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Inhibition of mammary cancer progression in fetal alcohol exposed rats by β -endorphin neurons

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Abstract

Background—Fetal alcohol exposure (FAE) increases the susceptibility to carcinogen-induced mammary cancer progression in rodent models. Fetal alcohol exposure also has decreased β -endorphin (β -EP) level and caused hyperstress response, which leads to inhibition of immune function against cancer. Previous studies have shown that injection of nanosphere-attached dibutyl cyclic adenosine monophosphate (dbcAMP) into the 3rd ventricle increases the number of BEP neurons in the hypothalamus. In this study, we assessed the therapeutic potential of stress regulation using methods to increase hypothalamic levels of β -EP, a neuropeptide that inhibits stress axis activity, in treatment of carcinogen-induced mammary cancer in fetal alcohol exposed rats.

Methods—Fetal alcohol-exposed and control Sprague Dawley rats were given a dose of N-Nitroso-N-methylurea (MNU) at postnatal day 50 to induce mammary cancer growth. Upon detection of mammary tumors, the animals were either transplanted with β -EP neurons or injected with dbcAMP-delivering nanospheres into the hypothalamus to increase β -EP peptide production. Spleen cytokines were detected using RT-PCR assays. Metastasis study was done by injecting mammary cancer cells MADB106 into jugular vein of β -EP-activated or control fetal alcohol exposed animals.

Results—Both transplantation of β -EP neurons and injection of dbcAMP-delivering nanospheres inhibited MNU-induced mammary cancer growth in control rats, and reversed the effect of fetal alcohol exposure on the susceptibility to mammary cancer. Similar to the previously reported

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immune-enhancing and stress-suppressive effects of β -EP transplantation, injection of dbcAMP-delivering nanospheres increased the levels of interferon- γ and granzyme B and decreased the levels of epinephrine and norepinephrine in fetal alcohol exposed rats. Mammary cancer cell metastasis study also showed that fetal alcohol exposure increased incidence of lung tumor retention, while β -EP transplantation inhibited lung tumor growth in both normal and fetal alcohol exposed rats.

Conclusions—Our results suggest that increase of β -EP production in the hypothalamus may serve as a potential therapeutic strategy for treating the cancer growth in patients with chronic stress and compromised immune function, such as the patients with fetal alcohol exposure.

INTRODUCTION

Fetal alcohol exposure occurs to the fetus when pregnant women drink alcohol, and causes a pattern of mental and physical defects collectively known as fetal alcohol spectrum disorders (FASD) (Jones et al., 1973). The prevalence of FASD is estimated to be 1 in every 100 live births, and it is the leading known cause of mental retardation in the western world (Sokol et al., 2003). In addition to damage in the central nervous system, FAE also causes decrease in the production of the stress-inhibiting polypeptide β -EP, hyper-active response to stress (Jacobson et al., 1999; Baldin, 2007; Hellemans et al., 2010) decreased immune function (Johnson et al., 1981; Redei et al., 1989; Taylor et al., 1999; Zhang et al., 2005; Gauthier et al., 2005), and increased incidences of different types of cancers including breast cancer (Severson et al., 1993; Hilakivi-Clarke et al., 2004; Mongraw-Chaffin et al., 2009; Polanco et al., 2010; Murugan et al., 2013). Clinical observations and animal researches have connected chronic stress with decrease of immune function (Leonard and Song, 1996; Zorrilla et al., 2001; Nunes et al., 2002; Moynihan, 2003) and increase of cancer incidence and growth (Moreno-Smith et al., 2010; Conti et al., 2011). On the other hand, interventions aimed at reducing stress and increasing optimism have been shown to enhance immunity and to reduce tumor growth in mammary and prostate cancer patients (Yermal et al., 2010; Rustøen et al., 2010; Galaway et al., 2012). β -EP neurons in the hypothalamus play an important role in the regulation of stress and immune functions. These neurons reside in the arcuate nucleus, and innervate the paraventricular nucleus (PVN) where they inhibit the activities of corticotrophin-releasing hormone (CRH) neurons and sympathetic nervous system (SNS) (Charmandari et al., 2006). We have recently reported that by transplanting *in vitro* produced β -EP neurons into the PVN, we could prevent the growth of carcinogen-induced prostate cancer (Sarkar et al., 2008) and mammary cancer growth and metastasis (Sarkar et al., 2011). Using the same method, we also inhibited the hyperactive stress axis in fetal alcohol exposed animals, and increased their immune function (Boyadjieva et al., 2009). Therefore we hypothesized that the increased susceptibility to mammary cancer growth in FAE animals is caused by the decrease of β -EP neuronal number in the hypothalamus. To test this hypothesis, we transplanted β -EP neurons into the PVN of fetal alcohol exposed rats, and tested their effect on mammary cancer growth and metastasis. Additionally, we have recently found that administering nanosphere-attached dibutyryl cyclic adenosine monophosphate (dbcAMP) into the 3rd ventricle could also increase β -EP neuronal number in the hypothalamus, suppress stress response and enhance immune function (Zhang et al., 2015). Therefore we tested whether injection of these dbcAMP-

