Comparative Medicine
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Vol 70, No 1
February 2020
Pages 6–15

Original Research

Betamethasone Treatment for Atopic Dermatitis in Gut Microbiota Transplanted Mice

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Gut microbiota composition correlates strongly with essential disease parameters in the oxazolone-induced mouse model for atopic dermatitis. The phenotype of this model can be transferred to germ-free mice with a gut microbiota transplant to achieve high and low responding mice. Therefore, the production of high responding mice through gut microbiota transplantation may be seen as a tool to reduce group sizes or increase power in intervention studies by increasing effect size. We sought to determine whether high responding mice respond to a common treatment in the same way as low responding mice. We hypothesized that while high responding mice would exhibit a higher clinical score than low responding mice before treatment, the clinical parameters would be similar in both groups after betamethasone treatment. Dermatitis was induced with oxazolone in barrier bred Swiss Webster mice, and a high responding and a low responding donor was selected based upon clinical and pathologic scores, as confirmed by monitoring a range of ear tissue cytokines. Feces from these donors were transplanted to pregnant germ-free Swiss Webster dams, and subsequently to their offspring. Although the overall effect of betamethasone on the clinical dermatitis score and ear thickness was rather small, the high responding recipients had significantly higher clinical dermatitis score and ear thickness than the low responding recipients before treatment, and these differences vanished after betamethasone treatment. We conclude that high responding recipients can be treated to a clinical level comparable with the low responding recipients.

Abbreviations: AD, atopic dermatitis; CDS, clinical dermatitis score; ET, ear thickness; GF, germ-free; GM, gut microbiota; HPS, histopathologic score; HR, high responding; KC/GRO, keratinocyte chemoattractant/growth related oncogene; LR, low responding; OXA, oxazolone; Th, T helper.

DOI: 10.30802/AALAS-CM-18-000136

Animal research should be done in accordance with the principles of replacement, reduction and refinement, also known as the 3R's.[31] To avoid using unnecessarily high numbers of animals, and to increase chances of differences between groups being significant, group sizes (n) must be estimated based upon significance level, power, effect size, and uncontrolled interindividual variation.[7,15] While significance level and power are based upon statistical concerns, and more or less governed by a tradition for a significance level of $P < 0.05$ and a power greater than 80%, both individual studies, and defined animal models may still be refined to increase effect size and/or reduce uncontrolled interindividual variation. This allows the researcher to reduce the number of animals used in each study.

In both human patients[33] and animal models,[6] the composition of the gut microbiota (GM) has a significant impact on the expression of several atopic diseases, such as atopic dermatitis (AD).[2] AD is a common chronic and relapsing skin inflammation that often presents in early childhood[18] characterized by xerosis and pruritus, with erythema in the acute phase, and excoriations and lichenifications in the chronic phase.[36] A dysfunctional epidermal barrier allows dendritic cells to present allergens, driving naive T-cells development into T helper (Th) cells type 2 and the production of immunoglobulin E (IgE).[3] IgE activates mast cells in the skin, resulting in increased production of interleukin (IL)4, IL5 and IL13.[19] In chronic AD, both IL5, and Th1-cytokines IL12 and interferon (IFN)γ, are present.[4,27] Other cytokines important for the development of AD in both humans and mouse models are IL33,[32] keratinocyte chemoattractant/growth related oncogene (KC/GRO),[38] IL21,[11] IL31,[8] and the Th17 related lineage including IL17, IL17F, IL21, and IL22.[9,17] The IgE mediated AD affects 70 to 80% of patients, while the AD of the remaining percentage of patients is non-IgE mediated.[18]

AD patients have a low GM diversity during the first months of life, before clinical signs develop.[1,5] In the oxazolone (OXA) induced mouse model, in which dermatitis is induced by repeated dermal application of OXA, the correlation between disease expression and GM composition is also extremely high.[21] One OXA challenge results in a Th1 dominated inflammatory response, while multiple OXA challenges, results in a shift into a more chronic, Th2 dominated inflammatory response, with multiple features of human AD. The clinical signs of the model include a persistent, chronic dermatitis with moderate to severe pruritus.[14,23] Both a high responding (HR) and a low responding (LR) phenotype may be transferred with the GM from conventional to germ-free (GF) mice.[37] HR inoculated mice exhibit increased ear thickness, a higher dermatitis score, and increased

Received: 26 Nov 2018. Revision requested: 31 Dec 2018. Accepted: 30 Apr 2019.
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concentrations of ear tissue cytokines, compared with LR inoculated mice.[37] Mice with LR phenotypes have a lower diversity in the GM composition than mice with a HR phenotype, and the LR phenotype may be explained by the presence of protective bacteria.[37]

The interindividual variation in both LR and HR inoculated mice does not seem to differ from the group of mice from which the donors are selected.[37] Therefore, the benefit of a smaller group size or increased power when using HR inoculated mice cannot be argued from a lower interindividual variation, but it may be argued if the increased clinical expression can be downregulated to a level similar to the LR inoculated mice, that is if the effect size is higher in the HR inoculated mice. Furthermore, it is crucial to know whether HR inoculated mice are able to respond to treatment or if they exert a more resistant phenotype to evaluate its validity as a model. The first line of treatment for human AD is topically administered corticosteroids. Of these topical corticosteroids, betamethasone is a commonly applied intervention control when the OXA induced mouse model is used for testing potential drug candidates against AD.[13] Corticosteroids interfere with immunologic cell activation and inhibit the production of inflammatory cytokines.[10,34]

Therefore, we hypothesized that HR inoculated mice would exhibit a higher clinical score than LR when untreated, but would respond to betamethasone treatment such that the clinical parameters would be similar to those of betamethasone treated LR inoculated mice. Thus, a larger effect size may be obtained if HR mice are used for intervention studies.

