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Faculty of Science

Role of adenosine deaminase in regulation of energy during bacterial infection in adult *Drosophila melanogaster*

Bachelor thesis

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Annotation

The expression of adenosine deaminase related growth factor A (ADGF-A) during immune response was studied in Drosophila melanogaster. The effect of increased extracellular adenosine signaling due to hemocytes specific ADGF-A knockdown was investigated by subjecting the flies to the chill coma experiments.

Declaration

I hereby declare that I have worked on my bachelor thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Zoja Lakovič

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I am grateful to my friends and family who always support me.

Abstract

Adenosine is well known for its signaling and regulatory role during immune response. Energy invested into the fight with the pathogen has to be tightly regulated since wasting of the energy reserves limits ability of individuals to survive other challenges and reproduce. Adenosine signaling is limited by extracellular adenosine metabolizing enzyme called adenosine deaminase. Adenosine deaminase related growth factor A (ADGF-A), an active adenosine deaminase in *Drosophila melanogaster*, expression was measured in infected flies and specifically in infected flies immune cells – hemocytes. In this work it was shown that ADGF-A is expressed mainly in hemocytes. Then the ADGF-A expression in hemocytes was silenced using RNAi. These flies were subjected to chill coma and their recovery and survival was compared to the wild type fly. Infection prolonged the time of recovery and resulted in lower survival rate of the wild type fly. The flies with lowered ADGF-A woke up on average significantly earlier than control, however ADGF-A downregulation did not affect their survival rate. Results indicate the selfish behavior of immune system that prioritizes its energy needs during immune response and that the stronger adenosine signaling can be beneficial for infected flies on the short run.

Abbreviations

ADA – adenosine deaminase

ADGF – adenosine deaminase related growth factor

Ado – adenosine

eAdo – extracelular adenosine

AdoR – adenosine receptor

cAMP – cyclic adenosine monophosphate

CCR – chill coma recovery

ATP – adenosine triphosphate

cDNA – complementary DNA

dsRNA – double-stranded RNA

FACS – fluorescence activated cell sorting

GAL4 – yeast protein, transcription activator

Hml – hemolectin

hpi – hours post infection

RISC – RNA-induced silencing complex

RNAi – ribonucleic acid interference

Rp49 – ribosomal protein L32

RT-qPCR – reverse transcription real time quantitative polymerase chain reaction

Sp – Streptococcus Pneumoniae

UAS – upstream activation sequence

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1 Introduction

Mounting the immune response requires high amount of energy that is available fast, therefore efficient regulation is needed (R. H. Straub et al., 2010). Adenosine signaling was shown to be the crucial regulator of energy release and metabolism in *Drosophila melanogaster* larval infection by parasitic wasp *Leptopilina boulardii* model (Bajgar et al., 2015). The goal of this thesis was to investigate if there is an involvement of adenosine signaling pathways in model of bacterial infection of adult flies as well. Until now only the effect of downregulating the adenosine signal (by adenosine receptor mutation or equilibrative nucleoside transporter down-regulation by RNA interference) during infection were studied (Bajgar et al., 2015). Now we wanted to see the effect that the increased adenosine signaling would have on the infected flies. In order to do that flies heterozygous for adenosine deaminase related growth factor A (ADGF-A) mutation were used, since homozygous mutation turned out to be mostly lethal in larval stage (Dolezal et al., 2005; Novakova & Dolezal, 2011). It was proven before that ADGF-A is expressed mainly in lymph gland (Zurovec et al., 2002), which is a hematopoietic organ of *Drosophila*, therefore tissue specific downregulation of ADGF-A was applied in hemocytes and its effects on the fly during the infection were studied.

1.1 Adenosine

Adenosine consists of a purine molecule adenine linked to the ribose sugar molecule via β-N_9-glycosidic bond. It is one of the main building block of nucleic acids and metabolite of energy storage molecule ATP. Adenosine is present inside the cells (intracellular adenosine) as well as in the extracellular matrix (extracellular adenosine – eAdo). It has several regulatory functions in the body, such as inducing sleep (Porkka-Heiskanen et al., 2002), protecting tissues by increasing oxygen supply/demand ratio, inhibiting inflammation and repairing the tissues by stimulating angiogenesis (Linden, 2005). Adenosine signaling through adenosine receptors has been shown to have an important role in regulation of immune response in humans as well as in *Drosophila melanogaster* larvae (Antonioli et al., 2018; Bajgar et al., 2015).

1.1.1 Adenosine signaling and metabolism

Signaling role of extracellular adenosine was first observed in 1929 by Drury and Szent-Györgyi who found out that some adenine compound in cardiac tissue extract slowed down the heart rate when injected in animal (Drury & Szent-Györgyi, 1929). Later this molecule was proven to be adenosine and has since been used for treating supraventricular tachycardia (DiMarco et al., 1985; Koeppen et al., 2009). Nowadays it is well known for its role in regulating immune system. It works as an immunosuppressant, protecting tissues from the damage that could occur due to uncontrolled inflammation (Haskó & Cronstein, 2004).

Usual concentration of adenosine in extracellular space is very low, in range of 30-200 nM, however it increases to micro molar levels as a response to physiological stress such as such as hypoxia, ischemia, inflammation or trauma (Antonioli et al., 2013; Bertil B. Fredholm, 2010).

The source of eAdo can either be extracellular ATP, which is converted to adenosine by a cascade of membrane-bound ecto-enzymes or it can be formed intracellularly and exported (B. B. Fredholm et al., 2011). In the cells ATP and methionine are reacted by S-adenosylmethionine synthetase into S-Adenosyl methionine (Cantoni, 1952) which is then demethylated into S-adenosyl homocysteine. Adenosine is formed from S-adenosyl homocysteine by S-adenosyl homocysteine hydrolase (Haskó et al., 2008). Concentration of ATP in the cells is four to five times higher as outside (B. B. Fredholm et al., 2011) so in case that the cell membrane is damaged, ATP leaks into extracellular space where it is converted by ecto-nucleoside triphosphate diphosphohydrolase to AMP which is then further catabolized into adenosine by ecto-5´-nucleotidase. Besides cell lysis ATP is transported from the cell via channels or exocytosis (Antonioli et al., 2013).

