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Hematoma size as major modulator of the cellular immune system after experimental intracerebral hemorrhage

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ABSTRACT

Inflammatory cascades are increasingly recognized as an important pathophysiological mechanism in intracerebral hemorrhage (ICH). In contrast, the effect of ICH on the systemic immune system has barely been investigated. We examined the effects of different hematoma volumes on immune cell subpopulations in experimental murine ICH. In C57BL/6 mice, ICH was induced by striatal injection of autologous blood (10, 30 or 50 μL). Control animals received the respective sham operation. Three days after ICH induction, differential blood leukocyte counting was performed. Lymphocyte subpopulations were further characterized by flow cytometry in blood, spleen, lymph node and thymus. Infectious complications were studied using microbiological cultures of blood and lungs. Only after large ICH a marked decrease of leukocyte counts and most lymphocyte subsets was observed in all organs. Despite this general leukocytopenia, a significant, up to 10-fold increase, was detected in the monocyte population after extensive hemorrhage. After moderate ICH induction, only specific lymphocyte subpopulations were differentially affected. Mature thymic cells were unaffected while immature CD4+CD8+ cells were depleted by over 90% after large ICH. A significant proportion of mice with extensive ICH (36.4%) developed spontaneous pneumonia and/or bacteremia while none of the sham operated mice had infectious complications. The ICH size determines the extent of systemic immunomodulation. Large ICH predisposes animals to infections.

Intracerebral hemorrhage (ICH) represents about 15% of all strokes in the industrialized countries [23] and is associated with a very high mortality and substantial morbidity [2]. Inflammatory mechanisms contribute substantially to cell damage and edema formation caused by cerebral bleeding [28,3,27].

Moreover, interactions between the injured brain and the systemic immune system have come into the focus of research in various fields of neurological diseases [6]. Importantly, a profound immunodepression has been described after extensive clinical and experimental ischemic stroke [18,5,15,16,10]. This immunosuppression-syndrome increases the susceptibility to infections [12] and is associated with a higher mortality and more severe neurological deficits [5]. In contrast, the immune system appears to be rather differentially modulated after smaller infarcts [10] and the risk of infections is low [7].

Despite the recognition of inflammation as a key factor for cerebral damage and neuronal death in ICH models [27], the impact of intracerebral hemorrhage on the systemic immune system is largely unknown. In patients with ICH, the overall peripheral leukocyte count exceeds that of healthy controls and correlates with hematoma size [24,1]. High leukocyte counts have been reported to be independent predictors of early neurologic deterioration in primary ICH [9,22]. Although multiple inflammatory pathways and their detrimental role have been described in hemorrhagic stroke, systemic immunodepression has not been investigated in intracerebral hemorrhage.

The purpose of the present study was to investigate the effects of ICH on various systemic immune cell populations. Specifically, we tested whether hematoma size is a major factor determining the extent of post-hemorrhagic immunomodulation.

The study was conducted in accordance with national and European guidelines for the use of experimental animals, and the protocols were approved by the institutional and governmental committees for animal care (Regierungspraesidium Karlsruhe, Germany). In all experiments, sexually mature male mice (C57BL/6,

