size based on preliminary results obtained for the 3 groups. As we compared the 3 patient groups, we used sample size calculation in Statistica, option several means. We set type I error rate (alpha) at 0.05, power goal at 0.80, RMSSE at 0.6, and the non-centrality parameter (delta) at 7.2. The analysis indicated that 15 patients in each group were required. Correlation between VAS values and the frequency of different leukocyte subpopulations and percentage of perforin expression was tested using Spearman’s rank correlation coefficient. A difference was judged to be statistically significant at p<0.05. All data are presented as the 25th–75th percentile values.

Results

Clinical parameters

Demographic and surgical data are shown in Table 1. There were no significant differences among Groups 1, 2, and 3 regarding age, sex, operation duration, or anesthesia duration.

Comparison of VAS scores at rest and on movement

Table 2 shows the VAS scores for Groups 1, 2, and 3 at rest and on movement at different time points (T1, T2, T3, and T4). VAS
The frequencies of T lymphocytes (determined as CD3+CD56- cells), NK cells (CD3+CD56+ cells), and NKT cells (CD3+CD56+ cells) did not differ significantly before surgery (T1) among groups (Figure 2A). At T2, the frequencies of T lymphocytes and their CD3+CD4+ and CD3+CD8+ subsets, as well as NK cells, were significantly lower in Group 3 than in Groups 1 and 2 (Figure 2B). At T3, the frequencies of CD3+ and the CD3+CD8+ subset were lower in Group 3 than in Group 2 (Figure 2C). The frequency of CD3+CD8+ cells was lower in Group 3 than in Group 2 at T4 (Figure 2D), while at T3 the frequency of NK cells was significantly lower in Group 3 than in Groups 1 and 2 at T3 (Figure 2C) and T4 (Figure 2D). The frequencies of NK cells and their subsets CD3+CD56+bright and CD3+CD56+dim (Figure 3C) did not differ significantly among groups (data not shown).

**Effects of different types of postoperative analgesia on perforin expression in peripheral blood lymphocytes**

Figure 3 shows the changes in perforin+ cell frequency at different time points. Perforin+ cell frequency was significantly lower in Group 3 than in Groups 1 and 2 at T3 and T4.

Perforin-expressing CD3+ cells and their CD3+CD8+ subset were lower in Group 3 than in Groups 1 and 2 at T3 (Figure 4C) and T4 (Figure 4D). Perforin-expressing CD3+CD4+ and NKT cells (Figure 4), as well as NK cells and their subsets (data not shown), did not differ significantly at any time point.

**Correlation between VAS values and frequencies and perforin expression of different leukocyte subpopulations**

VAS values were negatively correlated with the frequencies of CD3+, CD3+CD4+, CD3+CD8+, and CD3+CD56- cells at both T2 and T3 (Table 3). At T3 and T4, a negative correlation was found between VAS values and all perforin-positive, CD3+perforin+, and CD3+CD8+ perforin+ cells (Table 3).

**Discussion**

Pain management after TKR is mandatory but usually difficult [1], and there are currently no unique postoperative pain management approaches. Despite the reported impacts of different methods of postoperative analgesia in patients undergoing major thoracic and abdominal surgery on cellular- and molecular-level alterations in the immune response, few studies exist on patients undergoing TKR [13,19,22,23]. Here, we report reductions in the frequency of circulating T lymphocytes, their subsets CD3+CD4+ and CD3+CD8+ cells, and NKT

Table 2. Comparison of visual analogue scores (VAS) at rest and on movement in patients of all groups.

| Group | T1 | T2 | T3 | T4 | P value |
|-------|----|----|----|----|---------|
| VAS at rest | 0 (0–0) | 0 (0–1) | 0.5 (0–2.5) | 0 (0–0) | p<0.05 |
| VAS on movement | 0 (0–1) | 0 | 1 | 0 | p<0.05 |