Materials and Methods

Ethical Evaluation and Health Status. The Danish Animal Experiments Inspectorate at the Ministry of Environment and Food approved the experiments in accordance with the Danish Animal Experimentation Act (Order no 12 of 07/01/2016) and the Directive 2010/63/EU on Protection of Animals Used for Scientific Purposes by (license number 2017-15-0201-01262). Health monitoring was performed according to FELASA guidelines,[22] revealing none of the infections tested for. Humane endpoints as set in the license were excessive weight loss or inadequate weight gain, ulcerations and/or excessive interest from the animal in the area treated in the forms of scratching and rubbing.

Animals and study design. Ten outbred female 8 wk old Swiss Webster mice (Tac:SW, Taconic, Lille Skensved, Denmark) were housed in our AAALAC accredited barrier-protected facility with 5 mice in each of 2 open cages (1290D Eurostandard type III, Scanbur/Techniplast, Karlslunde, Denmark) with free access to an Altromin 1324 diet (Brogaarden, Lynge, Denmark), and tap water. Room temperature was 20 to 24 C°, humidity of 55% ± 10%, 15 to 20 air changes per hour and the light was on from 0600 to 1800. They acclimated for 6 d before they were sensitized on day -7 (Age = 9 wk) and challenged on days 0, 3, 5, 7, 10, 12, 14, 16, 18, and 20 (Figure 1). Selection of the HR donor mouse and the LR donor mouse was based on clinical dermatitis score (CDS) as the primary readout, with the additional criteria of ear thickness (ET) and histopathologic evaluation of sections from ear puncture biopsies (Figure 1). Cytokine concentrations were analyzed at the end of the study to verify correct selection of donor mice.

Male (n = 4) and female (n = 4) 7 wk old GF outbred Tac:SW mice were housed individually in our AAALAC accredited GF facility in open cages (1284L, Scanbur/Techniplast) in 2 HEPA-ventilated isolators (PFI-systems, Milton Keynes, United Kingdom) with free access to an irradiated Altromin 1314 diet (Brogaarden) and sterile water (B. Braun, Frederiksberg, Denmark). The room had a temperature of 20 to 24 C° and the pressure in the isolators was 110 Pa. The mice acclimated for 25 d. On day 26 to 29 and day 33 to 36 after arrival, one female and one male were housed together to mate. The pregnant GF dams and the recipients born in the 2 isolators were randomly assigned to receive GM from either the HR or the LR donor. GF status in the isolators was tested and confirmed by aerobic and anaerobic bacterial culturing before GM transfer. The recipients (n = 32, sex = 6 females and 7 males in the HR group, and 8 females and 11 males in the LR group) were weaned at 3 wk of age. Littermates were housed together in open cages with females and males separately. The recipients were sensitized on day -7 (Age = 4 wk), and challenged on days 0, 3, 5, 7, 10, and 12 (Figure 1).

All cages were provided with aspen chip bedding (Tapvei, Harjumaa, Estland) and enriched with a small size aspen chewing block (Tapvei), and a Cotton squares filt pillow (LBS-Biotech, Horley, United Kingdom). Beyond that, the donor mice were enriched with a cardboard house (LBS-Biotech) and a Mini Fun Tunnel (LBS-Biotech) and the GF mice were enriched with a red Mouse Igloo (Scanbur). All bedding and enrichment for the isolators were autoclaved before use. To assess animal welfare, the mice were observed daily and body weight was systematically monitored. The donor mice and the recipients were euthanized under anesthesia by cervical dislocation followed by decapitation.

Induction of Skin Inflammation. OXA (4-ethoxymethylene-2-phenyl-2-oxazolon-5-one, Sigma–Aldrich , St Louis, MO) was dissolved in 4:1 Acetone (GPR RECTAPUR, Radnor, PA) and olive oil (Organic extra virgin olive oil, Svansø, Denmark).[37] OXA was weighed into sterile microfuge tubes in advance, and stored in the fridge at 3 to 5 °C. A new OXA solution was prepared daily. A total amount of 20 µL of the OXA solution was applied onto the right ear with a pipette, 10 µl on the internal and 10 µL on the external surface of the pinnae. The pipette tip was changed between every 10 µL application. A 0.8% OXA solution was used to sensitize the animals and a 0.4% OXA solution to challenge the animals (Figure 1).

Clinical dermatitis score (CDS). The right ear of the donor and the recipient mice was macroscopically scored using a modified CDS.[26,37] The clinical signs scored included redness, visual ear thickening, excoriation/erosion, and incrustation/xerosis. Each clinical sign was scored from 0 to 3, where 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The same 2 individuals scored the clinical signs throughout the study in a blind and independent manner and a mean of the 2 scores was calculated. On day 21 the clinical signs of the donor mice were scored immediately after euthanasia, while the recipients were scored on day 7 and day 14 (Figure 1).

Ear thickness (ET). ET was measured on the right ear of the donor mice and the recipients using a micrometer (Mitutoyo Absolute Low Force Caliper, series 5). Every measurement was repeated twice on the right ear and a mean was calculated.[37] To ensure the same anatomic position on all animals, the same person performed all the measurements throughout the study. The person performing the ET measurement was blinded in regards to the GM status of the mice. ET of the donor mice was measured on day -7 in vivo and on day 21 after euthanasia (Figure 1) and of the recipients on day -7, 7, and 14 in vivo (Figure 1).

