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Repeated exposure to hair dye induces regulatory T cells in mice

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I.M.C. Rubin, S. Dabelsteen², Morten Mølle Nielsen¹, I. R. White³, J. D. Johansen⁴, C. Geisler¹ & C. M.

Bonefeld¹

¹Department of International Health, Immunology and Microbiology, ²Department of Odontology, Faculty of Health Sciences, University of Copenhagen, Denmark. ³St John's Institute of Dermatology, London SE1 7EH, UK. ⁴National Allergy Research Centre, Department of Dermato-Allergology, Copenhagen University Hospital Gentofte, Denmark.

Corresponding author: Charlotte Menné Bonefeld, Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3, DK-2200, Copenhagen, Denmark. Phone: + 45 3532 7866, FAX: +45 3532 7881, e-mail: cmenne@sund.ku.dk

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Exposure to hair dye results in the development of severe contact allergy in some instances. However, most consumers do not develop noticeable allergic responses. Whether this is due to an absent immune response or to the development of anti-inflammatory mechanisms is unclear. The aim of this study was to investigate the immune response in mice repeatedly exposed to hair dye.

Abstract

Background We have recently shown that commercial p-phenylenediamine (PPD)-containing hair dyes are potent immune activators that lead to severe contact hypersensitivity in an animal model. However, only a minority of people exposed to permanent hair dyes develop symptomatic contact hypersensitivity. This suggests that the majority of people exposed to hair dyes do not become sensitised or develop immunological tolerance.

Objectives To study the immune response in mice repeatedly exposed to PPD-containing hair dye in a consumer-like manner.

Methods A commercial hair dye containing PPD was tested in C57BL/6 mice. The local immune response was measured by ear swelling and by histological examinations. The immune response in the draining lymph nodes was analysed by flow cytometry.

Results The hair dye induced local inflammation as seen by swelling and cell infiltration of the treated ears. In addition, exposure to hair dye caused T cell activation as seen by T cell proliferation and production of IFN γ and IL-17 within the draining lymph nodes. The inflammatory response peaked at the fourth exposure to hair dye. From this point on, an up-regulation of regulatory T cells and IL-10 producing cells was seen.

Conclusions This study shows that PPD-containing hair dyes strongly affect the immune system. In addition to being potent skin sensitizers that activate inflammatory T cells, hair dyes also induce anti-inflammatory mechanisms. This might explain why many consumers can use hair dyes repeatedly without developing noticeable allergies, but it also raises the question whether the immune modulatory effects of hair dyes might influence the development of autoimmune diseases and cancers.

Abbreviations

PPD, p-phenylenediamine

BrdU, bromo-5-deoxyuridine

Treg, regulatory T cells

mAb, monoclonal antibody

Introduction

Permanent hair dyes have received much attention in recent years due to their content of the potent contact allergen p-phenylenediamine (PPD). PPD or PPD-related substances are used in more than two-thirds of the permanent (oxidative) hair dyes¹ and some consumers develop contact hypersensitivity and experience severe allergic reactions following hair dyeing^{2;3}. These patients often have a positive patch test reaction to PPD. It has been claimed that in-use hair dyes contain a large quantity of couplers that bind the free PPD and thereby inhibit the allergic potential of the product. In addition, it is claimed that the mixture of colour gel and the developer (oxidant) results in rapid polymerization of the hair dye chemicals and washing the hair after 30 minutes reduces the exposure time to the allergen⁴. However, we have recently shown that consumer available PPD-containing hair dyes are potent immune activators that lead to severe contact hypersensitivity and systemic increase of inflammatory cytokines in an animal model⁵.

