Adenosine A₁ receptors contribute to immune regulation after neonatal hypoxic ischemic brain injury

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Abstract Neonatal brain hypoxic ischemia (HI) often results in long-term motor and cognitive impairments. Post-ischemic inflammation greatly effects outcome and adenosine receptor signaling modulates both HI and immune cell function. Here, we investigated the influence of adenosine A₁ receptor deficiency (A₁R^{-/-}) on key immune cell populations in a neonatal

brain HI model. Ten-day-old mice were subjected to HI. Functional outcome was assessed by open locomotion and beam walking test and infarction size evaluated. Flow cytometry was performed on brain-infiltrating cells, and semi-automated analysis of flow cytometric data was applied. A₁R^{-/-} mice displayed larger infarctions (+33 %, $p<0.05$) and performed worse in beam walking tests (44 % more mistakes, $p<0.05$) than wild-type (WT) mice. Myeloid cell activation after injury was enhanced in A₁R^{-/-} versus WT brains. Activated B lymphocytes expressing IL-10 infiltrated the brain after HI in WT, but were less activated and did not increase in relative frequency in A₁R^{-/-}. Also, A₁R^{-/-} B lymphocytes expressed less IL-10 than their WT counterparts, the A₁R antagonist DPCPX decreased IL-10 expression whereas the A₁R agonist CPA increased it. CD4⁺ T lymphocytes including FoxP3⁺ T regulatory cells, were unaffected by genotype, whereas CD8⁺ T lymphocyte responses were smaller in A₁R^{-/-} mice. Using PCA to characterize the immune profile, we could discriminate the A₁R^{-/-} and WT genotypes as well as sham operated from HI-subjected animals. We conclude that A₁R signaling modulates IL-10 expression by immune cells, influences the activation of these cells in vivo, and affects outcome after HI.

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Introduction

Inflammation greatly affects pathogenesis and outcome after neonatal brain injury [1, 2]. Thus, infections and inflammation may aggravate subsequent hypoxic ischemic (HI) encephalopathy [1]. To study the interplay between key parts of the

immune system, we have developed a flow cytometry-based method to investigate immune populations previously suggested to influence outcome after neonatal HI [3] and demonstrated that brain inflammation is sustained for months after HI with involvement of both innate and adaptive immunity [3].

Adenosine is a known regulator of immunity and has substantial effects on other systems involved in brain homeostasis, including vascular reactivity, temperature control, and cell work [4]. Adenosine typically reduces inflammation by G_s -coupled adenosine A_{2A} and A_{2B} receptor signaling that stimulates formation of cAMP [5]. However, adenosine A_1 and A_3 receptors are coupled to G_i , mediating inhibition of cAMP [5] and thus potentially promoting an opposing effect. A balance between the receptor subtypes dependent on expression pattern and affinity for adenosine is therefore suggested to affect immunological phenotype [4]. It is known that neonatal hypoxic ischemia alters adenosine deaminase levels and A_1 receptor levels [6, 7], but the impact of adenosine A_1 signaling on immune cell function has not been evaluated in the post ischemic neonatal brain.

Notably, caffeine, a competitive antagonist of adenosine A_1 , A_{2A} , and A_{2B} receptors, is used in neonates against apnea of prematurity and has become a candidate for neuroprotection [8]. This amplifies the need for increased knowledge about the impact of adenosine receptor signaling in neonatal brain injury to better understand the effects modulatory therapy may have. Therefore, we examined if adenosine A_1 receptor deficiency in neonatal brain HI alters brain injury and the subsequent immune response.

Materials and methods

Animals and experimental groups

All experiments were approved by the regional ethics committee, Stockholms norra djurförsöksetiska nämnd, in accordance with local institutional guidelines. Animals were randomized to treatment group (sham or HI), and all evaluations were performed by investigators blinded to treatment and genotype.

Adenosine $A_1R^{-/-}$ congenic mice of C57/bl6 background described in detail previously [9] and wild-type (WT) C57/bl6 mice, of either sex, specific pathogen free (SPF) with free access to pelleted food were used. Pups were kept with their mother except during surgery and hypoxia. Animals were euthanized by injection of 240 mg/kg sodium pentobarbiturate i.p. and cardiac perfusion with 12 mL phosphate-buffered saline (PBS) to remove intravascular blood cells.

Hypoxic ischemic brain injury

HI and sham operation was performed at postnatal day 10, $n=47$ and $n=21$ respectively. We used a modified version of the Vannucci model [10] as described previously [11, 12], with unilateral electrocoagulation at 8 W of the right carotid artery via midline neck incision under isoflurane sedation and local bupivacaine infiltration. Pups rested 1 h with the dam after surgery, prior to 1 h of 10 % O_2 in 90 % N_2 at 36 °C skin temperature.