Feces Collection and Gut Microbiota Transfer. Feces from the donor mice were collected before the OXA application on day -7, 0, 3, 5, 7, 10, 12, 14, 16, 18, 20, and 21 into sterile microfuge tubes and stored at –80 °C. On the days of GM transfer fecal

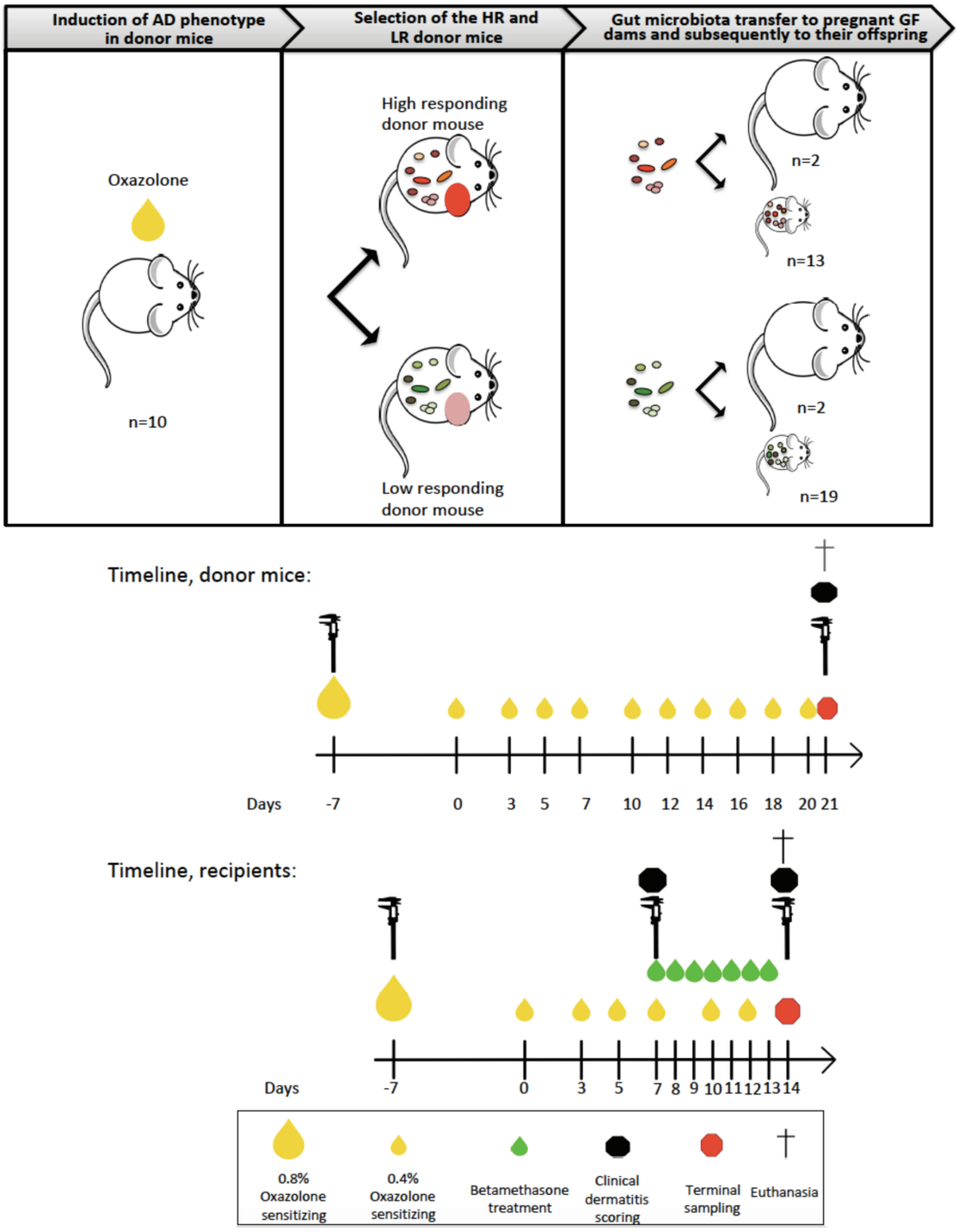

Figure 1. A classic parallel design was used in this study in which dermatitis was induced onto the right ear of 10 barrier-housed Tac:SW mice by sensitizing with a single application of 0.8% OXA solution on day –7 and maintained by challenges with 0.4% OXA solution on day 0, 3, 5, 7, 10, 12, 14, 16, 18 and 20. Based upon the clinical dermatitis score, ear thickness measurement and the histopathologic score one high responding (HR) and one low responding (LR) mouse were selected to be gut microbiota donors. Gut microbiota from the HR and the LR donor mice was orally transferred to germ-free (GF) Tac:SW dams in 2 isolators in their third week of pregnancy and subsequently to their offspring at 1 wk and 3 wk of age. Dermatitis was induced onto the ears of the recipient mice by application of a 0.8% OXA solution at day -7 and with a 0.4% OXA solution on day 0, 3, 5, 7, 10, and 12. The clinical signs were scored, and the ear thickness of the right ear measured, on day -7 and day 21 of the donor mice and on day -7, 7 and 14 of the recipients. The recipients were topically treated with a betamethasone solution on day 7, 8, 9, 10, 11, 12 and 13. The donor mice were euthanized on day 21 and the recipients on day 14 and samples for further analysis were collected.

pellets were thawed at room temperature. All fecal pellets were mixed with 1 mL Phosphate Buffered Saline (Sigma–Aldrich) in a sterile microfuge tube and given orally to the mice by a syringe (1 mL, KRUUSE, Langeskov, Denmark). The GM transfer was performed outside the isolators on a disinfected table.[20] Gloves were changed between cages. Pregnant GF dams were inoculated in their third week of pregnancy with a mixture of fecal pellets obtained from the donor mice at day 0, 10, and 16 (100 µL per mouse), while recipient mice were inoculated at one week of age with fecal pellets obtained from the donor mice at day 3, 12, and 18 (30 µL per mouse), and again at 3 wk of age with fecal pellet obtained from donor mice at day 7, 14, and 21 (30 µL per mouse). The recipients were in addition inoculated at one week of age by application of the GM inoculum onto the mammae of the dam (50 µL per dam).