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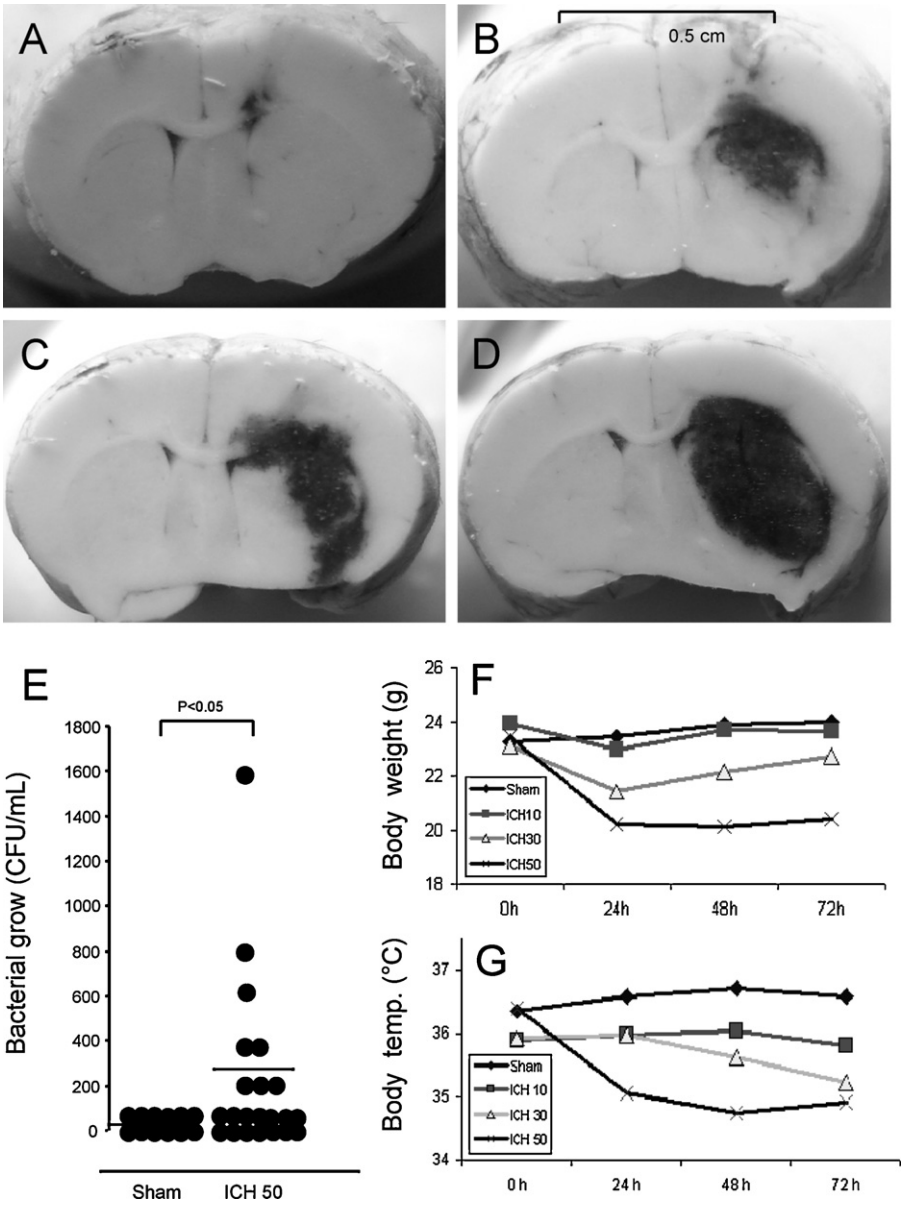

Fig. 1. Representative images of brain sections from similar anatomical regions at 3 days after sham operation (A), 10 μL (B), 30 μL (C) and 50 μL (D) intracerebral blood injection. Number of colony forming units per mL (CFU/mL) in blood and lung homogenates of mice after sham operation and 50 μL ICH induction (E). Mice body weight 24, 48 and 72 h after ICH induction (F). Rectal body temperature of mice 24, 48 and 72 h after ICH induction (G).

Charles River Laboratories, 12–14 weeks of age, body weight 23–27 g, $n = 132$) were used.

Mice were anesthetized with 1.0–2.0% halothane in 30% O_2/N_2O enriched air. ICH was induced by the injection of one of three different blood volumes (10, 30, or 50 μL) into the left striatum using a stereotaxic frame (Model 51650, Stoelting®, USA) with a mouse adaptor (Model 51625, Stoelting®, USA) as described previously [21]. Briefly, after localizing the injection point (3.5 mm deep, 2.0 mm lateral, 0.5 mm anterior to Bregma) 7 μL of autologous blood (from the retro-orbital plexus) was injected into the left striatum. After waiting for 10 min, the remainder of the desired blood volume was injected at a rate of 2 μL/min. thereafter; the cannula was left in place for 10 min before removal. Sham treatment was performed using the same surgical protocol except without injection of blood and leaving the cannula in place for 30 min.