Immunohistochemistry

Brains were collected after HI in WT and $A_1R^{-/-}$ ($n=47$ and $n=21$, respectively) at day 24 after behavioral evaluation. Three levels of each brain were collected, corresponding to bregma 1.32, −1.64, and −2.92 mm in the adult mouse brain using cryostat 10–12- μ m sections. Tissue sections were fixed for 10 min in 4 % paraformaldehyde. Endogenous peroxidase was blocked by 0.3 % H_2O_2 in 3 % Normal Horse Serum for 10 min. Microtubule associated protein-2 (MAP-2) staining VECTOR® M.O.M.™ Immunodetection Kit and VECTASTAIN® Elite ABC-Peroxidase Kit were used according to the manufacturer's specifications. Additional primary antibodies used were B220 (BD Biosciences, 557390; 1:1000) and IL-10 (Abcam, ab9969; 1:1000). The enzymatic coloration of immunoreactivity was performed by simultaneous full immersion in 3,3'-diaminobenzidine, DAB for all slides of the same marker to enable comparison. Imaging was performed with a Nikon eclipse E800 microscope with an Olympus DP70 camera with DP controller and version 3.1.1.267 acquisition software. ImageJ version 1.47t was used to analyze positive staining with color thresholding to select positive staining. IL-10 staining was evaluated for whole brain, whereas co-localization of B220 and IL-10 was investigated in the infarction zone. Percent MAP-2 stained area was manually selected in each hemisphere of brain slice, with investigators blinded to genotype and treatment. Infarction size was measured, and atrophy was calculated as tissue loss excluding the infarction compared to contralateral hemisphere.

Behavioral tests

Behavioral assessments were done at 24 days of age, 2 weeks after HI in WT and $A_1R^{-/-}$ ($n=47$ and $n=21$, respectively). Open field test (Kungsbacka Mät- och Reglerteknik AB, Fjärås, Sweden) was used to investigate explorative behavior during 30 min. The animals were taken directly from their home litters and put in the open field box without further handling. In addition, the beam walking test, where mice walk three times back and forth on a beam (10-mm wide, 600-mm long), was performed directly after the open field test. Mice were handled with care to assure a calm explorative state of

mind in the animal and the number of slips with the hind limbs recorded in a blinded manner when possible.

Telemetry

The Somedic metabolic system (Somedic; Hörby, Sweden) with implantable Minimitters was used to record VCO₂, body temperature, heart rate, simultaneously at 3-min intervals in adult mice WT and A₁R^{-/-} ($n=7$ and $n=4$, respectively). Briefly, the animal was anesthetized with isoflurane, the Minimitter implanted into the peritoneal cavity and the leads sutured in a lead II position across the heart. Analgesia was ensured by local application of 5 % Xylocaine (AstraZeneca, Södertälje, Sweden), and subcutaneous injection of Temgesic (0.3 mg kg⁻¹, Schering-Plough Europe, Brussels, Belgium) after the implantation. Each mouse was allowed at least 7 days of recovery before recording was initiated.

For hypoxic challenge, mice were first allowed to acclimatize in the airtight ventilation chamber with a constant flow of air at 20 °C for 2 h, and after which oxygen concentration was lowered to 10 % and kept constant at this level for 1 h.

FACS analysis

Randomization to sham or HI operation was performed at postnatal day 10 and samples were analyzed at day 17 in sham A₁R^{-/-} ($n=9$) and WT ($n=6$) and in HI A₁R^{-/-} ($n=16$) and WT ($n=3$). For B lymphocyte dynamics in WT, separate samples were analyzed at day 11 ($n=6$), day 12 ($n=7$), day 17 ($n=6$), day 24 ($n=6$), day 100 ($n=7$), and day 200 ($n=3$). The immune activation in WT after HI was equal to that previously reported, and statistical power to detect alterations was calculated from our previous data [3]. Day 17 was chosen to detect both innate and adaptive activation [3]. Gross morphology was assessed with the following scoring system: 1=no injury, 2=observable tissue loss, 3=small plaques, 4=large plaques, and 5=cystic lesions, $n=5$. Cell preparation was performed as described previously [3]. For antibodies used, see Supplemental Table 1. Flow cytometric data was collected on a FACS Aria (BD Biosciences).

Automated gating has been proposed as a solution to inconsistent results of manually analyzed flow cytometry data [13]. Even if there are attempts to standardize lymphocyte gating [14], there is no current standard for tissue resident immune cells since the conformation, ramification, and marker distribution within these cells become substantially altered. Thus, we deployed a data-driven automated gating approach that still allowed us to compare the results with known cell populations described with manual one- or two-dimensional gating in the literature (Supplemental figure II).

Flow cytometry analysis was made using FlowCore package for R [15]. Obvious debris in brain was removed using a polygon gate in forward and side scatter. This filter was

manually set to exclude maximal values outside the cytometer's range and events smaller than the lymphocyte cloud. Thus, all events of a size congruent with intact cells were included, since cell size is substantially more varied in the brain than in the spleen or lymph nodes. A curvfilter was used to filter out immune cell populations, and not a normal distribution filter, as infiltrating populations not necessarily fulfill the assumption of normality. Dead cells were excluded using DAPI stain. Since the brain in an unchallenged state is a fairly immune-privileged site [16], there were often no clear positive populations in controls, why the negative population was selected as reference and cells with higher expression than that were regarded as positive cells. Median fluorescent intensities of the selected populations were used to discriminate the positive and negative populations. The same filter settings were used for each marker in all brain samples. After automated batching, all gate settings were visually confirmed and no manual adjustments were required. 2.5×10^6 [6] events were collected from each brain hemisphere. The temporal activation of B220⁺ B lymphocytes experiments were done separately and analyzed as described previously [3].