Data are presented as median (25th–75th percentile). Statistical significance is shown by p-values between VAS on movement at T2 and T3 (a), T2 and T4 (b) in Group 1, at T2 and T4 in Group 2 (c), T1 and T2 (d), T1 and T3 (e), and T1 and T4 (f) in Group 3. Statistical significance between VAS scores at rest in Group 3 between T1 and T2 (g), T1 and T3 (h), and T1 and T4 (i) in Group 3 is shown. When comparing VAS values between groups, statistical significance is presented using p-values as follows: * between Group 2 and Group 3 at T2 between Group 1 and Group 3 at time T2, # between Group 2 and Group 3 at time T3, ° between Group 1 and Group 3 at T4, % and between Group 2 and Group 3 at T4. * p<0.05. VAS – visual analogue scale; T1 – before surgery; T2 – after surgery; T3 – 24 h after surgery; T4 – 72 h after surgery.

Scores at rest and on movement recorded at T1 did not differ significantly. In Group 1, the VAS score on movement was significantly higher at T3 and T4 than at T2. In Group 2, the VAS score on movement was significantly higher at T4 than at T2. VAS scores in Group 3 were significantly higher at T2, T3, and T4 than at T1 at rest and on movement. At T2, the VAS score was significantly higher in Group 3 than in Groups 1 and 2 at rest and on movement. At T3, the VAS score was significantly higher in Group 3 than in Groups 1 and 2 at rest and on movement. At T4, the VAS score was significantly higher in Group 3 than in Groups 1 and 2 on movement.
Figure 2. Frequencies of CD3+, CD3+CD4+, CD3+CD8+, and CD3+CD56+ cells in peripheral blood lymphocytes in Group 1 (■), Group 2 (□), and Group 3 (●) at time points T1 (before surgery) (A), T2 (immediately after surgery) (B), T3 (24 h after surgery) (C), and T4 (72 h after surgery) (D). Data are presented as median (□), 25th–75th percentile (□), non-outlier range (‖), and outliers (●). *p<0.05.

Figure 3. Frequencies of perforin-positive cells within peripheral blood lymphocytes in Group 1 (■), Group 2 (□), and Group 3 (●) at times points T1 (before surgery), T2 (immediately after surgery), T3 (24 h after surgery), and T4 (72 h after surgery). Data are presented as median (□), 25th–75th percentile (□), non-outlier range (‖), and outliers (●). *p<0.05.
not exclude the possibility that local anesthetics themselves can induce neuro-immunomodulation induced by locally produced pro-inflammatory and pain-inducing cytokines [28–32]. We can therefore propose that regional analgesia, particularly those with the cytotoxic CD3+CD8+ phenotype, could be responsible for elimination of cytotoxic mediators of cell-mediated immunity and its downregulation in peripheral blood is associated with increased incidence of systemic infection and poor patient outcome [24]. Here, a greater decrease in perforin was observed in patients receiving systemic multimodal analgesia than in those receiving epidural analgesia, experience lesser hyperalgesia than patients not receiving regional analgesia, including peripheral nerve blockade or epidural analgesia, experience lesser hyperalgesia than those receiving systemic multimodal analgesia [13,33]. In line with these results, techniques of regional analgesia, predominantly epidural postoperative analgesia, can significantly reduce the incidence of systemic infection and poor patient outcome [24]. However, we did not find significant fluctuation in NK cells (data not shown).

The present results agree with previous findings that patients receiving regional analgesia, including peripheral nerve blockade, experience lesser hyperalgesia than those receiving systemic multimodal analgesia [13,33]. In line with these results, techniques of regional analgesia, predominantly epidural postoperative analgesia, can significantly reduce the incidence of systemic infection and poor patient outcome [24]. However, we did not find significant fluctuation in NK cells (data not shown).