The immune response to contact allergens can be regulated by various mechanisms. It has been shown that ultraviolet B irradiation of the skin prior to allergen exposure, oral exposure to the allergen or skin exposure to low doses of the allergen can lead to allergen tolerance⁶⁻⁸. Although different mechanisms initiate the development of tolerance, they all lead to the induction of regulatory T cells (Treg)⁶⁻⁸. Different types of

CD4⁺ Treg have been described; naturally occurring CD4⁺CD25⁺Foxp3⁺ T cells, induced CD4⁺CD25⁺Foxp3⁺ T cells, and Tr1 and Th3 cells defined by prominent release of IL-10 and TGF- β , respectively^{9;10}. All of these CD4⁺ Treg are able to control the immune response to contact allergens by impeding the development and function of allergen-specific effector T cells^{6-9;11;12}. This is done either by cell-cell interactions or by the release of anti-inflammatory cytokines like IL-10 and TGF- β . Interestingly, nickel specific Treg cells that mediate tolerance to nickel in non-allergic individuals have been found¹³. In parallel with this, Treg cells specific for various type 1 allergens have been described in healthy controls¹⁴. This indicates that Treg cells play an important role in individuals that do not develop clinical signs of hypersensitivity. Additionally, IL-10 production by resident skin cells like keratinocytes, mast cells and Langerhans cells are involved in tolerance induction to contact allergens^{12;15;16}. Taken together, these studies demonstrate that different anti-inflammatory mechanisms can be induced following exposure to contact allergens and that these play an important role in regulating the response.

In contrast to our animal model where all mice developed severe allergic reactions, only a minority of people exposed to permanent hair dyes seem develop contact allergies to these products^{3;5;17}. Therefore, the majority of people exposed to hair dye must either not become sensitised or must develop immunological tolerance. The induction of tolerance could either be induce at the first contact with the allergen or develop over time in sensitized individual with no overt skin reaction. Differences in the exposure frequency might explain whether exposure to hair dye lead to sensitization or to tolerance induction. Thus, in our previous animal model, mice were sensitized with hair dyes on

three successive days, which rarely is the case for humans using hair dyes. These observations indicate that temporally spaced, regular exposure to hair dyes might lead to induction of immune regulatory mechanisms. In the present study, we investigated the immune response in mice exposed to hair dyes in a consumer-like manner.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Taconic and housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal-protection guidelines (licence number (2007/501-1357)). Four mice were used in each experimental group.

Contact sensitization

For testing the effect of regular use of hair dyes on the immune response, 25 µl hair dye mixture was applied to the dorsum of the mice ears. The hair dye was mixed accordingly; colour gel and oxidizer (1:1). The commercial hair dye product tested was from a leading international brand obtained from the retail market in the UK. The product had a black shade and contained PPD according to the ingredients label. Product name, manufacturer and outlets from where purchased have been disclosed to the BJD editors. The mixture was washed away after 30 min with cotton wool soaked in lukewarm water until no more colour could be removed from the ears. Control mice were exposure to olive oil:acetone (1:4). Groups of mice were giving 0.8 mg/ml bromodeoxyuridine (BrdU) in their drinking water 24 hours before the second, fourth and sixth exposure. The day after the second, fourth and sixth exposure, the mice were killed. Skin inflammation was quantified

by the increase in the thickness of the right ear as measured in a blinded manner using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Ears were removed and prepared for immunohistochemistry. The draining lymph nodes were removed for further analyses with flow cytometry. For PPD sensitization and challenging mice were exposed to either 0.1, 0.5 or 3% PPD dissolved in olive oil:acetone (1:4) on the dorsal aspect of the ears.

Immunohistochemistry

Mouse ears were either snap frozen in liquid nitrogen or fixed in 4% formalin and cut in 5 μ m sections. Paraffin embedded, formalin fixed sections were deparaffinised and stained with haematoxylin. Frozen sections were air dried and immunostained using a two-step immunofluorescence labelling. In brief, sections were blocked with 10% rabbit serum and incubated o.n. at 4°C with biotinylated anti-CD3 ϵ monoclonal antibody (mAb) (145-2C11; BD Biosciences, Denmark), washed and incubated one hour with FITC conjugated streptavidin, washed and mounted. Some of the frozen sections were deparaffinised and stained with anti-CD3 or anti-Foxp3 mAb (FJK-16s, eBioscience). These sections were first blocked with 0.5% Saponin + 10 mM Hepes (pH 7.4) + 10 mM Glycine (pH 8) and 10% goat serum in RPMI. They were then incubated overnight at 4°C with either with anti-CD3 ϵ or anti-Foxp3 mAb. The next day they were washed and incubated for one hour with FITC conjugated F(ab')₂ fragments of Goat Anti-Mouse IgG (Jackson Immuno Research Laboratories), washed in and mounted. Immunohistochemical detection of IL-10 was performed on frozen sections. Sections were fixed in icecold methanol for 5 min, air dried and blocked with 2% BSA in PBS. Staining with FITC-conjugated Rat Anti-Mouse IL-10 (BD PharmingenTM) was done o.n. at 4°C, washed and mounted.