To investigate IL-10 production in brain-infiltrating leukocytes after HI ($n=5$), cell suspensions from homogenized brain were prepared as described above. EasySep™ Mouse FITC Positive Selection Kit was used according to the manufacturer's specifications to purify CD45⁺ leukocytes. To stimulate IL-10 production, cells were incubated 5 h in complete RPMI (10 % FCS, 1 % glutamine, 1 % PeSt, 50 μM b-ME) culture medium containing PMA (50 ng/ml, final concentration), ionomycin (500 ng/ml final), LPS (10 mg/ml final), and monensin (2 mM final) [17]. LIVE/DEAD Blue VD (Life Technologies) and Fc-block (1:100) (Biolegend) was used to exclude dead cells and block unspecific binding. Please see Supplemental figure 1 for antibodies used. After staining of surface antigens BD Cytotfix/Cytoperm (BD Biosciences) was used to fixate and permeabilize cells for IL-10 detection. Data was acquired on an LSR FortessaII (BD Biosciences) and analyzed with FlowJo X 10.0.7r2 software.

Splenocyte production of IL-10 was investigated with flow cytometric staining as described above in cells from WT ($n=3$) and A₁R^{-/-} ($n=3$) spleens in the presence or absence of adenosine 66 nM A₁R agonist (CPA), 66 nM A₁R antagonist and two concentrations 100 nM and 20 nM of the pan adenosine agonist NECA compared to PBS. A balanced D-optimal experimental design with 100 % estimated D-efficiency was used and analyzed with linear regression in R version 3.2.2 (2015-08-14) package ggplot2 (1.0.1).

Statistical analysis

Mann-Whitney *U* test was used to analyze data on morphology and behavior data. ANOVA with Neuman-Keuls post hoc test when appropriate, was used to investigate key immune

cell populations previously identified [3]. Chi-square test was used to compare mortality. A principal component analysis (PCA) with the NIPALS algorithm was used when analyzing flow cytometry data. Expectation-maximization (EM) cluster analysis was used to predict genotype and treatment group. Correlation analysis was made with multiple linear regression or linear regression. Statistical calculations were made in StatSoft, Inc. (2011) STATISTICA, version 10 or R.

Results

Adenosine A_1 receptor-deficient mice ($A_1R^{-/-}$) displayed 44 % larger infarctions 1 week after HI (Fig. 1a) than WT controls. There was a nonsignificant trend towards higher mortality during hypoxia in $A_1R^{-/-}$ than WT (22.7 vs. 7.7 %). $A_1R^{-/-}$ had significantly increased numbers of slips in the beam walking test and increased locomotion in the open field test (Fig. 1b, c), which correlated with infarction size with a coefficient of 0.52 ($p < 0.001$) and 0.37 ($p < 0.01$), respectively. Thus, we concluded that $A_1R^{-/-}$ mice suffer from more severe injury after HI.

Temperature and heart rate regulation is affected by adenosine A_1 receptors [18] and reduction of cell work has been reported in many cell types and organ systems [4]. To assess the impact of A_1R deficiency on respiration, temperature, and circulation, we used a telemetry system which recorded temperature, heart rate, and CO_2 production during 1 h normoxia and subsequent hypoxia. During this time, no significant differences in those parameters were observed between WT and $A_1R^{-/-}$ mice (Supplemental figure 1).

Since adenosine modulates local immune responses and inflammation is a risk factor for adverse outcome after HI [1], we hypothesized that the local A_1 receptor-mediated regulation of immune cells may contribute to infarct formation.

Innate immunity

We found a significant increase in the number of CD11b-expressing cells in brains from HI-subjected $A_1R^{-/-}$ mice compared to WT ($p = 0.01$, $F = 6.8$). Neither CD11c⁺ antigen presenting cells nor Ly-6G⁺ granulocyte populations displayed any genotype-dependent changes (data not shown).

Adaptive immunity

B lymphocytes

In order to study the kinetics of B lymphocytes, we first investigated WT mice subjected to HI. We found a rapid and highly significant increase of B220⁺ B lymphocytes infiltrating both the affected and unaffected hemisphere (Fig. 2a). The number returned to baseline 100 days after HI. In parallel, the number of activated B lymphocytes increased as measured by the very early activation antigen, CD69 (Fig. 2b). Notably, the number of CD69-expressing cells significantly increased preferentially in the damaged hemisphere 2 days after HI and the number of activated (CD69⁺) B lymphocytes decreased over time (Fig. 2b).