Adenosine can be transported through cell membranes along its concentration gradient via equilibrative nucleoside transporters (ENTs), or concentrative nucleoside transporters (CNTs), which carry it against its concentration gradient, using the sodium ion gradient as the energy source to drive the transport. Once in the extracellular space it can transfer the signal to neighboring cells by binding to the adenosine receptors (Antonioli et al., 2013).

1.1.2 Adenosine receptors

Signaling of eAdo mainly proceeds by binding to the G-protein coupled membrane receptors on the surrounding cells. In vertebrates four different adenosine receptors have been classified and are denoted as A_1, A_{2A}, A_{2B}, and A_3 (B. B. Fredholm et al., 2011). A_1, A_{2A}, and A_3 receptors can be activated by usual concentrations of eAdo, while concentration over 10 µM, which occurs only under pathophysiological conditions, is necessary for A_{2B} activation (B. B. Fredholm, 2007; Bertil B. Fredholm et al., 2001; Haskó et al., 2008).

A_1 and A_3 receptors are coupled to G_i protein subunits, meaning that they work by inhibiting adenylyl cyclase, enzyme that is responsible for catalyzing the cyclic adenosine monophosphate (cAMP) production from ATP (Cronstein & Haskó, 2013). Signaling through A_{2A} and A_{2B} on the other hand increases cAMP levels in the cell since they are coupled to G_s subunit, which stimulates the activity of adenylyl cyclase and in turn increases cAMP production. A_{2B} and A_3 also contain G_q subunit which activates phospholipase C (Antonioli et al., 2013).

Concentration of eAdo and thus its signaling role is kept regulated by extracellular enzymes adenosine deaminases or by its uptake into cells via nucleoside transporter. In the cell it is then either phosphorylated to AMP by kinase enzyme or deaminated (Antonioli et al., 2014).

1.1.3 Adenosine deaminase (ADA)

Adenosine deaminase is an enzyme that catalyzes the irreversible conversion of adenosine or deoxyadenosine to inosine and deoxyinosine, by substituting the amino group for a keto group. It can be found in cytosol of cells or as a membrane bound ecto-enzyme (Cristalli et al., 2001).

Two groups of ADA are found in animals, ADA1 and ADA2. Adenosine deaminase plays an important role in human immune system. The deficiency of ADA1 is known to be the cause of 15-20% of cases of SCID (severe combined immune deficiency) disease in humans, which if not treated usually leads to death of patients in first two years (Whitmore & Gaspar,

2016). It mostly affects the development, viability, and function of lymphocytes (Hershfield, 2006).

ADA2 is coded in humans by CERC1 genes, which are a member of adenosine deaminase related growth factors (ADGF) family of genes (Maier et al., 2005; Zavialov & Engström, 2005). ADGFs are very conserved group of genes found in almost all organisms (Zavialov & Engström, 2005). In *Drosophila melanogaster* there are six homologs of human CERC1 genes – ADGF-A, ADGF-B, ADGF-A2, ADGF-C, ADGF-D, and ADGF-E. ADGF-A and ADGF-D are active adenosine deaminases and have shown mitogenic activity by depleting the level of eAdo (Zurovec et al., 2002). ADGF-A is expressed mainly in gut and lymph glands of *Drosophila* larvae (Dolezal et al., 2005; Zurovec et al., 2002). In this work the ADGF-A expression was studied during immune response in adult *Drosophila melanogaster* fly.

1.2 Drosophila melanogaster as a model organism

About a 100 years ago Thomas Hunt Morgan studied extensively the genetics of *Drosophila melanogaster*, commonly known as a fruit fly, and his discoveries in that field have led to *Drosophila* becoming the model organism in research of genetics, medicine, neuroscience and many other fields (Stephenson & Metcalfe, 2013). There are many reasons which make fruit fly so useful for the research. Most of the fly cell biology fundamentals have been conserved evolutionarily (Cauchi & van den Heuvel, 2006). It has been shown that as many as 77% of human disease genes have *Drosophila* homologue (Reiter et al., 2001). The discovery of Gal4/UAS system (Brand & Perrimon, 1993) and possibility to use RNAi makes tissue specific regulation of gene expression in fly easy. Another advantage of using *Drosophila* is its short reproduction cycle and relatively low costs comparing to the mouse model (Stephenson & Metcalfe, 2013). In order to study the immune response in *Drosophila* it is necessary to trigger it by infection. Many different bacteria, fungi and parasites that attack *Drosophila* also in the wild can be used, for example parasitic wasp (*Leptopilina boulardi), Listeria monocytogenes* (Neyen et al., 2014). Here in this work Gal4/UAS system was combined with RNAi to downregulate the expression of ADGF-A (increase the signaling of eAdo) and infection with

Streptococcus pneumoniae was used to trigger the immune response, then the flies were submitted to chill coma experiments.

1.2.1 Chill coma recovery

Under critically low temperature insect enter a state where all movement ceases, called chill coma, and it is possible for them to fully recover from that state. The recovery depends on temperature and duration of chill coma (Findsen et al., 2014) as well as on the energy stores and the age of the animal. Since flies need to be fit in order to recover from stress such as chill coma, chill coma assays can be used to evaluate fly fitness and it is a popular tool in aging research (Linderman et al., 2012). In this thesis it was used to evaluate the effect of increased eAdo signaling on fly performance under infection.