Treatment with Betamethasone. The right ear of the HR and LR recipients was topically treated with betamethasone (Betnovat 1 mg/mL kutan-emulsion, GlaxoSmithKline Pharma A/S, Brøndby, Denmark) diluted in sterile water (B. Braun) to a final concentration of 0.15 mg/mL. 10 µL was applied on the internal and 10 µL on the external surface of the pinnae (0.003 mg/ear/day) using a pipette. The tip was changed between every 10 µL application. The betamethasone solution was vortexed between cages. The HR and LR recipients were treated on day 7, 8, 9, 10, 11, 12, and 13, same time every day. On day 7, 10, and 12 the time gap between OXA challenge and betamethasone treatment was 4 h, to allow the ear to dry (Figure 1).

Serum IgE. On the day of euthanasia, the donor mice and the recipients were anesthetized with 0.1 mL/10 g body weight 'rodent mixture' (25% Midazolam (5 mg/mL, B. Braun, Melsungen, Tyskland) and 25% Hypnorm (0.315 mg/mL Fentanyl citrate/10 mg/mL Fluanisone, Vetapharma, Leeds, United Kingdom) dissolved in sterile water (50%)) injected subcutaneously. Blood was collected[37] from the retro-orbital sinus into microfuge tubes, using heparin coated capillary tubes (Brand, Wertheim, Tyskland), which were kept at room temperature for one hour and subsequently stored in the fridge at 3 to 5 °C overnight. The next day samples were centrifuged for 10 min at 10000G and serum was pipetted into new microfuge tubes and stored at –80 °C. On the day of analysis, samples were thawed at 4 degrees in the fridge. Serum IgE concentration was determined in a 1:20 dilution with a mouse IgE ELISA kit (Bethyl Laboratories, Montgomery, TX), following manufacturer's instructions. IgE concentrations from 9 HR recipients and 8 LR recipients, that were selected by drawing lots, are included in the statistics due to technical limitations.

Biopsies. Immediately after euthanasia, an 8 mm punch biopsy (Biopsy punch 8 mm, KRUUSE) was collected from the center of the pinnae of both ears of the donor mice and the recipients (Figure 1). The left ear was collected as a control. From the center of each biopsy, a 3 mm punch biopsy (Biopsy punch 3 mm, KRUUSE) was collected and fixed in 4% formaldehyde (Sarstedt, Nümbrecht, Germany). The remaining tissue of the 8 mm biopsy was weighed and snap-frozen in liquid nitrogen and subsequently stored at - 80 °C. The 8 mm punch biopsy from the right ear from donor mouse number 1 was lost during sampling due to technical issues and a new biopsy punch was collected from the most distal part of the right pinnae.

Histopathology. The 3 mm biopsies were processed and stained with haematoxylin and eosin as previously,[37] and scored histologically (Olympus BX51) excluding sections from 3 HR recipients and one LR recipient due to insufficient quality. The histopathologic score (HPS) for the donor mice was used to support the selection of the HR and LR donor mice, and using a modified HPS, the parameters evaluated were epidermal hyperplasia and spongiosis, visual dermal thickening, mast cell infiltration and granulation, dermal intercellular edema, and infiltration of lymphocytes, neutrophils and eosinophils. In addition to this, the HPS for the recipients also included hypergranulosis, regularity and irregularity of stratum spinosum, dysplasia and erosion of stratum basale, dermal hyperplasia, hemorrhage, fibroplasia, and intact cartilage.[24,29,30] All sections were independently and blindly scored by the same 2 persons on 10×, 20×, and 40× magnification. Histopathology was scored as 0 = none, 1 = mild, 2 = moderate, and 3 = severe, except for regularity or irregularity of stratum spinosum, where 0 = regular and 1 = irregular. Crusts, pustules, and hyperkeratosis were also scored but did not count in the total HPS due to a high risk for misinterpretation. The cartilage was assessed as intact or not, but it was not scored. A mean of the 2 scores was calculated. Mast cells, neutrophil granulocytes, eosinophil granulocytes and lymphocytes of the recipient mice were counted on a 60× magnification. A scoring scale was developed for the recipients based on the total number of cells in the sections (Table 1).

Cytokines. The remaining tissue from the 8 mm biopsy sampled from the right ear of the donor mice and the recipients was thawed on ice and then homogenised with a Polytron homogeniser (PT 1200 E, Kinematica AG, Lucerne, Switzerland)[37] in 300 µL lysis buffer. 10 mL of lysis buffer contained 100 µL Phosphatase Inhibitor I, 100 µL Phosphatase Inhibitor II, 100 µL Protease Inhibitor Solution, and 9.7 mL of Tris Lysis Buffer (Meso Scale Diagnostics, Rockville, MD). The mix was centrifuged (7500 G, 5 min) and the supernatant pipetted into microfuge tubes, which were frozen until the day of analysis. IFNγ, IL1β, IL2, IL4, IL5, IL10, IL12p70, IL13, KC/GRO, and tumor necrosis factor (TNF)-α levels were measured using the U-PLEX TH1/TH2 Combo Mouse assay kit (K15071K, Meso Scale Diagnostics) and IL17A, IL17C, IL-17E/IL25, IL17F, IL21, IL22, IL23, IL31, and IL33 using the U-PLEX TH17 Combo 1 Mouse assay kit (K15077K, Meso Scale Diagnostics) following the instructions from the manufacturer. The plates were read on a MESO QuickPlex SQ 120 (Meso Scale Diagnostics). Due to plate limitations, double determinations were run on all the recipients and 6 of the donor mice, while single determinations were run on 4 of the donor mice. Cytokine concentrations not detected in the kits were assumed to be too low to detect and therefore adjusted concentrations were used in the statistics for both the donor mice and the recipients. The adjusted concentrations were calculated by dividing the lowest detected concentration for each cytokine by 2.