Next, we compared brains from $A_1R^{-/-}$ and WT mice subjected to HI or sham procedures. Sham-operated $A_1R^{-/-}$ mice displayed significantly increased numbers of brain-infiltrating B220⁺ B lymphocytes compared to sham-operated WT (Fig. 3a). Surprisingly, the relative number of infiltrating B lymphocytes in $A_1R^{-/-}$ mice did not increase as in WT after the HI procedure (Fig. 3a). Upon activation, B lymphocytes upregulate expression of the co-stimulatory molecule CD86, a molecule providing the important signal 2 for T lymphocyte activation [19]. In WT brains, the number of B lymphocytes expressing CD86 increased significantly (Fig. 3b), substantiating the finding of increased CD69⁺ activation (Fig. 2b). In $A_1R^{-/-}$, however, the frequency of CD86⁺ B lymphocytes did

Fig. 1 Morphology and behavioral outcome in $A_1R^{-/-}$ and WT mice after HI brain injury ($n = 21$ and $n = 47$, respectively). **a** $A_1R^{-/-}$ mice had significantly larger infarctions than WT, measured as the area of lost MAP-2 immunohistochemical staining; **b** increased numbers of slips in

beamwalking tests; and **c** increased locomotion in open field tests (*open circles*) compared to WT (*filled squares*). As measured by Mann-Whitney *U* test (**a** and **b**) and ANOVA repeated measurements analysis (**c**), respectively. Bars represent SE and $*p < 0.05$

Fig. 2 Temporal B lymphocyte brain infiltration and activation pattern in WT C57/Bl6 mice after HI. **a** Number of brain-infiltrating B220⁺ B lymphocytes in the infarcted ipsilateral hemisphere (circles, light grey bars) and undamaged contralateral hemisphere (squares, dark grey bars). Two days after HI, a highly significant influx of B lymphocytes

was observed which then declined. **b** The number of activated CD69⁺ B lymphocytes increased 2 days after HI. $n=6$ day 11, $n=7$ day 12, $n=6$ day 17, $n=6$ day 24, $n=7$ day 100, and $n=3$ day 200. Error bars indicate SE. Data analysis performed by manual gating. *** $p<0.001$

not increase after HI (Fig. 3b). In contrast, the increase in MHC class II (ANOVA $p=0.002$ for treatment), presenting extracellular antigens, after HI was similar in A₁R^{-/-} compared to WT controls (Fig. 3c), indicating intact function.

Decreased activation in B lymphocytes might intuitively suggest neuroprotection; however, our findings imply the opposite since A₁R^{-/-} mice acquire larger lesions, and thus, the influx of B lymphocytes into WT brains could represent a protective B lymphocyte subpopulation. Therefore, we investigated IL-10 production in HI brains, a hallmark cytokine characteristic for regulatory B lymphocytes [20]. One and two weeks after HI, the majority

of brain-infiltrating B lymphocytes produced IL-10 (Fig. 4a, e), supporting an anti-inflammatory role of these cells. Up to 97 % of the B lymphocytes expressed IL-10 in brains with high damage score in gross morphology as measured by flow cytometry. The IL-10⁺ percentage was lower in less-injured animals due to background presence of CD19⁺IL-10⁻ cells that appeared unaffected by brain injury. On average, 59 % of CD19⁺ brain-infiltrating cells expressed IL-10 ($n=5$). In addition, the majority of brain-infiltrating CD11c⁺ and Gr-1⁺ cells (up to 94 and 97 %, respectively) produced IL-10 after HI, whereas the corresponding numbers were 14

Fig. 3 Differential B lymphocyte response to brain injury in A₁R^{-/-} mice 7 days after HI. S.C. sham contralateral hemisphere, S.I. sham ipsilateral hemisphere, HI.C. HI contralateral hemisphere, HI.I. HI ipsilateral hemisphere. **a** There was a robust B lymphocyte response in WT animals with increased percentage of live brain-infiltrating cells 1 week after HI. In A₁R^{-/-} mice, B lymphocytes failed to respond to the HI insult, although sham-operated animals had a significantly higher B lymphocyte infiltration than WT controls. **b** The number of CD86⁺ B lymphocytes

increased after HI, however, only in the WT and not in the A₁R^{-/-}. **c** Antigen presenting function of B lymphocytes from A₁R^{-/-} mice appeared similar as the HI-induced change in MHC class II expression was comparable to WT (ANOVA $p=0.002$ for treatment). Error bars indicate SE. A₁R^{-/-} sham-operated group $n=9$, A₁R^{-/-} HI group $n=16$, WT sham-operated group $n=6$, WT HI group $n=3$. * $p<0.05$ and *** $p<0.001$

Fig. 4 IL-10 production in the brain after HI brain injury measured by immunoreactivity. **a** Brain-infiltrating B lymphocytes from A1^{-/-} (displayed) and WT mice produced IL-10 coherent with regulatory B lymphocyte phenotype 2 weeks after HI. B220⁺ B lymphocytes (red), IL-10 (green), and nuclear DAPI staining (blue) 100× magnification. **b** There was significantly less total IL-10 in brains of A1^{-/-} than in WT, respectively. $n=21$ for A1^{-/-} and $n=47$ for WT. Analyzed with Student's *T* test. * $p<0.05$ arbitrary units. Error bars indicate SE. **c** At 20 nM pan adenosine receptor agonist NECA (a concentration similar to physiological adenosine concentrations), IL-10 production decreased in all investigated immune populations. In contrast, at 100 nM NECA