attached nanospheres could also reverse the adverse effects on stress, immune and mammary cancer growth in FAE animals.

MATERIALS AND METHODS

Fetal alcohol exposure

Adult Sprague Dawley rats were purchased from Charles River (Wilmington, MA) and maintained in a controlled environment with a 12 h light/dark cycle at Bartlett Hall Animal Research Facility of our institute. Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy. Female Sprague Dawley rats were mated with males and their vaginal smears were checked every morning. The presence of sperm in the vaginal smearing indicated mating and that day was designated as gestational day 1 (GD1). On gestational days GD7-GD21, pregnant rats were fed with daily chow ad libitum (AD), or a liquid diet (BioServ Inc., Frenchtown, NJ) containing alcohol (AF), or pair-fed with an isocaloric liquid control diet (PF, with the alcohol calories replaced by maltose-dextrin). The concentration of ethanol varied in the diet for the first 4 days from 1.7 to 5.0% v/v to habituate the animals with the alcohol diet. After this habituation period, animals were fed the liquid diet containing ethanol at a concentration of 6.7% v/v. At postnatal day 2 (PD2), AF and PF pups were cross-fostered by untreated lactating AD dams to prevent any compromised nurturing by the AF and PF moms. Litter size was reduced to 8 pups per dam. Pups were weaned on PD21, and housed by sex until the injection of MNU or MADB106 cells, when they were individually housed. MNU injection protocol was the same as previously described (Sarkar et al., 2011). Rats were then palpated weekly from 5 weeks after MNU injection. These animals were used in both the β -EP transplantation study and the nanosphere study.

β -EP cells preparation and transplantation

We isolated neural stem cells from 17 days old fetal rat brains of Sprague Dawley rats and then differentiated these cells into β -EP neurons in culture to use in this study. We used cAMP and pituitary adenylate cyclase-activating polypeptide (PACAP) to differentiate β -EP neurons from rat fetal neural stem cells, as we have previously described (Sarkar et al., 2008). To control for transplantation, we used cortical cells prepared from 17-day-old fetal rat brains. The justification for the use of cortical neurons as control is previously described (Sarkar et al., 2008). Prior to transplantation, differentiated β -EP cells were dissociated and resuspended at a concentration of 20,000 viable cells/ μ L in HEPES-buffered DMEM-containing serum supplement (SS; 30 nM selenium, 20 nM progesterone, 1 μ M iron-free human transferrin, 5 μ M insulin, 100 μ M putrescine and antibiotics), cAMP (10 μ M) and PACAP (10 μ M) for the transplantation. Cells were placed on ice throughout the grafting session. Animals were anesthetized and injected with cortical neurons or β -EP neurons in both sides of PVN of the hypothalamus using stereotactic procedures described previously (Sarkar et al., 2008).

Nanosphere injection to animals

Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, Butler Schein, Columbus, OH) and injected with 10 μ L plain nanospheres (control) or nanospheres

that deliver 70 nmol dbcAMP (cAMP) in the third ventricle using stereotactic instrument. To be specific, 10 μ L of nanospheres were injected into the brain of animals (about 300 g body weight) with the coordination of 2.0 mm behind the bregma, midline, and 8.0 mm below the cortex using a 10 μ L Hamilton syringe. Each injection was over 5-min duration. After the injection, the cannula was left in place for 3 min to prevent nanospheres from backflow during the removal of the cannula. The cannula was then slowly removed over a 3-min period. The skin was closed with wound clips.