In patients receiving peripheral nerve blockade, the maintenance of T lymphocytes, NKT cells, NK cells, and their subsets, as well as perforin expression, in the early postoperative period could be explained by prolonged blockade of C-fibers of afferent and efferent nerves with local anesthetics, thereby reducing central nervous system stimulation and subsequent neuro-immunomodulation induced by locally produced pro-inflammatory and pain-inducing cytokines [28–32]. We cannot exclude the possibility that local anesthetics themselves modulate the inflammatory response locally and systemically.

Figure 4. Frequencies of CD3+ perforin+, CD3+CD4+ perforin+, CD3+CD8+ perforin+ and CD3+CD56+ perforin+ cells within peripheral blood lymphocytes in Group 1 (●), Group 2 (□), and Group 3 (=) at times points T1 (before surgery) (A), T2 (immediately after surgery) (B), T3 (24 h after surgery) (C), and T4 (72 h after surgery) (D). Data are presented as median (●), 25th–75th percentile (□), non-outlier range (I), and outliers (○). * p<0.05.
Conclusions

Our results suggest that peripheral nerve blockade or epidural analgesia are better and more effective in maintaining perforin-expressing peripheral blood T lymphocytes with cytotoxic CD3⁺CD8⁺perforin⁺ phenotype and NKT cells than multimodal systemic analgesia after TKR in the early postoperative period.

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Conflicts of interest

None.

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Table 3. Correlation between VAS values and different lymphocytes subpopulation or their perforin expression after surgery (T2), 24 (T3), and 72 (T4) h.

|                   | VAS       | T2  |   |   |   | T3  |   |   |   | T4  |   |   |
|-------------------|-----------|-----|---|---|---|-----|---|---|---|-----|---|---|
|                   | r         | p   | r |   |   | r   | p |   |   | r   | p |   |
| CD3⁺ cells        | −0.538    | 0.038 |   |   |   | −0.0617 | 0.014 | 0.178 | 0.525 |   |   |
| CD3⁺CD4⁺ cells    | −0.083    | 0.001 |   |   |   | −0.0648 | 0.009 | 0.078 | 0.78  |   |   |
| CD3⁺CD8⁺ cells    | −0.078    | 0.001 |   |   |   | −0.063  | 0.012 | −0.448 | 0.093 |   |   |
| CD3⁺CD56⁺ cells   | −0.078    | 0.001 |   |   |   | −0.494  | 0.041 | −0.616 | 0.054 |   |   |
| CD56⁺ cells       | −0.028    | 0.92  | 0.124 | 0.659 | −0.454 | 0.089 |   |   |   |   |   |
| CD56dim⁺ cells    | −0.999    | 0.72  | 0.377 | 0.16  | −0.17 | 0.54  |   |   |   |   |   |
| Perforin⁺ cells   | −0.998    | 0.726 | −0.193 | 0.48  | −0.08 | 0.77  |   |   |   |   |   |
| CD3⁺perforin⁺ cells| −0.624    | 0.13  | −0.445 | 0.05  | −0.442 | 0.048 |   |   |   |   |   |
| CD3⁺CD4⁺perforin⁺ cells| −0.14    | 0.616 | −0.09 | 0.72 | −0.266 | 0.33 |   |   |   |   |   |
| CD3⁺CD8⁺perforin⁺ cells| −0.678    | 0.051 | −0.774 | 0.001 | −0.672 | 0.006 |   |   |   |   |   |
| CD3⁺CD56⁺perforin⁺ cells| −0.512    | 0.052 | −0.157 | 0.575 | 0.135 | 0.63 |   |   |   |   |   |
| CD56dim⁺perforin⁺ cells| −0.385    | 0.156 | −0.235 | 0.398 | −0.291 | 0.358 |   |   |   |   |   |
| CD56bright⁺perforin⁺ cells| −0.298    | 0.296 | −0.375 | 0.17  | −0.408 | 0.13 |   |   |   |   |   |
| CD56bright⁺perforin⁺ cells| 0.36      | 0.183 | −0.484 | 0.067 | −0.549 | 0.054 |   |   |   |   |   |

Statistical significance is shown as p and r values.

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