(comparable to brain adenosine concentration during HI), IL-10 levels increased. The presence of the A1¹R antagonist DPCPX disrupted the biphasic response, here exemplified as proportion IL-10⁺ of CD19⁺ B lymphocytes. Box and whisker plots indicate IQR±1.5 IQR. $n=3$ for both genotypes used. **d** Representative flow cytometry data from WT brain of an animal with large infarction after HI show that CD11c⁺ and GR-1⁺ cells produced IL-10 (most of them double positive (CD11c⁺Gr-1⁺)) while most CD11b⁺ cells did not. **e** Similarly, most CD19⁺ B lymphocytes produced IL-10, whereas almost no IL10⁺ TCRβ⁺ T lymphocytes were found

and 1 % of CD11b⁺ and TCRβ⁺ cells, respectively in the animals with the most severe gross morphology (Fig. 4d, e).

When comparing the overall brain hemisphere IL-10 expression levels by densitometric evaluation of immunoreactivity, brains from A1^{-/-} mice expressed significantly lower IL-10 levels as compared to WT (Fig. 4b). To functionally test if A1¹R signaling affects IL-10 production, we stimulated splenocytes from WT and A1^{-/-} animals to produce IL-10 in the presence or absence of A1¹R antagonist DPCPX and A1¹R agonist CPA. Indeed

A1^{-/-} B lymphocytes displayed a significantly decreased proportion IL-10 producing CD19⁺ B lymphocytes, paralleled by a similar, although not significant ($p=0.11$), trend in the presence of the A1¹R antagonist (Fig. 5). Inversely, the A1¹R agonist significantly increased the proportion IL-10⁺ cells not only in B lymphocytes, but in all investigated immune populations (Fig. 5). Furthermore, the unselective adenosine receptor agonist NECA decreased the proportion IL-10⁺ cells at low (20 nM) concentration while there was an increase at high concentration (100 nM). The 20 nM concentration

Fig. 5 IL-10 production in indicated immune cell populations in WT ($n=3$) and in the A₁R^{-/-} ($n=3$) assessed by flow cytometry after stimulation with PMA, ionomycin, and LPS in the presence or absence of A₁R agonist (CPA) or antagonist (DPCPX) and/or the influence of 20 or

100 nM of the pan adenosine receptor agonist NECA with monensin (2 mM) added during the last 5 h of culture. Error bars indicate SE. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

of NECA was selected to represent basal levels and the 100 nM concentration is within the reported concentrations of the endogenous agonist adenosine after HI [21]. Intriguingly, this biphasic pattern in all IL-10⁺ cells was disrupted by antagonism of A₁R indicating that A₁R regulation of IL-10 expression is dependent on agonist concentration (exemplified by CD19⁺ cells, Fig. 4c).

T helper and T regulatory lymphocytes

There were no significant alterations in CD4⁺ T helper cells in the A₁R^{-/-} brains compared to WT (Fig. 6a, b). Since A₁R^{-/-} mice displayed less IL-10, we investigated if regulatory T lymphocytes were affected by A₁R deletion. Brains from

A₁R^{-/-} mice demonstrated no significant alterations in CD4⁺FoxP3⁺ T regulatory cells compared to WT (data not shown). Thus, T regulatory cells cannot explain the altered outcome in the A₁R^{-/-}.

Brain-infiltrating cytotoxic CD8⁺ T lymphocytes (Fig. 6c) and the activation of these cells, as measured by the very early activation antigen CD69 (Fig. 6d), increased significantly in WT animals after HI. To our surprise, the number of CD8⁺ (Fig. 6c) and CD8⁺CD69⁺ (Fig. 6d) T lymphocyte numbers remained unaffected by HI in A₁R^{-/-} brains. In summary, the CD4⁺ T lymphocyte response to HI in A₁R^{-/-} mice was largely unaffected, whereas CD8⁺ T lymphocytes appeared unable to respond to the injury in A₁R^{-/-} mice.

Fig. 6 T lymphocyte response in the brains of A₁R^{-/-} and WT mice after HI. S.C. sham-operated contralateral hemisphere, S.I. sham-operated ipsilateral hemisphere, H.I.C. HI contralateral hemisphere, H.I.I. HI ipsilateral hemisphere. **a, b** The CD4⁺ T helper response to brain HI was unaffected by genotype 1 week after the lesion (ANOVA $p < 0.01$ for treatment). **c** Cytotoxic CD8⁺ T lymphocytes in A₁R^{-/-} brains were

unable to respond to HI in contrast to WT. **d** The number of CD8⁺CD69⁺ brain-infiltrating T lymphocytes was unaffected by HI in the A₁R^{-/-}. Error bars indicate SE. A₁R^{-/-} sham-operated group $n = 9$, A₁R^{-/-} HI group $n = 16$, WT sham-operated group $n = 6$, WT HI group $n = 3$. * $p < 0.05$ and *** $p < 0.001$