Flow cytometry

Single cell suspensions were prepared from the draining lymph nodes and the cells were counted. To determine the distribution of T and B cells, the cells were incubated with fluorochrome-conjugated anti-CD4 (RM4-5), anti-CD8a (53-6.7), or anti-CD19 (ID3) mAb all from BD Biosciences. Cells were incubated with the indicated mAb diluted in ice-cold PBS containing 2% FCS and 0.1% NaN₃ for 30 min on ice. After washing, cells were analyzed on a FACSCalibur (BD Biosciences, Denmark) with CellQuest software. To determine the number of CD4+CD25+Foxp3+ Treg cells, the cell suspensions were surfaced stained with fluorochrome-conjugated anti-CD4 and anti-CD25 mAb followed by intracellular staining with anti-Foxp3 mAb using the Foxp3 kit from eBioscience.

T cell proliferation and intracellular cytokine staining

Proliferating cells were identified by intracellular BrdU staining using the BrdU flow kit from BD Pharmingen according to the instruction of the manufacture. In brief, the cells were adjusted to 1×10^7 cells/ml and plated in 96-well plates (100 μ l/well). The cells were stained for surface expression of CD4 and CD8 as described above. After washing, the cells were fixed, permeabilized and treated with 300 μ g/ml DNase for 60 min at 37°C. Finally, the cells were stained with anti-BrdU mAb and analyzed by flow cytometry. To determine allergen-induced cytokine staining, cells were stimulated for 4 h at 37°C with 10 ng/ml PMA and 500 ng/ml ionomycin as described¹⁸. Following surface staining, cells were co-stained with anti-BrdU and anti-cytokine mAb and analysed by flow cytometry.

Statistical analysis

Statistically significant differences were determined by two-sample equal variance student's t-test. Analysis were performed on data from two individual experiments (n=4) comparing hair dye exposed mice with control mice at the different time points.

Results

To analyse the immune response in mice regularly exposed to hair dyes we used the experimental set-up outlined in Figure 1a. We found that mice exposed to hair dye in this consumer-like manner gradually developed considerable skin inflammation as measured by ear swelling and cell infiltration (Fig. 1b and c). After the second exposure only a minor increase in ear thickness and cell infiltration was seen. However, after the fourth exposure the ears had swollen to a thickness of 130 – 140% of the ears of mice in the control group. In accordance, a heavy infiltration of neutrophile granulocytes was found in the dermis of the dyed ears (Fig. 1c). Interestingly, the inflammatory response seemed to peak at around the fourth exposure, where after it levelled out; indicating that a balance between pro- and anti-inflammatory mechanisms had been reached.

The immune response in the draining lymph nodes reflected the local inflammatory response in the exposed ears. An increase in proliferation of both CD4+ and CD8+ T cells, as determined by BrdU+ cells, was seen in the mice exposed to hair dye (Fig. 2a and b). As for the local inflammation, a minimal response was seen after the second exposure. However, significantly increased T cell proliferation was seen after the fourth exposure. We further determined the ability of the hair dye to induce effector T cells as assessed as BrdU+ cells producing either IFN γ or IL-17. After the second exposure a small increase in the number of CD4+BrdU+IFN γ + T cells (Th1) was seen compared to control

mice. The amount of Th1 cell was gradually increasing with the number of hair dye exposures (Fig 2c). In contrast, both the numbers of CD8+BrdU+IFN γ + (Tc1) and CD4+BrdU+IL-17+ (Th17) T cells peaked after the fourth exposure compared to control mice and thereafter fell a little (Fig. 2d and e). Thus, the kinetics of both the local immune response in the ear and the immune response in the draining lymph nodes indicated that immune regulatory mechanisms that control the inflammatory response are induced after repeated exposure to hair dyes.