1.2.2 Gal4/UAS

Gal4/UAS is a system that is very useful for gene regulation in research organisms such as *Drosophila*. It consists of two parts, Gal4, which is a yeast protein an acts as a transcription activator, and UAS – upstream activated sequence. The targeted gene is inserted downstream of UAS and can only be turned on after Gal4 binds to UAS. Therefore the targeted gene can be separated from its transcriptional activator in two distinct transgenic lines, one line carrying the Gal4 gene (driver line) and the other one UAS and the gene of interest (responder line). Gal4 has no endogenous target in *Drosophila*, thus it can be expressed in the driver line without any effect. Only after crossing the two lines it can bind to the UAS and activate the targeted gene expression (Brand & Perrimon, 1993). Gal4/UAS allows the spatial and temporal control of gene expression. Temporal control can be achieved by using temperature-sensitive Gal80 ($Gal80^{Ts}$) protein which binds to Gal4 and inhibits its activity when the temperature is below 29°C. If the temperature is raised above that $Gal80^{Ts}$ losses it suppressive function (Martín & Alcorta, 2017). Using the tissue specific promoter for Gal4 enables to turn on the desired gene only in specific tissue. One of the possibilities to silence the gene expression is to combine Gal4/UAS system with RNAi.

1.2.3 RNAi

RNA interference (RNAi) is a process used by many organisms to block gene expression post-transcriptionally by degradation of mRNA. Firstly the double stranded RNA (dsRNA) binds to Dicer, ribonuclease protein, which cuts it into smaller fragments that are mostly 21 base pairs long. These small RNAs are called small interfering RNAs (siRNA). Antisense strand of siRNA then forms ribonucleoprotein complex called RNA-induced silencing complex (RISC) and guides it to the complementary mRNA strand. Once bound, RISC catalyzes cleavage of mRNA which is then rapidly degraded (Bernstein et al., 2001; Hammond et al., 2000; Yamamoto-Hino & Goto, 2013).

In this thesis Gal80^{Ts}Gal4/UAS RNAi construct with hemolectin (Hml) as tissue specific promoter was used in order to silence the ADGF-A gene expression specifically in immune cells where hemolectin is produced. Using the temperature-sensitive Gal80 made it possible to postpone the gene knockdown until the desired time.

1.3 Extracellular adenosine role in *Drosophila melanogaster*

Studying flies with ADGF-A gene mutations provided many interesting insights of eAdo signaling and role in *Drosophila* (Bajgar et al., 2015; Dolezal et al., 2005; Zuberova et al., 2010).

It was shown that the mutant larvae with decreased ADGF-A activity had increased concentration of adenosine and deoxyadenosine in the larval hemolymph, which lead to disintegration of fat body, the abnormal development of hemocytes (causing melanotic tumors), delaying of the development, and, in the end, death of the larvae or pupae (Dolezal et al., 2005).

The further study of Zuberova et al. (2010) revealed that the larvae death is caused by change in metabolism of carbohydrates and that eAdo is working as an anti-insulin hormone. Increased eAdo concentration caused released of glucose from the stores and decreased the accumulation of dietary glucose into glycogen and trehalose storage and thus increased the hemolymph glucose.

That further led to the research of eAdo role on the energy relocation during infection. Since immune response is energetically costly, energy allocation to immune system is needed. Bajgar *et. al.* (2015) investigated the role of eAdo in this metabolic switch in fruit fly larvae infected with parasitic wasp. While normally developmental growth is delayed in infected larvae and glucose consumption by immune cells is increased, this was not observed in the flies with the mutation of fly adenosine receptor, AdoR, showing that the eAdo signaling is indeed needed for the metabolic changes, and the lack of it decreases the resistance to the infection. Adenosine as well causes energy storage suppression, however through some AdoR independent signaling pathway. Knocking down the adenosine transported ENT2 in immune cells showed similar effect as blocking the AdoR signaling, indicating that immune cells are the source of eAdo during infection and thus the way immune system signals the relocation of energy to itself (Bajgar et al., 2015).

Such behavior of immune system has been called *selfish immune system* (Dolezal, 2015; Rainer H Straub, 2014). During the infection immune system becomes privileged in a way that its needs for energy are more important than energy needs of other parts of organism. This is similar to selfish brain theory, claiming that the brain when allocating the energy, puts the priority in covering its own needs before the rest of organism (Peters et al., 2004). Since adenosine is produced when the energy source ATP is decreased and it suppresses energy consuming processes it seems like a possible source of this selfish signal (Dolezal, 2015).

In this thesis the effect of adenosine deaminase silencing specifically in immune cells was studied in hope that it would lead to better understanding of the connection of adenosine signaling to the selfish response of the immune system during the infection.

2 Aims

- Characterize expression of adenosine deaminase in the flies in response to infection of *Streptococcus pneumoniae*.
- Characterize expression of adenosine deaminase in the adult fly hemocytes in response to infection of *Streptococcus pneumoniae*.
- Examine the efficiency of RNA interference treatment in hemocytes using the RNAi/GAL4/UAS system.
- Characterization of adenosine deaminase hemocyte specific down-regulation on ability to recover from chill coma.

3 Materials and methods

3.1 Fly stocks

Flies were grown in glass vials sealed with a cotton plug, on a standard diet of mixture of cornmeal (8%), yeast (4%), agar (1%), and saccharose (5%). The vials were kept at 25 °C and, 65 to 70% moisture in incubator, under 12 h light, 12h dark cycle. ADGF-A RNAixHmlGal4 flies were kept at 18 °C and then transferred 24 hours before infection to 29 °C in order to trigger RNAi production in hemocytes.