Classification of genotype and treatment from immune patterns

We noted distinct patterns of immune cell activation in $A_1R^{-/-}$ and WT. Since many of the factors included in the study are mechanistically entangled, we used a principal component analysis to reduce the dimensionality and discriminate between the main effects, as described previously [22]. Three significant principal components described 69 % of the variance in the damaged and contralateral hemisphere. Under the assumption that the components describes distinct underlying factors, component 1 best described genotype effects and component 3 best described treatment effects since these factors most influenced loadings on each component, respectively (Fig. 7a). Component 2 displayed approximately equal loadings for genotype and treatment. HI and $A_1R^{-/-}$ genotype displayed positive loadings on this component whereas sham and WT had negative. Thus, brain immune activation in $A_1R^{-/-}$ is more similar to HI than WT, which is coherent with our finding of more severe injury in the $A_1R^{-/-}$ animals.

In order to illustrate the capability of the model, we attempted to classify genotype and treatment group belonging (sham or HI) from the immune parameters alone. If the

immune pattern observed was due to random variation, it is extremely unlikely that genotype and treatment groups could be classified from the immune pattern seen. Thus, failure to group the cases into the correct treatment and genotype groups would falsify the hypothesis of a causative relationship. When plotting individual animals against components 1 and 3, it was possible to accurately distinguish $A_1R^{-/-}$ from WT mice (genotype) and treatment (HI or sham; Fig. 7a). To investigate whether these clusters could be mathematically separated, a generalized EM clustering was applied to components 1 and 3. Ninety percent of the cases were classified correctly. Two of the three misclassified cases were HI treated and had negative loading on component 3 similar to sham-operated animals. Not all HI-treated animals obtain brain injury after HI, and those two cases likely have minimal or no damage to the brain. We conclude that the model is adequately able to discriminate between genotype and injury size-induced effects.

Of note, genotype had no influence (loading) on component 3 (Fig. 7a), and therefore, this component was uncorrelated to genotype effects. Instead, variations in injury size within the HI model best describe the variation in component 3, since this is the major source of uncontrolled variation in our dataset. Thus, we exploited this finding and assumed that

Fig. 7 Principal component analysis (PCA) of the main factors affecting overall variability in the data. x- and y-axis represent loadings on each component respectively. **a** When components 1 and 3 were plotted against each other (see “Results” for details), treatment (sham or HI) and genotypes ($A_1R^{-/-}$ to WT) could be discriminated, identifying a specific immune profile for each of the four combinations. Expectation-maximization (EM) clustering could correctly classify genotype and treatment from immune cell profile alone in 90 % of the cases; ovals represent 95 % CI for each cluster. Open black circles are sham-operated $A_1R^{-/-}$, filled red circles HI exposed $A_1R^{-/-}$, open black squares sham-operated WT, and filled red squares are HI exposed WT. **b** To visualize the separate influence of treatment and genotype on major immune populations in the damaged brain, we plotted the two first

components from a consecutive PCA including only cell populations significantly affected by genotype, together representing 66 % of the total variation. As the components are uncorrelated, any line passing through the origin is equally valid as axis, and the black axis (line) from $A_1R^{-/-}$ to WT describes the genotype effect. Immune populations that affects outcome due to deletion of A_1R would be expected to correlate both with genotype and injury, similar to the $\%CD86^+B220^+$ population. CD11b expression is mainly dependent on injury size and $CD8^+$ T lymphocytes preferably dependent on genotype as suggested by the PCA. No variables correlated positively with sham operation. $A_1R^{-/-}$ sham-operated group $n=9$, $A_1R^{-/-}$ HI group $n=16$, WT sham-operated group $n=6$, WT HI group $n=3$

component 3 could be used as an approximation of injury size. Given this assumption, the fraction of CD86 expressing B220⁺ B cells in the brain of A₁R^{-/-} animals after HI correlated negatively to injury ($p=0.03$, $r^2=0.36$). For comparison, CD8⁺ T lymphocytes displayed a nonsignificant trend towards a positive correlation with injury.

To investigate the immune populations in the damaged brain specifically, we performed a second PCA rendering new components (Fig. 7b). To limit noise and increase resolution, we only included immune populations significantly affected by treatment and/or genotype.

The new first two components explained 66 % of the variance. Treatment (sham or HI)-induced effects were related to innate immunity, and genotype effects were related to adaptive immunity such as CD8⁺ T lymphocytes (Fig. 7b), confirmed by multiple regression ($p<0.001$, $b^*=-0.74$ for treatment and $p=0.38$, $b^*=0.11$ for genotype). Activated CD86⁺B220⁺ B lymphocytes were related to both HI and genotype ($p<0.001$, $b^*=-0.55$ for genotype and $p=0.04$, $b^*=0.3$ for treatment).

Together, the PCA and regression analysis indicate that immune activation profile is influenced by the combination of A₁R^{-/-} genotype and HI treatment and not by differences in injury size (between genotypes) alone.

Discussion

The major findings were that A₁ receptor deficiency in neonatal mice is associated with larger brain damage after hypoxic ischemia, worse neuromotor outcome, and altered post-ischemic activation of immune cell populations.