To study regulatory cells in the draining lymph nodes we consequently determined the number of conventional Foxp3+ Treg and IL-10+ Tr1 cells. We found a gradual increase in the number of both CD4+CD25+Foxp3+ Treg and CD4+BrdU+IL-10+ Tr1 cells up to the fourth exposure to hair dye (Fig 3a and b). At this point Treg and Tr1 significantly outnumbered the inflammatory IFN γ - and IL-17-producing T cells, and the Treg constituted 14% of the total number of CD4+ T cells. Thus, in addition to activating pro-inflammatory T cells the hair dye induced activation and propagation of different types of regulatory T cells. To investigate whether this could be ascribed to PPD alone or whether activation of immune regulatory mechanisms required the complete hair dye mixture we exposed mice, either to the hair dye or to various concentrations of PPD ranging from 0.1% to 3%. After two weeks, the mice were again exposed to hair dye or to the same concentration of PPD as initially used. Twenty-four hours after the second exposure we determined the number of Treg in the draining lymph nodes. We found that PPD induced only a minor up-regulation of CD4+CD25+Foxp3+ Treg whereas the hair dye induced a significant increase in the number of Treg (Fig. 3c). Finally, we looked for Treg in the exposed ears. Foxp3+ cells were not detected in the ears at any time (Fig. 3d). In

contrast, after the fourth and sixth exposure a significant number of IL-10 positive cells were found in the exposed ears (Fig. 3e).

Discussion

Permanent hair dyes contain potent skin sensitizers including PPD and PPD-related substances, and we have recently shown that commercial hair dyes induce massive immune activation in an animal model⁵. However, only a minority of those exposed develops contact hypersensitivity. In a questionnaire study 5.3% of individuals exposed to hair dyes reported skin reactions compatible with an allergic reaction, which indicates that regulatory immune responses may be induced following exposure to hair dyes³. In this study we tested how exposure to permanent hair dye in a consumer-like fashion, affected pro- and anti-inflammatory immune responses in a mice model. We found that exposure to hair dye gradually induced an inflammatory reaction in the exposed ears and the draining lymph nodes. However, after the fourth exposure a balance between pro- and anti-inflammatory mechanisms was reached concordant with a massive increase of Treg in draining lymph nodes and IL-10 producing cells in the inflamed ears. This balance between pro- and anti-inflammatory mechanisms might explain why only 5.3% of individuals exposed to hair dyes report allergic reactions. Only 15.6% of these individuals contacted healthcare services after the reaction³. It is therefore likely that more individuals develop mild skin reactions after the first exposures to hair dye before the balance is induced. However, this needs to be investigated further in humans.

We have recently shown that PPD-containing hair dyes induce major allergic responses in a mice model using a modified version of local lymph node assay⁵. In this model we exposed mice to hair dyes on three consecutive days but in the present model

we exposed the mice to hair dyes in a consumer-simulated manner. In contrast to our previous model, we found that after the second exposure to hair dye only a minor skin inflammation was seen, however, the inflammation gradually increased and peaked at the fourth exposure. Interestingly, the maximal increase in ear thickness, which was found after the fourth exposure, were only half the size of what we found using our previous model. We found that proliferation of both CD4⁺ and CD8⁺ T cells develops with the same kinetics as the change in ear thickness, and as for the change in ear thickness, T cell proliferation was less prominent compared with the old model⁵. Thus, the interval between exposures to hair dye clearly affects the immune response.