Statistics. GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, San Diego, CA) and Microsoft Excel for Mac 2011 version 14.7.7 (Microsoft Corporation, Redmond, WA) were used. P values below 0.05 were considered significant. The D'Agostino-Pearson normality test was used to test for Gaussian distribution of quantitative data. Differences between the LR and the HR recipients were analyzed with an one-tailed unpaired t test for parametric data and an one-tailed Mann–Whitney test for ordinal or non-Gaussian quantitative data. A paired t test or the Wilcoxon test was used to determine differences

Table 1. Histopathologic scoring scale for counts of mast cells, neutrophil granulocytes, eosinophil granulocytes and lymphocytes in Tac:SW recipient mice with oxazolone induced dermatis on the ear after betamethasone treatment.

	Score			
Histopathologic score for cell counts	0	1	2	3
Granulated mast cells	0	1–40	41–80	>80
Neutrophil granulocytes	0	1–40	41–80	>80
Lymphocytes	0	1–13	14–26	>26
Eosinophil granulocytes	0	—	—	—

	Selected donor mouse			**HR**							**LR**
	Mouse no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	Clinical dermatitis score	3	6.5	6.5	5	6	5	2.5	4.5	4.5	2
	Histopathologic score	9	11.5	15	10	10.5	11	10.5	13.5	9.5	11
	Ear thickness (mm)	0.18	0.38	0.47	0.43	0.39	0.34	0.28	0.32	0.25	0.19
Cytokines TH1/TH2 kit (pg/mL)	IFNγ	1.76	2.01	3.96	3.05	1.51	4.05	*0.19*	2.22	0.95	0.80
	IL10	15.10	34.73	56.11	28.43	36.31	17.21	50.35	33.29	21.22	13.13
	IL12p70	—	—	—	—	—	—	—	*24.49*	*0.22*	—
	IL13	*0.80*	—	*0.51*	*1.57*	*0.51*	—	*0.34*	*0.54*	—	—
	IL1β	129.18	670.57	1446.34	257.21	2730.65	1010.41	429.44	262.62	772.49	86.28
	IL2	1.98	5.40	1.06	4.27	*0.46*	4.12	*0.51*	1.76	1.15	0.95
	IL4	42.50	147.79	356.84	153.30	591.68	199.03	350.31	112.71	247.16	61.19
	IL5	*0.36*	*0.21*	0.52	*0.20*	0.72	*0.47*	*0.38*	*0.33*	*0.43*	*0.20*
	KC/GRO	13.78	175.46	199.61	28.12	100.44	72.24	102.54	22.97	64.38	41.07
	TNFα	64.66	146.77	143.57	67.85	139.93	128.81	67.95	63.04	82.86	36.93
Cytokines TH17 kit (pg/mL)	IL17A	*0.12*	0.24	0.45	*0.08*	0.27	0.32	0.24	0.30	0.40	*0.07*
	IL17C	0.32	—	—	0.34	—	1.38	1.48	2.41	0.83	—
	IL17E/IL25	—	*0.34*	*0.14*	—	*0.20*	*0.31*	*0.44*	*0.78*	*0.51*	—
	IL17F	35.79	40.68	33.94	*15.61*	28.71	33.33	31.18	64.01	44.04	*15.61*
	IL21	5.44	5.32	5.71	8.62	4.32	7.05	4.58	6.12	4.64	4.85
	IL22	*0.19*	*0.64*	1.35	*0.49*	*0.72*	*0.83*	*0.73*	*0.77*	1.08	*0.61*
	IL23	*2.76*	*3.01*	*3.45*	*3.67*	*2.47*	*2.58*	*3.95*	*2.04*	*3.17*	*2.79*
	IL31	40.37	82.36	111.73	79.86	146.76	67.26	72.73	84.86	96.48	91.10
	IL33	2596.90	7997.91	10360.76	6220.87	6326.30	7721.86	7701.01	4195.73	8726.88	4038.86

Figure 2. The selection of one high responding (HR) and one low responding (LR) gut microbiota (GM) donor Tac:SW mouse after induction of dermatitis with oxazolone. The clinical dermatitis score, ear thickness and the histopathologic score were used to select mouse number 3 as the HR GM donor and mouse number 10 as the LR GM donor. Subsequently cytokine concentrations in ear biopsies from the right ear were measured. Values highlighted in green are <25% percentile and values highlighted in red are >75% percentile. Concentrations written in italics indicate adjusted concentrations below the limit of the detection in the assay. Hyphens (-) represent concentrations not detected in the assay.

within a group between different days. Differences in cytokine concentrations between LR recipients, HR recipients, and GM donor mice were calculated with One Way ANOVA for parametric data or the Kruskal-Wallis test for ordinal or non-Gaussian quantitative data. Dunn or Tukey multiple comparisons were applied as post hoc test. Raw data were used for calculating the difference in ET before and after induction of skin inflammation. From all other data presented on ET the measurement from day -7 was subtracted to reduce risk of bias. Area under the curve was calculated for the weight and significance levels were calculated with an unpaired *t* test. Correlations were calculated with the nonparametric one tailed Spearman correlation.