Three different strains of *Drosophila melanogaster* were used in the experiments:

1. **w^{1118}** – fly that resembles wild type except for a mutation in the *White* gene, was used as a control and as a genetic background
2. **ADGF-A /+ (Karel x W)** – fly heterozygous for ADGF-A null mutation (Dolezal et al., 2003), it was used to lower the ADGF-A in whole fly (only one allele is present in these flies)
3. **ADGF-A RNAi x HmlGal4 $Gal80^{Ts}$** – GAL4/UAS system (Brand & Perrimon, 1993) was applied using GAL4 driver specific to hemocytes in order to achieve RNAi transcription and thus reduce ADGF-A specifically in hemocytes
4. **Hml Gal4>UAS GFP** – fly strain producing green fluorescent protein in immune cells which enable us to sort the cells out from the suspension of the other cells.

3.2 Infection

In order to trigger immune response the flies were infected with *Streptococcus pneumoniae*. Seven to ten days old male flies (300 per genotype) were anaesthetized with CO_2 Flowbuddy Flow Regulator (Genesee Scientific, 7l/min) and injected with 50 nL of PBS buffer containing 20 000 bacteria, using Eppendorf Femtojet Microinjector. Flies used for control were injected with 50 nL of PBS buffer only. After infection the flies were kept in polystyrene vials at 29 °C. Every three hours three flies of each type were collected and frozen in and then used later for RNA extraction.

3.3 Hemocytes isolation

Fluorescence-activated cell sorting (FACS) method was used to isolate hemocytes. 200 flies per sample were divided in three microtubes and homogenized in 200 µL of PBS. Then the homogenate was sieved through nylon cell strainer (⌀ 40 µm) 200 µL of PBS, which was used to clean the microtubes, were added. Subsequently, samples were centrifuged for 3 min at 6 °C, 3500 rpm, Supernatant was discarded, pellet was resuspended in 1 mL of PBS and the whole procedure was repeated twice more. Samples were transferred into polystyrene vials and sorted based on endogenously produced GFP under the Hemolectin Gal4 promoter, using S3^{TM} cell sorter (BioRad). Cells were sorted into 100 µL of TRIzol Reagent (Ambion) and then the RNA was isolated.

3.4 RNA isolation

Whole flies or the hemocytes were transferred in Eppendorf microtubes and homogenized with the pestle in 200 µL of TRIzol Reagent (Ambion). Subsequently 800 µL of TRIzol Reagent were added to each tube and solutions were incubated for 5 minutes at room temperature. Afterwards 200 µL of chloroform were added, the solution containing tube was vortexed for at least 20 seconds and incubated for 3 minutes. Then it was centrifuged at 4 °C for 15 minutes at 14 000 RPM. The upper aqueous phase containing RNA was transferred to a new tube, 500 µL of isopropanol were added to it and then it was kept on ice for 10 minutes. Afterwards the samples were centrifuged again (10 min, 4 °C, 14 000 RPM), supernatant was discarded and 500 µL of 75% RNAse free ethanol were used to wash the sample. After centrifuging at 4 °C, 14 000 RPM for 1 minute the ethanol was discarded, RNA sample was let to air dry and in the end dissolved in DEPC-treated water.

3.5 Reverse transcription

4 µL of each sample of previously isolated RNA were mixed with 1 µL of oligo-dT Primer (5´-d(TTTTTTTTTTTTTTTTTTTT)-3´), 4 µL of dNTP, and 5 µL of dH_2O. Tubes with the mixture were then transferred to the Eppendorf's MiniSpin and centrifuged shortly, then they were kept in thermoblock at 65 °C for 5 minutes and then put on ice for 1 minute. This was followed by the addition of 4 µL of 5X SSIII buffer, 1 µL of DTT, and 1 µL of SS III reverse transcriptase into each tube. Samples were again transferred to thermoblock and incubated first for 60 minutes at 50 °C and then for 15 minutes at 75 °C.

3.6 RT qPCR

Real time quantitative PCR measurement was performed using cDNA samples that were obtained by reverse transcription of mRNA. Samples were first diluted with 250 µL of dH_2O. 3 µL of each sample were pipetted into 96-well plate and then 2.5 µL of PCR Ultra H_2O (TopBio), 0.25 µL of forward primer, 0.25 µL of reverse primer, and 6 µL of SYBR GREEN Supermix (TopBio) were added to each well. PCR was measured according to the protocol found in *Table 1*. Ribosomal protein 49 (Rp49) gene expression was measured and used to standardize gene expression of AGFA-A.

Following primers were used:

ADGF-A	Forward	5'ATGTCATATAGCGTGGGAAC3'
	Reverse	5'ATGTGCGAGCCAAATACGG3'
Rp49	Forward	5′AAGCTGTCGCACAAATGGCG3′
	Reverse	5′GCACGTTGTGCACCAGGAAC3′

Table 1: Temperature program of PCR. After initials denaturation for 3 minutes the cycle of denaturation, annealing and elongation was repeated 40 times.

94 °C	3 min	Denaturation	1 cycle
94 °C 56 °C 72 °C	15 sec 30 sec 35 sec	Denaturation Annealing Elongation Fluorescence detection	40 cycles

3.7 Chill-Coma recovery

The method for the analysis of chill coma recovery described in the literature (Jean David et al., 1998) was followed. The infected and buffer injected flies were taken 24 hours after injection and placed in clean vials. The vials were closed and put on ice in order to avoid the use of CO_2 for anesthesia and prevent the possible effects it could have on the recovery time and survival of the flies. Aluminum foil was placed on ice and once the flies stopped moving they were put on the foil and from there transferred one by one with tweezers to the 96-well plate. Afterwards the wells were closed with a lid and the plate was covered with ice and left for 16 hours at 8 °C. Then the plate was put on white paper at room temperature and time of recovery was noted for each fly. The time when the fly stood up on her legs was considered as a moment of recovery. After 150 minutes all the flies that have not moved yet were counted as dead.

3.8 Analysis of results - statistical methods and software

The data was processed using GraphPad Prism version 7.04. For statistical evaluation of qPCR results two-way ANOVA with Tukey's multiple comparison test was used. Data obtained from chill coma experiments was evaluated using one-way ANOVA and Tukey's and Dunnett's post-hoc test for multiple comparison.