MNU-induced mammary cancer

In order to induce mammary cancer, 50 days old ovary intact virgin Sprague Dawley rats were injected i.p. with a dose of MNU (50 mg/kg body weight), and palpated weekly starting from the fifth week after injection. In the β -EP study, rats that were newly found to have a tumor with a diameter larger than 0.5 cm were randomly transplanted with either cortical neurons as control or *in vitro* differentiated β -EP neurons (20,000/6 μ L/per PVN) into both PVN. Animals continued to be palpated for 14 weeks, or until one dimension of the tumors reached 3 cm. In the nanosphere study, rats that were newly found to have a tumor with a diameter larger than 0.5 cm were injected with either plain nanospheres (control) or nanospheres containing 70 nmol dbcAMP (cAMP), and continued to be palpated until 10 weeks after the discovery of tumor or until tumor diameter reached 3 cm. Then the rats were sacrificed, and tumors were collected for histology diagnosis, spleens were collected for cytokine mRNA detection, and plasma samples were collected for measurement of stress hormones. Tumor growth was measured using a caliper, and the volumes of tumors were calculated as $(\pi/2 \times \text{Length} \times \text{Width}^2)$.

Lung metastasis study

Fetal alcohol exposed female rats were also transplanted with cortical neurons as control or *in vitro* differentiated β -EP neurons (20,000/6 μ L/per PVN) into both PVN, and injected with 100,000 MADB106 mammary carcinoma cells suspended in 0.2 mL of RPMI medium into the jugular vein. At the time of tumor inoculation, 1 mL of vein blood was drawn into a 1 mL syringe containing 0.05 mL EDTA (100 mg/mL) for the test of plasma corticosterone levels. These animals were sacrificed after 4 weeks. Tumor growth on the surface of the lungs was counted as an indicator of extent of metastasis.

RNA extraction and PCR

Total RNA was isolated from about 30 mg spleen tissue, using RNeasy Mini Kit (Qiagen, Valencia, CA). With the Superscript III First-Strand Synthesis SuperMix (Invitrogen, Grand Island, NY) for RT-PCR, 100 ng total RNA was reverse transcribed and relative quantification of mRNA levels was performed by real-time RT-PCR (SYBR Green; Applied Biosystems, Foster City, CA), using Applied Biosystems 7500 fast real-time PCR system. The following primer sequences were used. Perforin F: 5'-GCATCGGTGCCCAAGCCAGTC-3', R: 5'-GCCAGCGAGCCCCTGCTCA-3'; Granzyme B F: 5'-CGTGCATCAGAAGTGGTGTG-3', R: 5'-GAGGCTGTTGTTACACATCCGG-3'; IFN- γ F: 5'-AGAGCCTCCTCTTGGATATCTGG-3', R: 5'-

GCTTCCTTAGGCTAGATTCTGGTG-3'; TNF- α F: 5'-CCAGGTTCTCTTCAAGGGACAA-3', R: 5'-CTCCTGGTATGAAATGGCAAA-3'; POMC F: 5'--3', R: 5'--3'; GAPDH primers were rodent GAPDH control reagents from Applied Biosystems. Measurement of GAPDH RNA levels served as an internal control for all experiments. Amplification was performed for 1 cycle of a sequential incubation at 50 °C for 2 min, 95 °C for 10 min, and subsequent 40 cycles of a consecutive incubation at 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 35 sec. The individual gene expression value was calculated after normalization to GAPDH.

Plasma analysis of hormones

Plasma was analyzed for corticosterone levels by a competitive ELISA (IBL, Minneapolis, MN) according to manufacturer's direction. All samples were run on one 96-well plate. Blood used for catecholamine measurement was mixed with EDTA (2 mg/mL) and ascorbic acid (100 μ g/mL, Sigma). Plasma was collected and epinephrine and norepinephrine levels were analyzed using Adrenaline & Noradrenaline ELISA assay kit (Eagle Biosciences, Nashua, NH) following manufacturer's recommendations.

Statistical Analyses

Differences in average body weight, tumor volume, tumor number, hormone levels and cytokine levels were assessed using two-way ANOVA followed by Bonferroni post-test. To evaluate tumor type and lung tumor incidence, Chi-square tests were performed.