Furthermore, we found that Th1, Tc1 and Th17 cells are induced following exposure to hair dye. All of these effector T cells are involved in allergic responses to contact allergens^{12;18-20}. Whereas the Th1 response correlated with the number of exposures to hair dye, both the Tc1 and Th17 responses peaked after the fourth exposure, where after they declined. Th17 cells have been identified in nickel allergic individuals¹⁸. However, the precise role of Th17 cells in contact allergy is still not clear. Tc1 cells are one of the major effector cells in mediating contact hypersensitivity. It would therefore be expected that the Tc1 response would increase following repeated exposure to the allergen. The decrease in Tc1 cells and properly also Th17 cells indicated that repeated exposure to hair dyes leads either to T cell exhaustion, apoptosis and/or induction of immune regulatory mechanisms. Treg cells can inhibit contact hypersensitivity^{11;12;21}, and we found that repeated exposure to hair dyes lead to a marked increase in CD4⁺CD25⁺Foxp3⁺ T cells as well as Tr1 cells within the draining lymph nodes. The PPD concentration within the tested hair dye product is not stated by the company. However, a maximum of 2% PPD is

allowed in the final hair dye mixture within the EU. The increased number of Treg therefore seemed to be induced by the mixture of reactive chemicals and irritants within the hair dye as up to 3% pure PPD only lead to a minor increase in Treg. In contrast to the draining lymph nodes, we were unable to detect any Foxp3+ cells in the hair dye exposed skin. This is in agreement with a previous study showing that CD4+CD25+ Treg in mice do not migrate to the site of inflammation, but mediate their suppressive function from the draining lymph node²¹. Repeated exposures to hair dyes also induced a local infiltration of IL-10 producing cells. In addition to Treg, keratinocytes, Langerhans cells and mast cells can produce IL-10 after exposure of the skin to contact allergens and this can limit the allergic response^{12;15;16}. Identification of the specific cell types producing IL-10 in the inflamed skin needs further investigation.

The ability of Treg to suppress immune responses is important for the prevention of allergic and autoimmune diseases. However, a developmental plasticity of Treg has been shown. Thus, Treg stimulated with IL-6 can differentiate into Th17-like cells^{5;22}. We have previously shown that exposure to hair dyes leads to a systemic increase of IL-6⁵. It could therefore be speculated that repeated use of hair dyes increases the risk of contacting autoimmune diseases because of hair dye-induced Treg to Th17 cell conversion. In contrast to the beneficial role of Treg cells in allergic and autoimmune diseases, their presence in cancer microenvironments leads to suppression of anti-cancer immune responses^{23;24}. Whether there is a correlation between the use of hair dye and development of cancers is very controversial²⁵⁻²⁷, and the focus of that discussion has been on the possible carcinogenic effects of hair dyes²⁵. However, our data suggest that further stud-

ies on the correlation between regular use of hair dyes and suppression of anti-cancer immune responses are needed.

In conclusion, this study shows that PPD-containing hair dyes strongly affect the immune system. In addition to being potent skin sensitizers that activate inflammatory T cells, hair dyes also induce anti-inflammatory mechanisms. This might explain why many consumers can use hair dyes repeatedly without developing overt allergies, but it also raises the question whether the immune modulatory effects of hair dyes might influence the risk of development of autoimmune diseases and cancers.

Acknowledgements

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Figure Legends

Figure 1. The inflammatory response to hair dye depends on the time of exposure to the dye. (a) The experimental setup. (b) Change in ear thickness following different exposures to hair dye. Results are presented as mean $\pm$ SEM. ** $p < 0.005$ (c) Haematoxylin staining of histological ear sections.

Figure 2. T cell response following repeated exposure to hair dye. Cellular response in draining lymph nodes from mice that have been repeatedly exposed to hair dyes were analysed by flow cytometry. (a) Proliferating CD4⁺ T cells (CD4⁺BrdU⁺ cells). (b) Proliferating CD8⁺ T cells (CD8⁺BrdU⁺ cells). Allergen-induced cytokine production after in vitro stimulation with PMA/ionomycin. (c) Th1 response (CD4⁺BrdU⁺IFN- γ ⁺ cells). (d) CD8⁺ T cell response (CD4⁺BrdU⁺IFN- γ ⁺ cells). (e) Th17 response (CD4⁺BrdU⁺IL-17⁺ cells). Results are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.005$.