Three days after hemorrhage induction, anesthetized mice were perfused transcardially with saline, and decapitated. Brains were removed and immediately frozen. Later, 40 μm thick coronal cryosections were cut every 400 μm. Unstained sections were scanned at 600 dpi, and the hematoma area was measured using a public domain image analysis software (Scion Image). The total hemorrhage volume was obtained by integrating measured areas and distance between sections.

Three days after hemorrhage induction 0.6 mL of blood was collected by puncture of the femoral vein and transferred into a standard EDTA test tube. Samples were analyzed immediately after collection in the Core Laboratory Facilities of the University Hospital Heidelberg for a complete blood count (ADVIA 120, Siemens Healthcare Diagnostics). In a different set of mice spleen, lymph node and thymus were collected after sacrificing the mouse and processed to a single-cell suspension. After erythrocytes lysis, leukocytes were counted in a Neubauer hemocytometer.

In the same mice, 500 μL of venous blood was drawn into heparinised tubes and leukocytes isolated using a Ficoll-Hypaque TM gradient. Lymphatic organs (spleen, mesenteric lymph nodes and thymus) were collected after transcardial perfusion with hep-

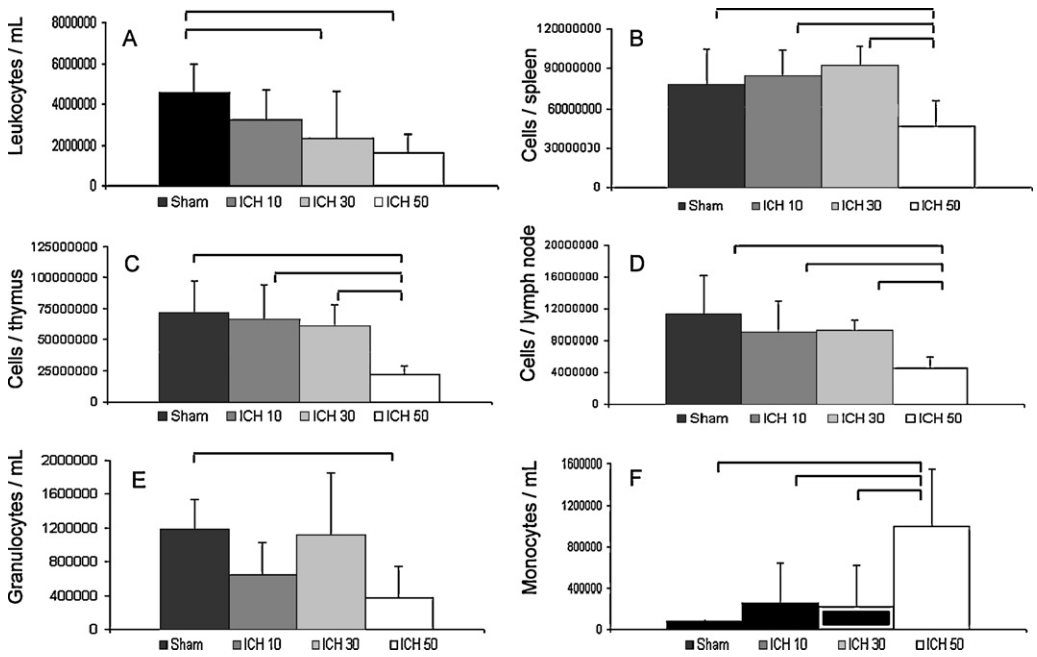

Fig. 2. Total leukocyte counts in blood (A), spleen (B), thymus (C) and mesenteric lymph nodes (D) at 3 days after ICH induction. Blood neutrophil (E) and monocyte counts (F) 3 days after sham surgery or intracerebral injection of 10 μL, 30 μL, or 50 μL blood. $N = 12$ per group, horizontal lines indicate $p < 0.05$ between groups. Results are expressed in cells per mL (blood) or cells per organ (spleen, thymus and lymph nodes).