RESULTS

β -EP neuronal transplantation into the hypothalamus suppresses mammary tumor growth and metastasis in both normal and fetal alcohol exposed animals

To make our study more relevant to clinical practice, we used a "detection-and-treat" paradigm instead of "prevention". To be more specific, fetal alcohol exposed or control animals were first injected with a dose of carcinogen, and observed for appearance of tumor. Tumors started to appear as early as 5 weeks after MNU injection, and the occurrence of new tumors in previously tumor-free animals surged at 9~10 weeks after MNU injection. Once the tumor was detected by palpation, the animal was then treated with neuronal transplantation, and followed up by observation of tumor growth. Body weights of these animals in different treatment groups didn't show a significant difference, although there seemed to be a trend for AF + control and PF + control groups to be lower than the other groups (Fig. 1A). This trend may imply growth deficiency in AF + control caused by fetal alcohol exposure. The reduced body weight of animals in PF + control groups may be related to the food restriction during fetal development, because this group was pair-fed the same amount of diet as the AF group. A significant effect of β -EP neuronal transplantation on tumor volume started at 6 weeks after the transplantation, with the tumor volume in all the β -EP-transplanted groups suppressed to a similar low level (Fig. 1B). In animals transplanted with cortical neurons, fetal alcohol exposure significantly increased the tumor volume from 6 weeks to 9 weeks after detection of the tumor compared to AD and PF rats, which is consistent with the previous publication (Polanco et al., 2010). After 9 weeks, the

tumor volume in AF animals had too much tumor burden and were sacrificed. β -EP transplantation suppressed the tumor numbers in all the treated groups (Fig. 1C).

We have previously shown that β -EP neuron transplants have potent effect on eliminating MADB106 cell metastasis from the lung (Sarkar et al., 2011). Here we determined the effect of fetal alcohol exposure on MADB106 cell metastasis, as well as the effect of β -EP neuronal transplantation on inhibition of metastasis in fetal alcohol exposed animals. Examination of the plasma drawn during cell inoculation showed that fetal alcohol exposure significantly increased basal corticosterone level of the rats, while this hyper-activity of stress axis is eliminated by β -EP transplantation (Fig. 1D). Four weeks after tumor cell inoculation, 70~80% of AD and PF animals that had control cell transplantation developed tumor in the lung, while 100% of AF animals developed tumor in the lung (Fig. 1E). β -EP neuronal transplantation significantly reduced the incidence of lung metastasis in all the AF, PF and AD groups (Fig. 1E), although it didn't completely eliminate the occurrence of tumor in AF and PF groups. Count of the surface tumor granule numbers revealed the same trend of β -EP transplantation inhibiting the lung retention of tumor cells (Fig. 1F, G). Animals with β -EP transplants had little or no tumor granule on the lung, while animals with control cell transplants had an average of 20~40 granules on the surface of the lung. AF + control animals had a trend of increased granule number over PF + control animals, but the difference was not statistical significant.

Injection of cAMP-delivering nanospheres inhibited the higher incidence of mammary cancer in fetal alcohol exposed rats

We tested the effect of cAMP-delivering nanospheres on the stress hormones, immune function and mammary cancer growth in fetal alcohol exposed female rats. We have previously shown that dbcAMP acts as a neurotropic factor for immature β -EP neurons (De et al., 1994). Also dbcAMP has been found to be effective in enhancing the differentiation of neural stem cells (NSCs) to neurons in culture and in vivo (Kim et al., 2002). In our previous study, after dbcAMP-delivering nanosphere injection into the third ventricle, we observed the increase of β -EP positive neurons (Zhang et al., 2015). We also checked the cAMP-responsive neurons that are localized in the arcuate nucleus, such as TH neurons, and didn't find significant change in the neuronal number (Zhang et al., 2015). These results are in accordance with our previous observations, and indicate that dbcAMP treatment seems to induce the differentiation of hypothalamic NSCs specifically into β -EP neurons. In this study, we tested whether by increasing the β -EP neuronal number and peptide production in the hypothalamus using a reagent that promotes NSCs to differentiate into β -EP neurons, immune clearance of mammary cancer can be achieved.