Adenosine A₁ receptors have profound effects on heart rate, temperature [18], and metabolism [4] under certain conditions. It is also well known that temperature markedly affects the impact of an ischemic insult [23]. Therefore, it was possible that the differences in ischemic injury could be related to differences in body temperature. However, there were no significant alterations in temperature, heart rate, and CO₂ production before or during hypoxia in A₁R^{-/-} mice.

Previously, an adult global brain ischemia model showed an increased neuronal loss after treatment with A₁ receptor antagonist but no alteration in brain injury in the A₁R^{-/-} compared to WT mice [24]. In a model of HI in neonatal rats, A₁ receptor antagonism during or after neonatal ischemic brain injury did not affect lesion size compared with controls; however, treatment given before HI increased mortality [25]. The apparent inconsistency may be due to differences between the models [26], species, and maturation stages. A₁ receptors are known to inhibit excitatory neurotransmission and removing this brake can have negative effects in hypoxia [9]. It is not impossible that such an effect could have contributed to the alteration in outcome and that this in turn has influenced the

immune response. However, our statistical analysis strongly suggests that this is not the only explanation and that genotype has a more direct influence on the immune reaction to HI.

There were large differences between genotypes in post-ischemic immune activation. The increase of CD11b⁺ cells was greater in A₁R^{-/-} brains than in WT, and an amplified CD11b⁺ cell response may contribute to the larger injury seen in A₁R^{-/-} mice [27, 28].

Few in vitro studies provide direct evidence that A₁R signaling critically influences macrophage function while in vivo studies do [4]. Therefore, an indirect mechanism involving other cell types is implied, rather than a direct A₁R signaling effect on CD11b⁺ cells.

We and others have recently shown that the adaptive immune system including T lymphocytes is activated long after a brain injury [3, 29]. Of note, mice without mature B and T lymphocytes are protected against brain injury [29]. Reconstitution of B lymphocytes produces a nonsignificant trend towards even less injury [30], whereas reconstitution with CD3⁺ T lymphocytes restored susceptibility to injury [30], confirming previous reports of detrimental effect of T lymphocytes [31, 32]. Since the kinetics of B lymphocyte activation after HI brain injury was previously unknown, we first investigated signs of activation with regards to expression of the very early activation antigen CD69, the co-stimulatory molecule CD86 and MHC class II. In WT brains, we found the expected increase in CD86 and MHC II expressing B lymphocytes after HI. In contrast, CD86 expression in B lymphocytes in A₁R^{-/-} brains did not increase. The low activation was unexpected with regard to the larger brain damage seen in A₁R^{-/-} animals. In addition to their antibody-producing function, B lymphocytes are capable to present antigens and activate T lymphocytes [20]. In our model, this function seemed intact in the A₁R^{-/-} mice, as measured by normal MHC II expression compared to WT. Recently, B lymphocytes have also been shown to harbor suppressor activities [20], a regulatory subset which interestingly has been shown to be of importance in an experimental stroke model [33].

If the impaired B lymphocyte response seen in A₁R^{-/-} brains represented a reduced regulatory B lymphocyte response, this would fit with the increased infarction size observed. Therefore, we co-stained for B lymphocytes and the immunosuppressive cytokine IL-10. Indeed the majority of brain-infiltrating B lymphocytes expressed IL-10, the hallmark effector cytokine of regulatory B lymphocytes [20]. This finding was confirmed by flow cytometry of brain-infiltrating cells where up to 97 % of the B lymphocytes in the damaged hemisphere expressed IL-10. Except B lymphocytes, a large majority of CD11c⁺ and Gr-1⁺ cells in the brain after HI also produced IL-10, whereas only a few percent of CD11b⁺ and TCRβ⁺ cells stained positive, establishing a differential expression of IL-10 between these cell types. In addition, densitometric evaluation of IL-10 revealed that brains

from $A_1R^{-/-}$ mice had overall decreased IL-10 levels after HI. Of note, IL-10 has previously been described as a protective cytokine in the neonatal brain [34]. To address whether the observed effects were due to acute A_1R signaling, IL-10 production was investigated in WT and $A_1R^{-/-}$ in the presence and absence of A_1 agonist and antagonist, as well as selected concentrations of the pan adenosine agonist NECA. Significantly less IL-10⁺ B lymphocytes were found in $A_1R^{-/-}$ compared to WT, paralleled by a similar trend in the presence of the A_1R antagonist DPCPX. The same pattern was observed in T lymphocytes, while CD11b⁺, CD11c⁺, and Gr-1⁺ cells were unaffected by A_1 receptor blockade alone. Interestingly, all investigated populations significantly increased their IL-10 production in the presence of the A_1 agonist CPA and 100 nM concentration of the pan adenosine receptor agonist NECA (a concentration set to mimic the local levels of endogenous adenosine seen during HI). At 20 nM NECA (probably giving a stimulation comparable to adenosine under physiological conditions), the opposite response was observed with significantly decreased IL-10 production in all investigated populations, a significant interaction counteracted by addition of A_1R antagonist DPCPX. Thus, A_1R antagonist-mediated regulation of IL-10 depends on the level of adenosine receptor agonist present.