Results

Clinical signs before oxazolone sensitization, and animal welfare. No signs of skin inflammation were observed on the ears of the donor mice before OXA sensitizing on neither donor nor recipient mice, and no significant difference was observed in ET between the LR recipients and the HR recipients ($P = 0.4018$). All mice were observed daily, and gained weight from start to the end of the study with no significant differences between the LR and HR recipients, neither before ($P = 0.8932$), nor after betamethasone treatment ($P = 0.8276$). None of the mice had to be euthanized due to the humane endpoint criteria.

Selection of donor mice. Topical OXA application resulted in a significant increase in CDS ($P = 0.001$) and ET ($P < 0.0001$) with development of microscopic skin inflammation in the affected ears. Based on the CDS, ET, and the HPS on the day of euthanasia, one HR donor mouse and one LR donor mouse were selected as GM donors (Figure 2). The HR donor mouse had cytokine concentrations above the 75% percentile in 11 out of 19 cytokines measured. Two of the cytokine concentrations were below the 25% percentile. The LR donor mouse had cytokine concentrations below the 25% percentile in 13 out of 19 cytokines (Figure 2).

Clinical signs after oxazolone sensitization. Before betamethasone treatment on day 7, the clinical signs of the recipients were scored and ET measured. Topical OXA application resulted in a significant increase in CDS and ET in both the LR recipients (CDS: $P < 0.0001$, ET: $P < 0.0001$) and the HR recipients (CDS: $P < 0.0001$, ET: $P < 0.0001$). HR recipients had significantly higher CDS ($P = 0.0439$) and ET ($P = 0.0353$) than did the LR recipients (Figure 3 A). Before the betamethasone treatment, a strong correlation between CDS and ET in both LR ($r = 0.7865$, $P = >0.0001$) and HR recipients ($r = 0.8144$, $P = 0.0005$) was evident.

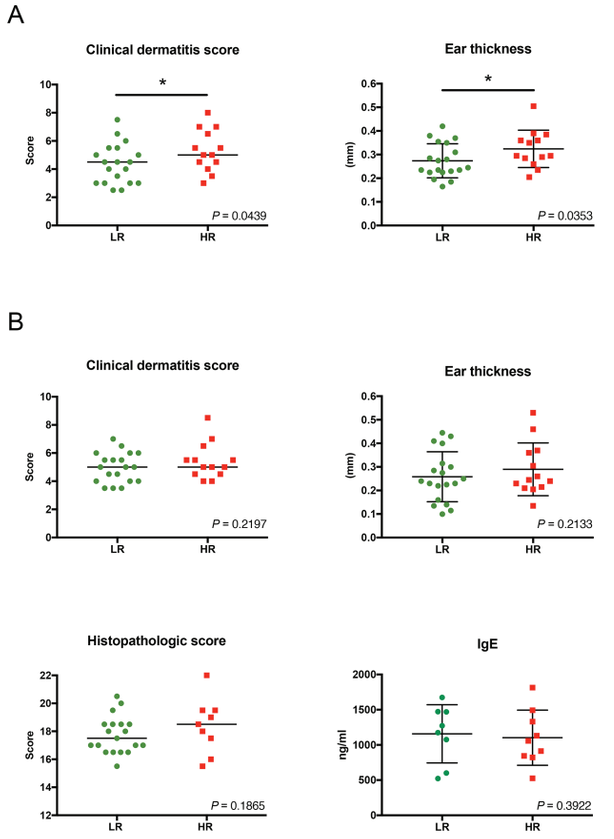

Figure 3. A. Clinical dermatitis score (* P = 0.0439) and ear thickness (* P = 0.0353) in high responding (HR) and low responding (LR) Tac:SW recipient mice before betamethasone treatment. B. Clinical dermatitis score (P = 0.2197), ear thickness (P = 0.2133), histopathologic score (P = 0.1865) and serum IgE (P = 0.3922) in high responding (HR) and low responding (LR) recipients after betamethasone treatment.

Clinical signs after betamethasone treatment. HR and LR mice more similar after betamethasone treatment. After betamethasone treatment, no significant difference were found between the LR and HR recipients in CDS (P = 0.2197), ET (P = 0.2133), HPS (P = 0.1865), and IgE (P = 0.3922) (Figure 3 B). Among the cytokines the only significant differences were for KC-GRO (P = 0.0204), and IL33 (P = 0.0355), for which HR recipients had significantly higher concentrations (Figure 4). When comparing the CDS and ET before and after betamethasone treatment, no significant differences were found (LR recipients: CDS P = 0.077, ET P = 0.3282; HR recipients: CDS P = 0.354, ET P = 0.2152) (Figure 5). HR recipients were regulated to the same level as the LR recipients, but the difference in clinical effect between the LR and HR recipients was not significant (CDS: P = 0.1246, ET: P = 0.3153). For obvious reasons, it was not possible to monitor cytokines in the ears before betamethasone treatment. However, LR recipients showed a strong correlation between several of the cytokines and CDS after betamethasone treatment (IL10: P = 0.0288, r = 0.4427; IL1β: P = 0.0277, r = 0.4462; IL2: P = 0.0009, r = 0.6685; IL4: P = 0.0358, r = 0.4223; KC/GRO: P = 0.0031, r = 0.6029; TNFα: P = 0.0006, r = 0.6888; IL31: P = 0.0303, r = 0.4383; IL33: P = 0.016, r = 0.4932), whereas among the HR recipients only KC/GRO (P = 0.0268, r = 0.5516) and IL21 (P = 0.0193, r = -0.585) showed correlation with CDS (Table 2). ET correlated with IL1β (LR recipients: P = 0.0393, r = 0.4133; HR recipients: P = 0.001, r = 0.7912), IL4 (LR recipients: P = 0.0158, r = 0.4941; HR recipients: P = 0.0237, r = 0.5659), and TNFα (LR recipients: P = 0.0331, r = 0.43; HR recipients: P = 0.0012, r = 0.7802) in both LR and HR recipients (Table 2).