After injection of MNU, which induces mammary cancer growth, we palpated the rats every week, and only took rats that had detectable tumors for injection of nanospheres. Tumor growth was then observed every week by palpation until 10 weeks after the first detection of tumor, when animals were sacrificed and tissues were collected. We found that injection of cAMP-delivering nanospheres significantly decreased tumor size (Fig. 2A) and number of tumors (Fig. 2B) in both control and fetal alcohol exposed animals. By histopathological analysis, we found that injection of cAMP-delivering nanospheres decreased the rate of

malignant tumors, which were increased in the case of fetal alcohol exposed animals (Fig. 2C). Moreover, nanosphere injection even cleared out some already initiated tumors in AD and PF groups (Fig. 2C). Examination of plasma hormone level showed that fetal alcohol exposed rats had increased levels of epinephrine and norepinephrine, while injection of cAMP-delivering nanosphere decreased the levels of epinephrine and norepinephrine back to normal (Fig. 2H, I). Measurement of splenic cytokines showed that IFN- γ (Fig. 2D) and granzyme B (Fig. 2E) that are essential cytokines for natural killer (NK) cell function against cancer, are decreased in fetal alcohol exposed animals, and increased in cAMP-delivering nanosphere injected animals. The level of perforin wasn't changed (Fig. 2F). The level of pro-inflammatory cytokine TNF- α was increased in fetal alcohol exposed animals, but inhibited by injection of cAMP-delivering nanospheres (Fig. 2G). These data suggest injection of the cAMP-delivering nanospheres inhibited carcinogen induced mammary cancer growth in both normal and fetal alcohol exposed animals, possibly by decreasing stress and increasing innate immune function.

5.4 Discussion

The increased incidence of tumorigenesis in fetal alcohol exposed offspring may be due to multiple factors, including: i) defects in immune function, which leads to impaired immune surveillance; ii) abnormal hormone levels, such as stress hormones, which suppress immune function; and iii) altered organ development and gene expression in the affected tissue by developmental imprinting. We have focused on the effect of FAE on neuroendocrine regulation of the immune function. Fetal alcohol exposure causes apoptotic death of neurons in the central nervous system, including the β -EP neurons (Chen et al., 2006; Zhang et al., 2015). Lower numbers of β -EP neurons or peptide production have been also found in brains of patients with schizophrenia, depression, and obese patients (Bernstein et al., 2002; Pankov et al., 2002; Kuhn and Sarkar, 2008), and these pathological conditions were correlated with higher incidences of cancers and infections (Bernstein et al., 2002; Pankov et al., 2002; Irwin and Miller, 2007; Grinshpoon et al., 2005; Giovannucci and Michaud, 2007). β -EP, as an endogenous opioid polypeptide compound, is produced by the pituitary gland and the hypothalamus in vertebrates during exercise, excitement, pain, consumption of spicy food and orgasm, and it resembles the opiates in its abilities to produce analgesia and a feeling of wellbeing (Akil et al., 1984; Goldfarb and Jamurtas, 1997; Dokur et al., 2005). Central administration of β -EP is shown to increase immune function, such as NK cytolytic activity and lymphocyte proliferative response to mitogens (Boyadjieva et al., 2001; 2002), β -EP neuron transplants also have been found to increase peripheral NK cell and macrophage activities, elevate plasma levels of anti-inflammatory cytokines and chemokines and reduce plasma levels of inflammatory cytokines and chemokines in response to tumor challenge (Sarkar et al., 2011). It has recently been shown that anti-inflammatory chemokine MCP-1 might act on tumor microenvironment to promote alcohol-induced mammary tumor growth and angiogenesis (Wang et al., 2012). Hence, β -EP neuron transplants may affect tumor growth and progression by inducing anti-inflammatory milieu in the tumor microenvironment.

We have previously demonstrated the anticancer activity of β -EP neuronal transplants in mammary gland, lung, prostate and liver tissues. In all cases we found β -EP neuronal

transplants enhance circulatory levels of innate immune cells and their cytolytic functions as well as reduce inflammatory cytokines levels. In the case of liver, the influence of β -EP neurons on the immune systems in the tumor microenvironment might have been mediated via the direct communication between the hypothalamus and the liver as well as via the communication between the hypothalamus and the lymphoid organs. Similar mechanism may also exist in case of mammary gland, since mammary gland receives neural input from the hypothalamus (Franke-Radowiecka et al., 2015) and contains a large amount of lymphatic vessels.