Figure 3. Repeated exposure to hair dyes leads to the induction of anti-inflammatory mechanisms. The induction of anti-inflammatory mechanisms in draining lymph nodes from mice that have been repeatedly exposed to hair dye were analysed by flow cytometry. (a) Allergen-induced IL-10 production after in vitro stimulation with PMA/ionomycin ($CD4^+BrdU^+IL-10^+$ cells). (b) The amount of $CD4^+CD25^+Foxp3^+$ T cells. (c) The percent of $CD4^+CD25^+Foxp3^+$ T cells after 2th exposure to 0.1% PPD, 0.5 % PPD, 3 % PPD or hair dye. $((\text{Number of } CD4^+CD25^+Foxp3^+ \text{ cells})_{\text{treatment}}/(\text{number of } CD4^+CD25^+Foxp3^+ \text{ cells})_{\text{control}} \times 100\%)$. Results are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.005$. Immunohistological staining of ear and spleen sections (d) Foxp3 staining. (e) IL-10 (green) and DAPI (blue) staining.

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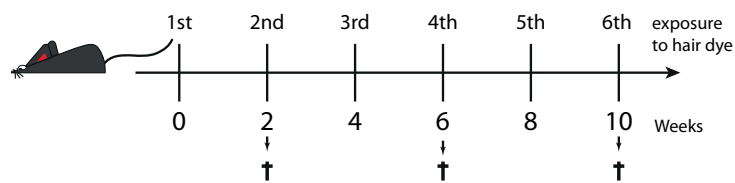
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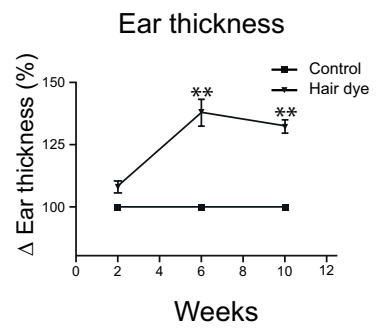
Figure 1

(a)

Experimental setup



(b)



(c)