arinised saline and processed to single-cell suspensions. Cells were stained for Anti-Mouse CD3 (Clone 17A2), CD4 (Clone RM 4-5), CD8 (Clone 53-6.7), CD25 (Clone 7D4), B220 (Clone RA3-6B2), PanNK (Clone DX5) and Foxp3 (Clone FJK-16s) and the appropriate isotype control by following the manufacturer's protocols (eBioscience). Flow cytometry was performed on a Beckton Dickinson FACScan and analyzed by CellQuest Pro software.

In a separate set of animals two treatment groups were compared: Sham ($n = 12$) and 50 μL blood injection ($n = 22$). Sterile blood samples were taken by cardiac puncture after thoracotomy. Lobes of lungs were collected, minced and homogenized under sterile conditions. 100 μL of all specimens was serially diluted in PBS and plated onto blood agar plates (BD) and MacConkey agar plates (Biomerieux). Blood samples were additionally tested for growth of anaerobic bacteria. Samples were taken, directly diluted in Schaedler broth and rapidly plated onto KV agar (BD) and Schaedler agar (Biomerieux). After 24 h and 48 h of incubation at 37 °C agar plates were analyzed for growth of colonies by a technician blinded to treatment groups. Isolates were counted and differentiated further by routine microbiological means.

All values are expressed as mean ± standard deviation (SD). Comparison of mean values was performed by ANOVA for multiple comparisons with post hoc Tukey test using SPSS software. Comparison of ordinal variables was performed by X^2 test. A p value < 0.05 was considered statistically significant.

Mean striatal hemorrhage volume 72 h after stereotactic blood injection was $5.2 \pm 2\,mm^3$ after 10 μL, $15.5 \pm 3\,mm^3$ after 30 μL, and $26.79 \pm 15\,mm^3$ after injecting 50 μL of blood ($p < 0.01$, $n = 12$ per group, Fig. 1A–D). Mean rectal temperature 72 h after blood injection was 36.4 ± 1.5 °C in the sham group, 36.0 ± 1.3 °C in the 10 μL group, 35.3 ± 0.6 °C in the 30 μL group and 34.5 ± 0.7 °C in the 50 μL group ($p < 0.05$, $n = 8$ per group, Fig. 1G). Weight was 24.0 ± 1.6 in the sham group, 23.6 ± 2.9 in the 10 μL group, 22.7 ± 1.9 in the 30 μL group and 20.4 ± 2.2 in the 50 μL group ($p < 0.05$, Fig. 1F). Mortality was 0% (0/12) in the sham group, 0% (0/12) after 10 μL, 14.2% (2/14) after 30 μL and 25% (4/16) after 50 μL blood injection ($p = 0.218$).