The data presented here show that by increasing the β -EP neuronal number and peptide production in the hypothalamus, either by transplantation of *in vitro* produced β -EP neurons or by injection of a reagent that promotes NSCs to differentiate into β -EP neurons, the hyper-active stress axis was suppressed and mammary cancer growth and metastasis was inhibited in fetal alcohol exposed animals. These effects may possibly be due to the impact of β -EP on the suppression of the HPA axis and the sympathetic nervous system, and regulation of cytokine levels, such as increasing IFN- γ and granzyme B levels, and decreasing TNF- α levels. Our study identified the possible role of β -EP neuronal deficiency in the abnormal stress and immune function, as well as increased incidence of cancer in fetal alcohol exposed animals. Furthermore, procedures that enhance β -EP production in the hypothalamus could suppress tumor growth in AF animals to a level that is lower than AD animals with cortical cell transplantation, indicating that the amount of β -EP increased by the treatments not only replenished the loss of β -EP neurons caused by FAE, but also supplemented additional β -EP neurons to these animals. Normal animals could also benefit from the increased amount of β -EP neurons in the hypothalamus and gain resistance to mammary tumor growth and metastasis. Therefore these data suggest a potential therapeutic method for treating stress, immune or neoplastic problems in patients with FASD.

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MNU-induced mammary cancer progression

Figure 1. Effect of β -EP neuronal transplant in the hypothalamus on mammary tumor growth and metastasis in both control and fetal alcohol exposed rats

Alcohol-fed (AF), pair-fed (PF) or *ad libitum*-fed (AD) rat offsprings were used during the adult period. For the carcinogen-induced mammary cancer model, animals were administered with a single dose of MNU (50 mg/kg body weight) at 49 days of age. After the MNU injection, animals were palpated every week for detection of tumors. Once an animal was found to get tumor, the animal was randomly assigned to transplantation of either cortical neurons or β -EP neurons. After the cell transplantation, animals were palpated

every week until 14 weeks after tumor detection, or until the tumor exceeded 3 cm in one dimension. A. Body weight of animals. B. Average tumor volume per animal in each group. C. Average tumor number per animal in each group. For the mammary cancer metastasis study, rats were transplanted with β -EP cells or cortical cells at young adult age. After recovery, they were then inoculated with MADB106 mammary carcinoma cells via jugular vein, and sacrificed after 4 weeks. D. Corticosterone levels in plasma collected at time of tumor inoculation. E. Percentage of animals with tumor in the lung in each group. F. Average tumor numbers in the lung of each animal in each group. Data in panels A~E were analyzed using 2-way ANOVA followed by Bonferroni posttest and in B and C, all groups with β -EP were significantly different from all the groups with control, $p<0.0001$. In panel B, AF+control was significantly different from AD control, as indicated by a. In panel D, AD+control was significantly different from all other groups, $p<0.0001$. F was analyzed using Chi-square test. *: $p<0.05$, **: $p<0.01$, comparing to the control counterparts. a: $p<0.05$ comparing to AD+ β -EP group.

Figure 2. Effect of dbcAMP-delivering nanospheres injection on mammary cancer growth, spleen cytokine levels and plasma catecholamine levels

Alcohol-fed (AF), pair-fed (PF) or *ad libitum*-fed (AD) female rat offsprings were injected with a single dose of MNU at the age of 49 days old, and were palpated every week for detection of mammary tumor. Once an animal was found to have tumor, it was randomly assigned to receive injection of control nanospheres or nanospheres containing dbcAMP. Afterwards, these animals were continued to be palpated every week until 10 weeks after tumor detection, or until one dimension of the tumor reaches 3 cm. A. Average volume of tumors on each animal in each treatment group. B. Average tumor number on each animal in each group. C. Percentage of each histological tumor type that developed in each group. D. IFN-γ expression in the spleen. E. Granzyme B expression in the spleen. F. Perforin expression in the spleen. G. TNF-α expression in the spleen. H. Epinephrine concentration in the plasma. I. Norepinephrine concentration in the plasma. Data in panels A, B and D-I were analyzed using two-way ANOVA, followed by Bonferroni posttest. In A and B, all the groups with cAMP were significantly different from all the groups with control. But there wasn't difference between groups with cAMP or between groups with control. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, comparing to AD+control group. a: $p < 0.05$ comparing to AD+cAMP group. C was analyzed using Chi-square test.