4 Results

4.1 Adenosine deaminase expression level is increasing in response to bacterial infection in whole flies

Firstly, it was investigated how the expression of ADGF-A in whole flies is changing in response to infection with *Streptococcus pneumoniae (Sp)*. ADGF-A mRNA was measured in w^{1118} fly in three hours intervals after *Sp* infection. The qPCR results can be seen in Figure 1. The data clearly shows that ADGF-A mRNA increased significantly in response to infection. From 9 hpi its expression is significantly higher in infected flies comparing to the control. This result suggest that during immune response more ADGF-A protein and its enzymatic activity is needed.

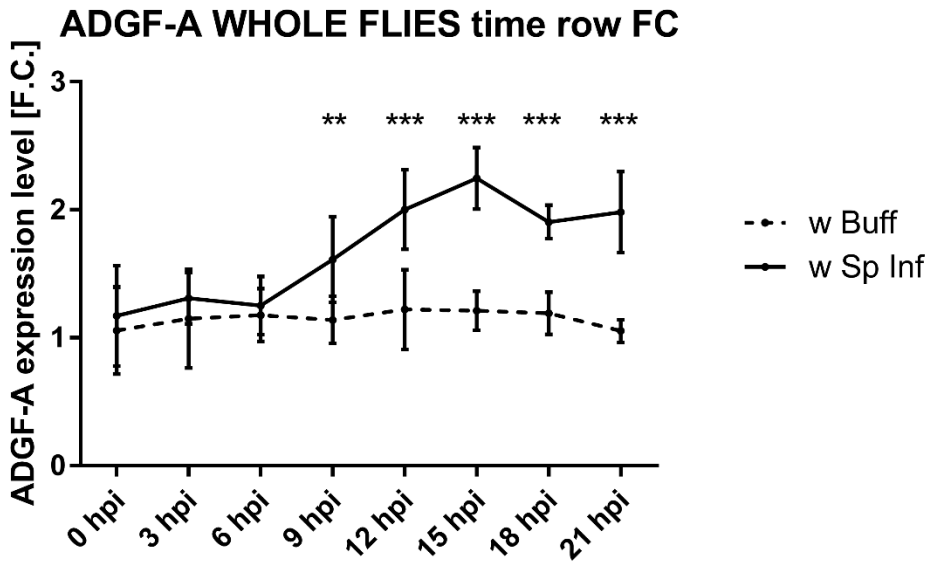

Figure 1: ADGF-A expression in w^{1118} flies after infection with *Streptococcus pneumoniae*, measured every three hours post infection. The lines show fold change of ADGF-A expression in comparing to the expression in uninfected flies hemocytes at 0 hpi, dashed line representing buffer injected control flies and solid line the infected flies. SEM are represented by the error bars and significant differences were tested by Two way ANOVA, Post Hoc comparison by Tukey's test, and are marked with asterisk (* represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001).

4.2 Adenosine deaminase expression level is increasing in response to bacterial infection particularly in immune cells

After proving that ADGF-A expression increases after infection in whole fly, it was of interest to investigate further which cells specifically are responsible for that. Hemocytes were isolated and ADGF-A mRNA amount was measured specifically in them.

Figure 2 shows that the ADGF-A is increased in response to infection directly in isolated immune cells. The increase starts at 3 hpi, which is earlier comparing to the data from the whole animal, probably because before it was masked by additional tissues.

Comparison between whole fly and hemocytes only expression is depicted in Figure 3. It can be seen that ADGF-A expression is higher in hemocytes of control as well as infected flies comparing to the expression in the whole fly. In uninfected fly hemocytes ADGF-A expression is on average 10-fold higher as in whole fly, suggesting hemocytes are its main producers. In infected fly hemocytes increase is even higher, at 15 hpi increase as big as 43-fold can be observed (comparing to the uninfected whole fly expression at 0 hpi).

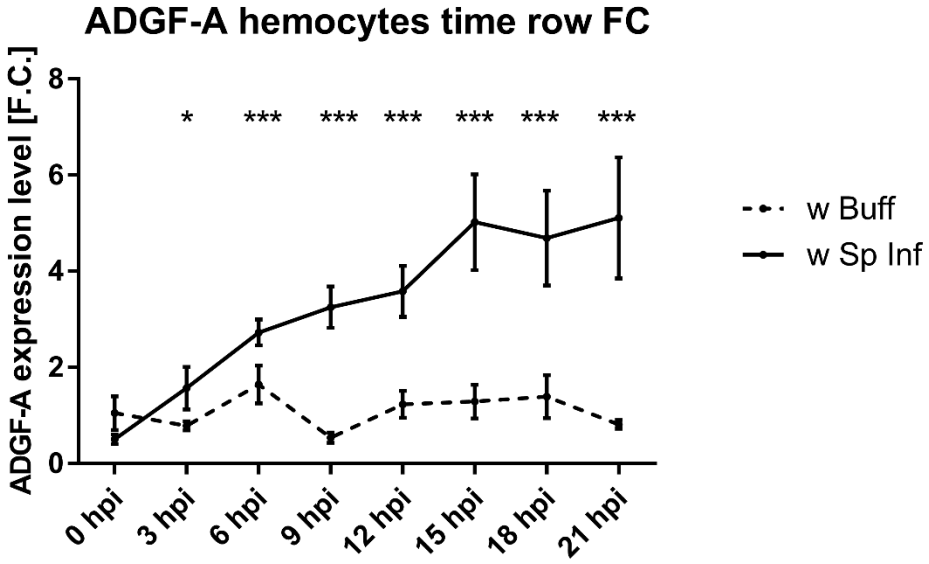

Figure 2: ADGF-A expression in hemocytes of w^{1118} flies after infection with *Streptococcus pneumoniae*. The lines show fold change of ADGF-A expression in hemocytes comparing to the expression in uninfected w^{1118} flies hemocytes at 0 hpi, dashed line representing buffer injected control flies and solid line the infected flies. SEM are represented by the error bars and

significant differences were tested by Two way ANOVA, Post Hoc comparison by Tukey's test, and are marked with asterisk (* represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001).