Discussion

The aim of this study was to transfer a HR, and a LR phenotype in OXA induced AD mice with the GM to GF mice and subsequently study their response to a common treatment. We hypothesized that the clinical parameters would be similar in both LR and HR inoculated mice after treatment with betamethasone. As previously described,[37] we observed that HR recipients exhibited higher CDS and ET, compared with LR recipients, and in this study, after betamethasone treatment these differences could no longer be shown in either recipient cohort. Also, there were no differences in HPS, and all cytokine concentrations, except for KC-GRO and IL33, for which HR recipients had still significantly higher concentrations compared with LR recipients.

The average effect of betamethasone on the clinical signs was rather weak. However, the cytokine concentrations were generally low in the recipients compared with donor mice. Th1 cytokines IL1β, TNFα and the Th2 cytokine IL4 have previously been shown to be important for the OXA mouse model[37] and to correlate strongly to GM.[21] The concentrations of IL1β, TNFα, and IL4 in donor mice were above the 75% percentile in the HR donor mouse and below the 25% percentile in the LR donor mouse, thereby showing correlations also existed in the donor mice. This emphasizes the importance of these cytokines and most importantly, their relationship to the GM composition, in this AD model, supporting previous findings.[37] We have previously observed correlation between ET and IL1β, IL4, TNFα and KC/GRO in this model, and this was also observed in this study, although LR recipients did not show correlation between ET and KC/GRO. The positive correlations in the recipients correspond well to the pathophysiology of AD. The cytokines IL17A,[25,28] IL17F, IL22, IL17C, IL21,[17] and IL5[18] correlated negatively to CDS, ET, and HPS. Th2 cytokines including IL4 and the Th1 cytokine IFNγ work as negative regulators on IL17A,[28] which may explain the negative correlation of this cytokine. IL17F, IL21, and IL22 are also secreted from Th17 cells and our results could therefore indicate that these Th17 cytokines also are negatively regulated. The negative correlations may eventually also be explained by the betamethasone treatment having suppressed the cytokine production. As the cytokine concentrations were generally lower in the recipients compared with the donors, it is reasonable to assume that the decreased cytokine concentrations are an effect of the betamethasone treatment,[34] and these data together with the changed correlation pattern in the HR recipients, indicates that CDS and ET has a prolonged recovery, and even though the inflammation is dampened, it takes time for the ears to heal.

The HR recipients had significantly higher concentrations of IL33 than the LR recipients, in spite of the betamethasone treatment. Human AD patients overexpress IL33[35] and in another mouse model, IL33 was overexpressed in acute AD skin lesions.[32] Our results, therefore, indicate that the same may apply in the OXA model, especially in mice with a HR phenotype. The HR recipients also had significantly higher concentrations of KC/GRO than the LR recipients, and after betamethasone treatment KC/GRO still correlated to CDS comparably to LR recipients, although cytokine CDS correlations generally were not present in the HR recipients to the same extent as in the LR recipients. KC/GRO is important in the recruitment of neutrophils to inflammatory sites.[38] Some individual mice in the HR