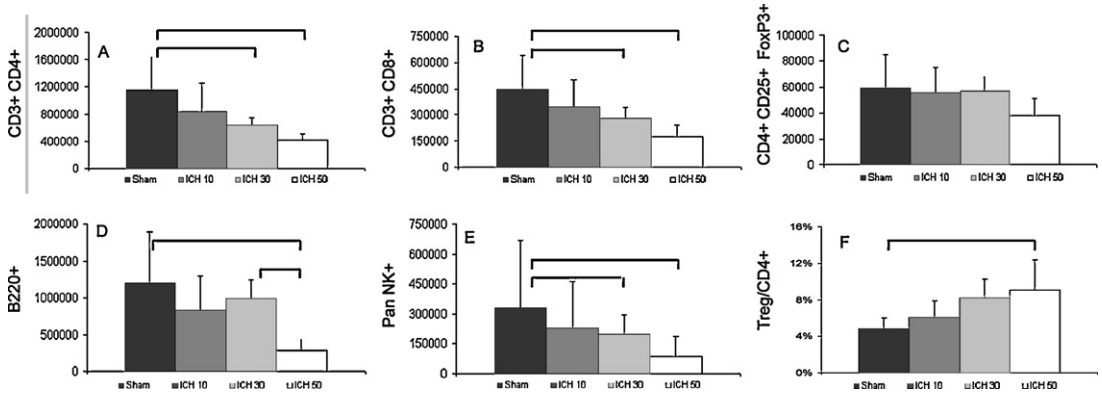

Fig. 3. Flow cytometric analysis of blood lymphocyte subpopulations. T_{helper} cells (A, CD3+CD4+), $T_{cytotoxic}$ cells (B, CD3+CD8+), T_{reg} cells (C, CD4+CD25+FoxP3+), B cells (D, B220+), Natural Killer cells (E, PanNK+), and the percentage of T_{reg} cells within the total CD4+ population (F). $N = 12$ per group, horizontal lines indicate $p < 0.05$ between groups. Results are expressed as cells per mL whole blood.

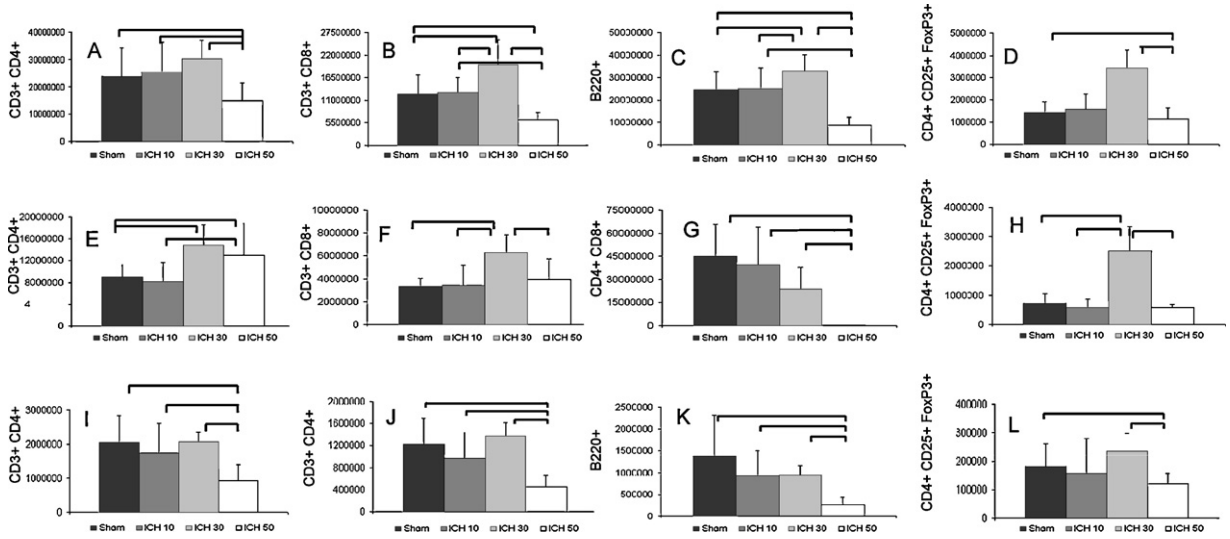

Fig. 4. Flow cytometric analysis of spleen lymphocyte subpopulations, T_{helper} cells (A, CD3+CD4+), $T_{cytotoxic}$ cells (B, CD3+CD8+), B cells (C, B220+) and T_{reg} cells (D, CD4+CD25+FoxP3+). Thymic lymphocyte subpopulations T_{helper} cells (E, CD3+CD4+), $T_{cytotoxic}$ cells (F, CD3+CD8+), T_{helper} cells (G, CD4+CD8+) and T_{reg} cells (H, CD4+CD25+FoxP3+). Lymph node subpopulations, T_{helper} cells (I, CD3+CD4+), $T_{cytotoxic}$ cells (J, CD3+CD8+), B cells (K, B220+) and T_{reg} cells (L, CD4+CD25+FoxP3+).