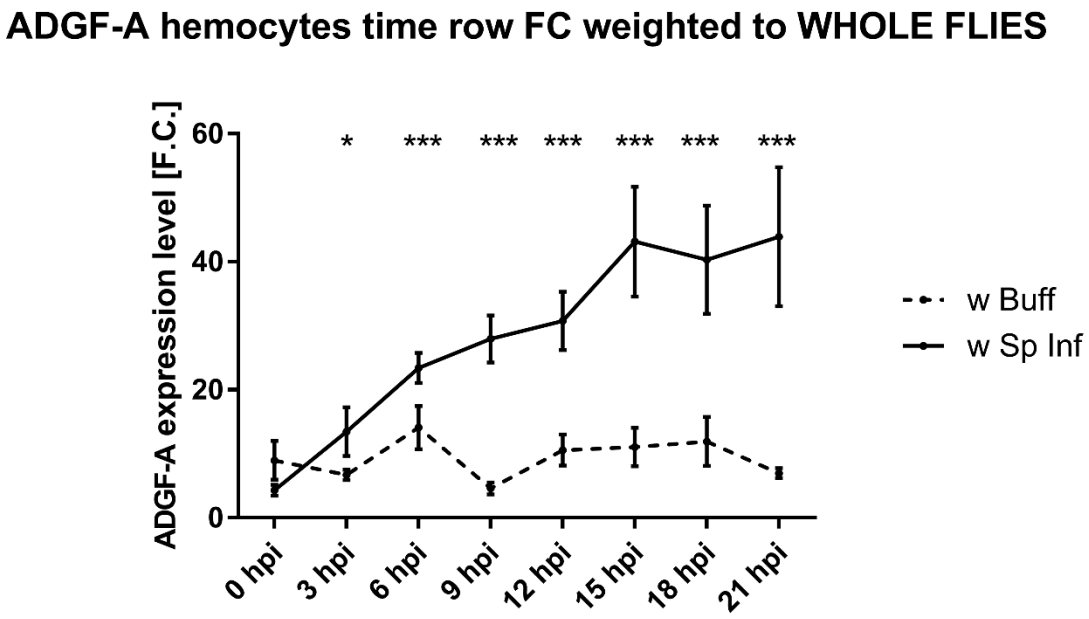

Figure 3: Comparison between expressions of ADGF-A in whole flies to the expression in hemocytes. Data shows fold change increase comparing to the ADGF-A expression in uninfected whole fly at 0 hpi, dashed line representing buffer injected control flies and solid line the *Sp* infected flies. SEM are represented by the error bars and significant differences were tested by Two way ANOVA, Post Hoc comparison by Tukey's test, and are marked with asterisk (* represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001).

4.3 Hemocyte specific expression of adenosine deaminase RNAi is very efficient tool for regulation of adenosine deaminase expression

Following the results obtained in previous section the role of ADGF-A expression in hemocytes was studied further. At first the successfulness of RNAi at silencing the ADGF-A gene expression in ADGF-A RNAi*HmlGal4 flies was evaluated. Expression of ADGF-A was determined by measuring mRNA amount with RT-qPCR. W^{1118}, Karel*w and ADGF-A RNAi*HmlGal4 flies were injected either with PBS buffer for control or infected with *Streptococcus pneumoniae (Sp).* Samples were taken 6 and 18 hours post infection (hpi).

It can be seen in Figure 4 ADGF-A expression in w^{1118} and Karel*x flies has increased during infection and is about 1.5-fold higher at 18 hpi than at 6 hpi. Comparing to both of these

types of flies the expression of ADGF-A in ADGF-A RNAi*HmlGal4 flies was significantly lower at all stages, in infected as well as in control flies.

These results confirm that the RNAi is working and the genetic construct can be used to further evaluate the effect of missing ADGF-A mRNA in hemocytes. Moreover, increased mRNA expression in response to infection (in w^{1118} and Karel*w) suggest the role of ADGF-A in response to infection, which was then further evaluated in next experiments.