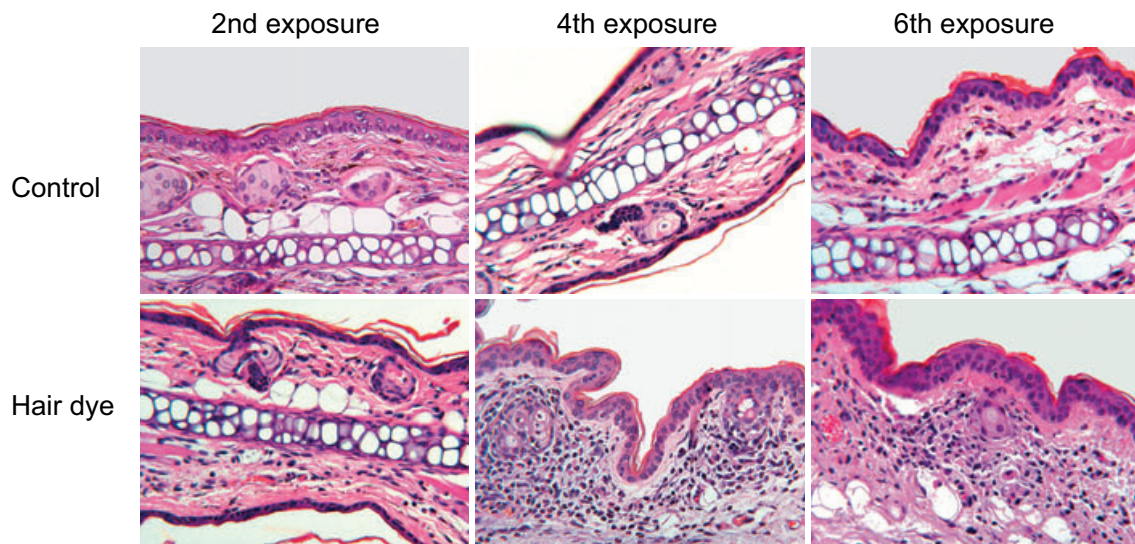


Figure 2

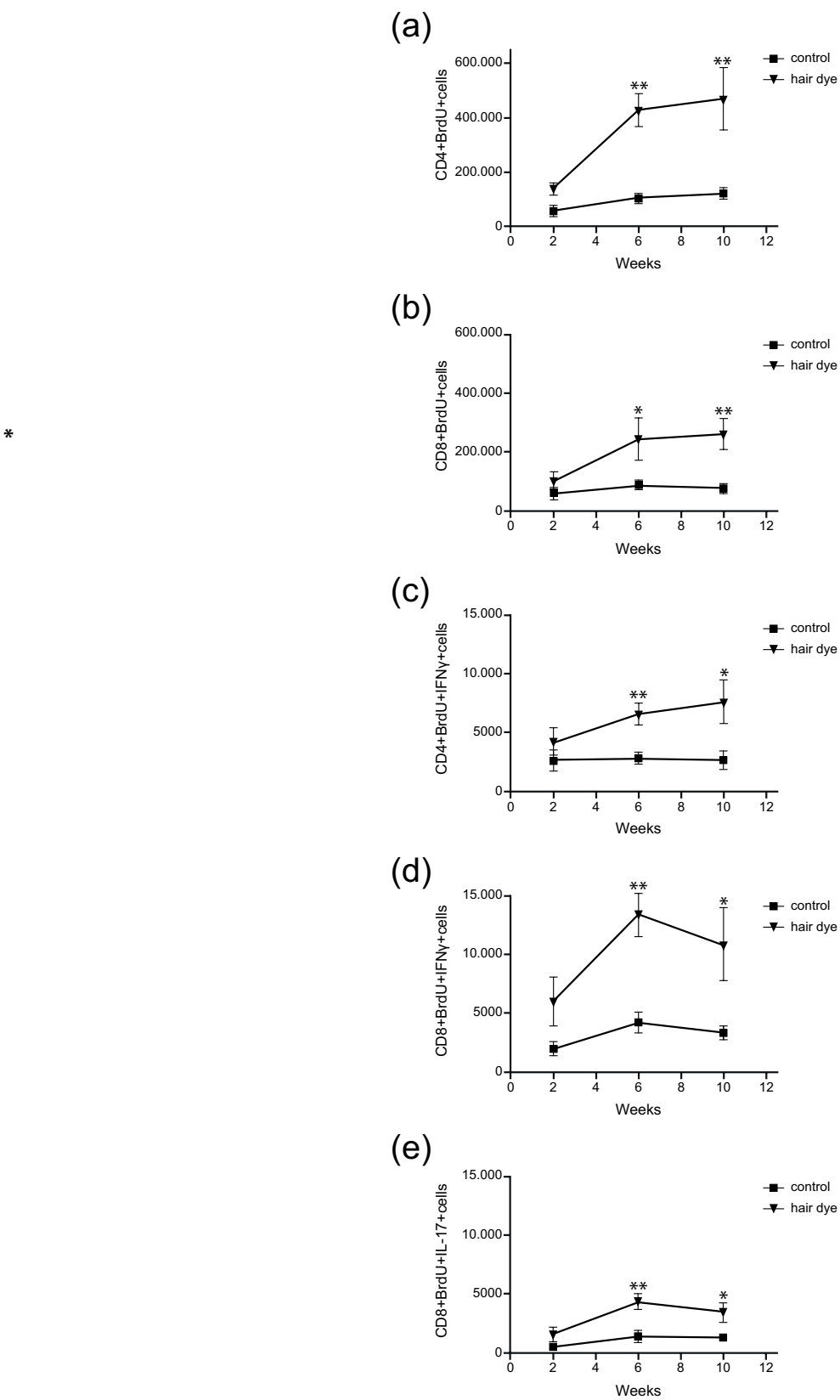


Figure 3