We further studied the microbiological status of mice 3 days after the operation in sham ($n = 12$) and 50 μL group ($n = 22$). Sham treated mice did not have bacteremia (0/12) or lung infection (0/12) after 48 h of incubation. In contrast, after large ICH, 27.3% (6/22 $p = 0.08$) of mice had bacterial proliferation in the lung homogenates and 22.7% (5/22 $p = 0.11$) in the blood (*Escherichia coli*, *Acinetobacter*, *Aerococcus viridans*) with up to 1500 CFU/mL. The composite incidence of blood and/or lung infection was 36.4% (8/22 $p < 0.05$, Fig. 1E) in the ICH 50 μL group.

Cell counts of leukocytes and their subpopulations in non-operated (WT) mice were within the published range of normal values for C57BL/6 mice [14]. For each group, blood of 12 mice was analyzed for complete blood counts. Leukocyte counts (Fig. 2A, $p = 0.029$) and total lymphocytes (Fig. 3, $p < 0.01$) were significantly reduced 72 h after ICH. In contrast, monocytes were significantly elevated in all groups after ICH and this effect was especially profound after 50 μL blood injection (Fig. 2F, $p < 0.001$).

The detected effects on immune cells in lymphatic organs differed significantly among groups between the ICH models. While sham operation and injection of 10 μL and 30 μL of blood did not significantly affect cell counts in spleen, thymus and lymph nodes, 50 μL blood injection induced a significant decrease of total cell counts in spleen (Fig. 2B, $p < 0.001$), thymus (Fig. 2C, $p < 0.001$) and lymph nodes (Fig. 2D, $p < 0.001$).

We compared absolute numbers of helper T cells (T_{helper}, CD3+CD4+), cytotoxic T cells ($T_{cytotoxic}$, CD3+CD8+), B cells (B220+), regulatory T cells (T_{reg}, CD4+CD25+Foxp3+), Natural Killer cells (PanNK+) and the percentage of regulatory T cells within the CD4+ population in blood, spleen, thymus and lymph node. T and B cells in all organs were significantly reduced ($p < 0.001$) on the third day in all ICH groups (10, 30 and 50 μL blood injection) compared to sham. T_{helper} and $T_{cytotoxic}$ cells were reduced in blood ($p < 0.001$, Fig. 3), spleen ($p < 0.01$, Fig. 4), thymus ($p < 0.001$, Fig. 4) and lymph nodes ($p < 0.001$, Fig. 4). T_{reg} cells were reduced in spleen ($p < 0.001$, Fig. 4) but not in blood ($p = 0.35$, Fig. 3). The proportion of T_{reg} cells within the entire CD4+ cell population was increased in blood ($p < 0.001$, Fig. 3F). B lymphocytes (B220+) and Natural Killer cell counts were reduced in blood ($p < 0.05$, Fig. 3).

To our knowledge, this is the first experimental study examining the impact of intracerebral hemorrhage on systemic immune cells. Our major new findings are that: (1) Extensive intracerebral hemorrhage induces a profound reduction of most systemic immune cells in blood and lymphatic organs whereas smaller hematomas induce a differential response in different immune cell populations. (2) After large hematomas animals are susceptible to infections.