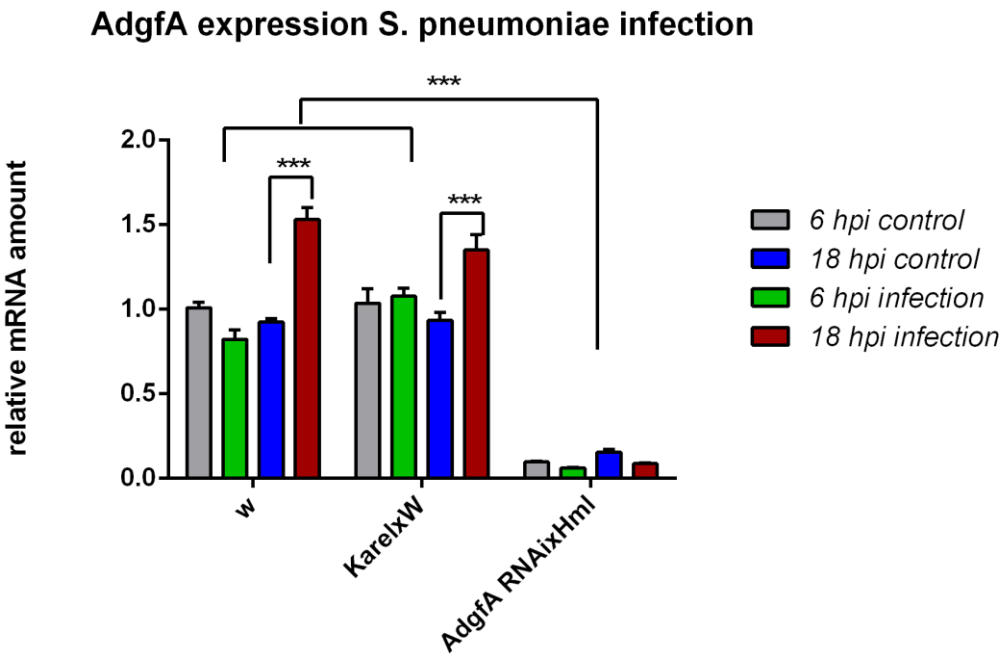

Figure 4: Expression of ADGF-A mRNA in w^{1118} fly, Karel*W and ADGF-A RNAi*HmlGal4 at 6 hpi (noninfected control flies are presented by grey bars, flies infected with *Sp* by green bars) and at 18 hpi (noninfected control flies blue bars, flies infected by *Sp* red bars). SEM are represented by the error bars and significant differences were tested by Two way ANOVA, Post Hoc comparison by Tukey's test, and are marked with asterisk (* represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001).

4.4 Adenosine deaminase hemocyte specific downregulation affects time necessary for recovery from chill induced coma in flies

In chill coma experiments flies were put on ice for 16 hours and after that the time they needed to wake up was recorded. The results are represented in Figure 5. Infection affected the recovery time in W^{1118}, where infected flies needed on average 19 minutes more to wake up

comparing to the control. In the other types of flies there was only slight, non-significant increase in recovery time of infected flies.

Flies with lowered ADGF-A in whole body (Karel x W) woke up on average significantly earlier than w^{1118} control. Downregulating ADGF-A in hemocytes as well decreased the time of recovery significantly comparing to the ADGF-A RNAixW which was used as control. Dunnett's test was used to compare the recovery times of w^{1118} uninfected flies to these of all other flies and it showed significantly lower times for Karel x W and ADGF-A RNAi*HmlGal4 flies.

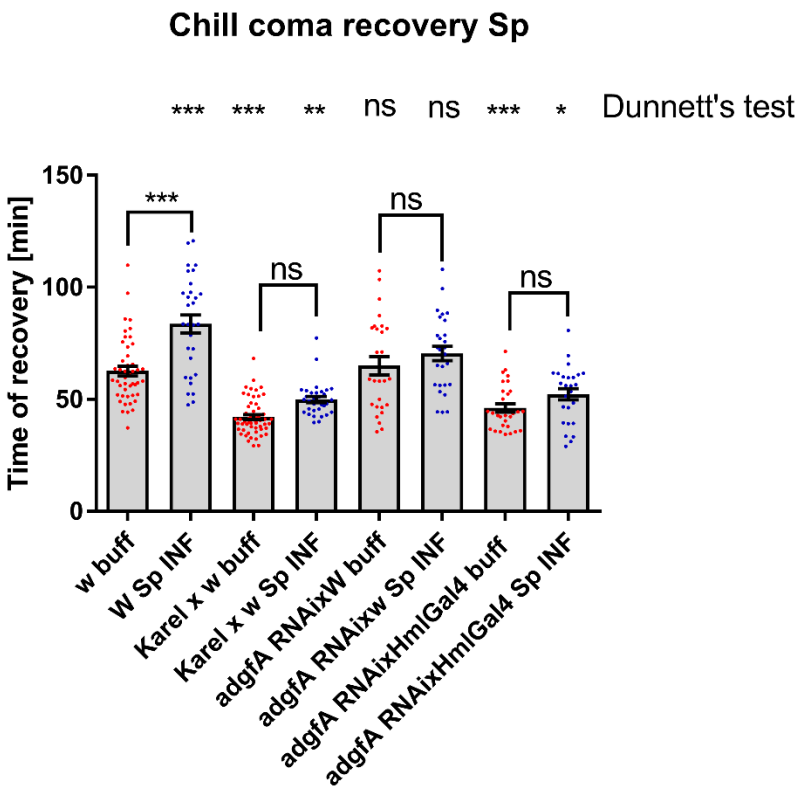

Figure 5: Chill coma recovery time. Red dots represent time of recovery of each individual control fly, blue dots time of recovery of each individual infected fly. Bars represent mean recovery time for each type of fly, SEM are represented by the error bars and significant differences were tested by One way ANOVA, Post Hoc comparison by Tukey test and are marked with asterisk (represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001). Dunnett's test was used to compare results of each type of flies to the w^{1118} type buffer results (first column).*

4.5 Silencing of adenosine deaminase expression in hemocytes does not significantly affect survival rate of *Sp* infected individuals

Following the measuring of the recovery time, the survival rate of flies after the chill coma was evaluated. Percentages of flies that did not wake up after chill coma and were presumed dead are presented by bars in Figure 6. The percentage of w^{1118} dead flies increased from 29% in uninfected to 47% in infected. For Karel x W this increase was from 24% to 42%. Other flies showed no significant increase when comparing the death rate of infected flies to uninfected.

Dunnett's test was performed to compare the all results to the percentage of dead w^{1118} uninfected flies (w buff). The results showed that the ADGF-A mutation in flies did not significantly affect the survival of the flies.