There was no change in infiltration of CD4⁺ T lymphocytes and Foxp3⁺ regulatory T lymphocytes (data not shown) when comparing WT and $A_1R^{-/-}$ brains. However, in light of the increased brain damage in $A_1R^{-/-}$ mice and the unaffected CD4⁺ T lymphocyte compartment, we were surprised to find the infiltration of CD8⁺ cytotoxic T lymphocytes greatly diminished. Since adenosine A_1R signaling protects against neuronal excitotoxicity [4], damaged cells in $A_1R^{-/-}$ could be shifted towards necrosis rather than delayed apoptosis [35], thus limiting the need for cytotoxic killing of injured cells. Another possibility is that adenosine A_1R -mediated inhibition of chemokine/cytokine function may lead to a dysregulated lymphocyte activation and entry into the brain. The fact that CD86 expression on B cells was negatively correlated with the PCA-derived estimate of injury size even within the $A_1R^{-/-}$ group supports this notion, rather than solely an impaired function of B regulatory cells per se. One potential candidate able to mediate this effect would be the chemokine fractalkine (CX3CL1) that has been reported to be neuroprotective in an A_1R -dependent manner [36, 37]. Interestingly, the fractalkine receptor (CX3CR1) is found on all of the affected immune populations in our study but not in the unaffected populations [38, 39, 40]. Moreover, vascular expression of A_1R has been associated with regulation of cell migration and extravasation of leukocytes [4] which potentially also could contribute to the clinical outcome.

Multicolor flow cytometry is a powerful tool; however, traditional gating methodology is subjected to investigator-dependent bias and increased intra- as well as inter-

experimental variability [41]. Here, we have utilized techniques from screening (semi-automated data-driven analysis) and data mining (PCA), combined with traditional hypothesis-based methodology to enable multifactorial hypothesis testing. The data-driven probabilistic analysis provided an unbiased method of evaluating larger data sets consistently, and principal component analysis enabled us to identify and discriminate between variables explaining treatment modality and genotype and limit the need for more extensive experiments.

Intriguingly, there was less difference in inflammatory cell activation between WT and $A_1R^{-/-}$ after HI than after sham operation. This is in line with earlier work where A_1R signaling was proinflammatory at low adenosine concentrations, but with higher concentrations, as seen after HI, A_2 receptor-mediated anti-inflammatory effects prevailed [42], thus limiting the impact of A_1R deletion. This could at least partly explain why a partial blockade of A_1R with the antagonist DPCPX in vivo had no effect on neonatal brain HI [5, 25]. Theoretically, a rescue of some A_1R function via adenosine A_3 receptor signaling may occur, since A_3 receptors are coupled to G_i as well. However, in no instance has A_1R deletion been associated with an upregulation of adenosine A_3 receptors [43]. Furthermore, a rescue of function would require A_3 receptors expressed in sufficient number in the appropriate locations. This distribution, together with A_1 receptors, is still largely uncharted since it seems to differ between cell type, model system, location, and functional status of the cells, in agreement with the dynamic nature of G protein-coupled receptors [4, 44]. Hence, an A_3R -mediated compensatory effect would be minor, if present at all.

The overall aim of this study was to understand the net immunological effect of A_1 receptor deletion, rather than to investigate single immune populations, although this may be a challenge considering the intricacy and coupled interactions of adenosine signaling and immunity. To assess all the underlying factors of the immunological phenotype in our model, we applied PCA, which allows discrimination of factors with covariates. In the setting of our model, we suggest that the current approach using PCA is a new, valuable, and cost-effective method in order to separate experimentally entangled but uncorrelated factors, such as genotype, lesion size, and immune activation.

The PCA suggested that treatment (sham or HI) was correlated to innate immune cells such as CD11b-expressing cells which is in agreement with the previous literature [45]. Genotype correlated better with post-ischemic adaptive immunity, including B and cytotoxic T lymphocytes suggesting that A_1R signaling has a greater impact on these populations in our model.

The correlation of diminished B lymphocyte activation and infiltration to greater brain damage in $A_1R^{-/-}$ mice was surprising. However, most brain-infiltrating B lymphocytes

express IL-10 and $A_1R^{-/-}$ animals displayed decreased levels of IL-10 in the brain and have impaired regulatory B lymphocyte function. IL-10 can diminish macrophage activation [46] and may thus represent a link between the dysfunctional regulatory B lymphocytes and the increased activation of CD11b⁺ myeloid cells seen in $A_1R^{-/-}$ mice after HI.

Conclusion

A_1R -deficient mice displayed worse outcome and an altered immune response after neonatal brain HI. We present evidence of A_1R -mediated dysfunction of regulatory B lymphocytes and indications of impaired cytotoxic T lymphocyte function in $A_1R^{-/-}$. We postulate that the loss in inhibition contributes to the exaggerated activation of myeloid cells and that this in turn leads to increased lesion size and adverse behavioral function.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no competing interests.

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