Hematoma size had a major impact on immune system alterations as well as on body temperature and weight. While mice after 50 μL blood injection were substantially impaired, mice undergoing 10 μL had only subtle hypothermia and weight loss. Large ICH blood volumes led to a profound decrease of circulating lymphocytes. In contrast, mice with a moderate hematoma size (ICH30) had even a slightly higher number of splenocytes, thymocytes and lymph node cells. These results are consistent with previous studies in experimental and clinical ischemic stroke in which the infarct size was a major determinant of the degree of lymphopenia [10,7,4]. The thymus as the primary lymphatic organ has a major role in lymphatic homeostasis and de novo T cell proliferation after lymphopenia [8]. Our analysis of T progenitor cells in the thymus reveals that T cell development is impaired in thymi after experimental ICH. Immature CD4+CD8+ cells were massively reduced in the thymus following ICH, while in contrast mature single positive CD4+ and CD8+ thymocytes, respectively, were not altered after large hemorrhagic lesions. This is consistent with previous studies in experimental cerebral ischemia in which CD4+CD8+ cells were hohgly susceptible to postischemic apoptosis [18,10].

In contrast to alterations of blood leukocytes and other lymphocyte subpopulations, the number of T_{reg} cells remained surprisingly less affected after ICH, thereby increasing the proportion of regulatory T cells within the CD4+ population. Interestingly, similar results were observed after brain ischemia [10]. Indeed, T_{reg} have been shown to be important, multi-targeted protective cells that limit neuroinflammatory brain damage after ischemia [11].

In contrast to most other cell populations, monocyte counts in blood were increased after extensive ICH in accordance with previous findings in clinical [19,26] and experimental brain ischemia [20]. The pathophysiological relevance of this finding is currently unclear. On the other hand, monocytes can serve as a prognostic marker for the risk of acquiring infections after ischemic and hemorrhagic stroke [26].

In clinical studies, about 20% of patients suffering from a large intracerebral hematoma die within 30 days after ICH as a consequence of pneumonia or sepsis [1,13]. In previous experimental studies, extensive brain ischemia predisposed mice to bacterial pneumonia and sepsis [17,10] whereas smaller infarcts did not

increase susceptibility to infections. The proportion of animals with large infarcts that developed infections differed among investigators. A potential factor for these discrepancies may be the different bacterial flora of experimental animals. The prevalence of infections found in the large hematoma group in the present study is in accordance with our previous study in mice after extensive cerebral infarction [10]. Beyond immunological factors including ICH-induced lymphopenia the increased susceptibility to infections may also be influenced by a deterioration of the general physical condition after large ischemia or extensive ICH which is reflected in a substantial weight loss and hypothermia.

Following the assumption of hypothalamic involvement after an ICH, a direct brain-immune interaction and lymphopenic immunodepression with atrophy of lymphatic organs in this experimental model might most likely be due to direct cholinergic, anti-inflammatory pathways to the lymphytic organs [25]. Our study confirms this fact with a robust reduction of blood leukocytes, splenocytes, lymph node cells and thymocytes after an ICH (Fig. 2). This issue might be of relevance translational aspects, since a large number of immunomodulatory drugs are meanwhile available that might have a therapeutic benefit in human ICH. Several previous articles discussed the importance of the central nervous system and especially cholinergic pathways in the interaction with the systemic immune response [25,19]. Other reports focused on the role of the hypothalamic–pituitary–adreanal axis and the sympathetic nervous system in immune system modulation after CNS injuries [5,12]. It is feasible to speculate about possible direct brain-immune interactions via humoral mediators or neuronal pathways after ICH, however, further studies need to be undertaken to properly address this issue.

A potential limitation of this study is that we analyzed the effects on the cellular immune system only at one time point after ICH. However based on previous studies in ischemic [10] and hemorrhagic [29] stroke the greatest impact on the systemic immune system after lesion induction has been observed in the time frame investigated in our experiments.

Our study shows that intracerebral hemorrhage has a profound impact on the systemic immune system and that the extent of systemic immunomodulation is determined by hematoma size. Compared to ischemic infarction, relatively smaller hematoma volumes induce more severe immune alterations resulting in bacterial infections.

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