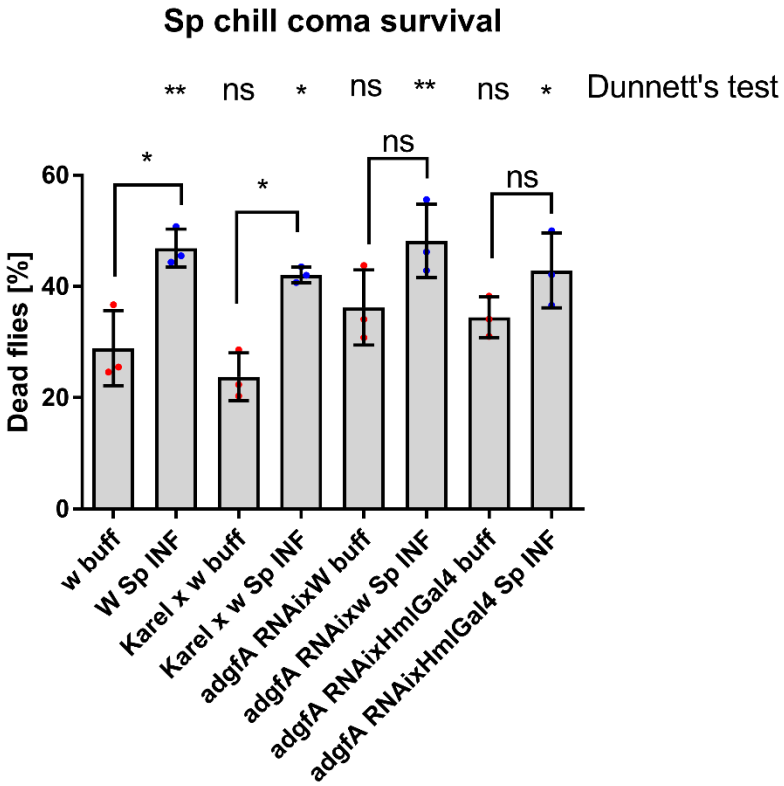

Figure 6: Percentage of flies that did not survive chill coma. Each measurement was repeated three times, red and blue dots represent results of each measurement and bars represent the mean of each three results. SEM are represented by the error bars and significant differences were tested by One way ANOVA, Post Hoc comparison by Tukey test and are marked with asterisk (* represents p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001). Dunnett's test was used to compare results of each type of flies to the w^{1118} type buffer results (first column).

In summary the results have showed that ADGF-A expression increases in response to the infection and that hemocytes are mainly responsible for this increase. RNAi tool was proven to be very efficient in silencing the ADGF-A expression in hemocytes, so the ADGF-A RNAi*HmlGal4 flies were used further in chill coma recovery experiments. Chill coma assay showed that the w^{1118} flies need more time to recover after infection and that the downregulation of ADGF-A improved the recovery time. The chill coma survival analysis however did not show any significant increase in survival rate of ADGF-A RNAi*HmlGal4 flies.

5 Discussion

The results obtained during this work highlight the importance of adenosine signaling during immune response. Firstly, it was shown that the gene expression of ADGF-A, adenosine deaminase enzyme which is responsible for regulation of adenosine concentration (Zurovec et al., 2002), is increased during immune response in adult flies infected with *Streptococcus pneumoniae*.

While ADGF-A acts systematically thorough lowering the signaling of extracellular adenosine, it was previously shown by using the ADGF-A mutant containing one copy of the GFP reporter, that ADGF-A is expressed in high concentration in immune cells (Novakova & Dolezal, 2011). The results obtained by RT-qPCR measurement of ADGF-A expression in hemocytes are in accordance with the literature, showing that the amount of ADGF-A mRNA was significantly higher in hemocytes comparing to the whole fly. Thus it was interesting to see the effect that downregulation of ADGF-A specifically in hemocytes would have. HmlGal4/UAS RNAi construct was prepared for that purpose and the results obtained from RT-qPCR measurements (shown in Figure 4) confirmed its efficiency. No decrease in ADGF-A mRNA could be observed in Karel*w, heterozygous mutant fly, since its mRNA is still expressed, however half of it possesses mutation and it does not produce functional protein (Dolezal et al., 2003). So it still has downregulated ADGF-A, but it cannot be observed by RT-qPCR results.

The Karel*w and HmlGal4/UAS RNAi flies were then subjected to chill coma experiments.

It was reported before that infection with *Listeria monocytogenes* or *Streptococcus pneumoniae* has negative effect on chill coma recovery (CCR), most probably due to the energy being used for immune response (Linderman et al., 2012).

While infection prolonged the time of recovery in w^{1118}, it showed no effect on CCR in ADGF-A deficient flies. In general recovery time was improved in these flies. It was shown before that the increased adenosine signaling due to the ADGF-A mutation leads to hyperglycemia and lowering of the glycogen storage (Zuberova et al., 2010), thus shorter

recovery times of ADGF-A deficient flies could be due to them having more energy directly in circulation and it can then be easier accessible during the recovery.

We could expect that this missing energy stores would on the other hand result in dying, however analysis of chill coma survival rate showed no increase in dying of ADGF-A deficient flies. The lower energy stores will probably show effect later in life and these flies would be dead in the next few days. Further experiments are needed to study this more properly.

"Selfish" behavior of immune system has been discussed before, with adenosine being immune system's autonomous signal for energy redistribution to itself (Bajgar et al., 2015; Dolezal, 2015). Fast energy release from the stores is needed to recover from chill coma and it could be due to the immune response using all the energy for itself that the infected flies performed worse than noninfected in CCR.

The outcome of this work suggest that in short term horizon it could be beneficial to downregulate adenosine deaminase to make adenosine signaling stronger. This provides release of more energy, which is then readily available for fly immune system to fight the infection or for flies to respond to other types of stress such as chill coma. However, this up-regulation of adenosine signaling is not so beneficial during the development (Zuberova et al., 2010), therefore ADGF-A role plays an important role in balancing the adenosine signaling pathway.

6 Conclusion

The first set of experiments confirmed that the ADGF-F expression is increased in response to infection directly in immune cells. Then the RNAi Gal4/UAS construct was proven to be efficient in silencing the ADGF-A expression in hemocytes and it was used in further experiments evaluating the recovery and survival of chill coma. The recovery of infected flies was impaired due to the metabolic changes induced by infection. ADGF-A silencing makes the flies better in recovery from chill coma but the higher circulating energy will probably cause them problems later in life and it should be investigated in the next sets of experiments.

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