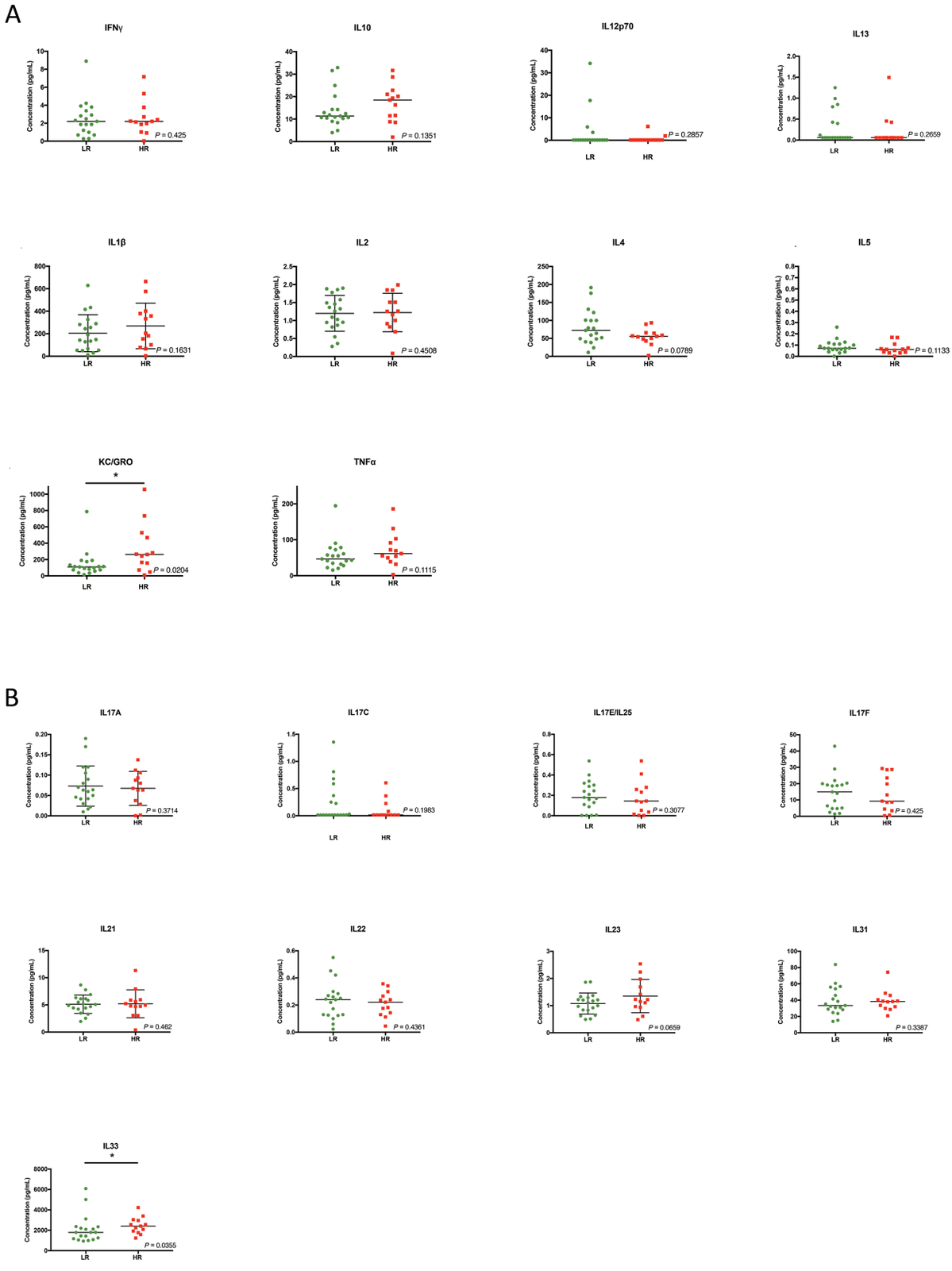

Figure 4. Levels of cytokines related to type 1 and type 2 helper, regulatory, natural killer and cytotoxic t cells, macrophages and neutrophils (A) and T helper cell type 17 related cytokine levels (B) in high responding (HR) and low responding (LR) Tac:SW recipients after betamethasone treatment. Asterisks (*) show significant differences calculated between the LR and HR recipients.

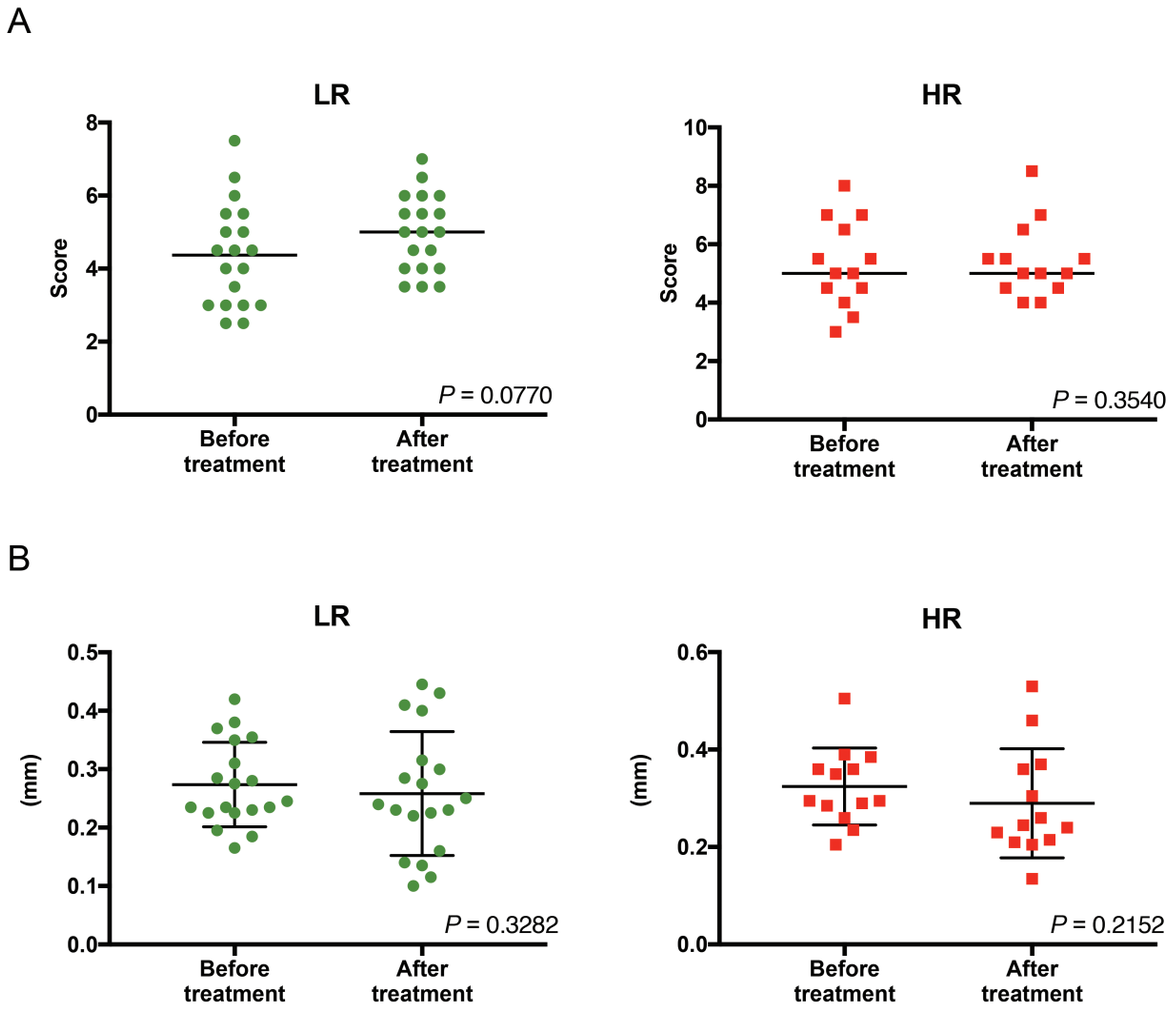

Figure 5. Comparison of the clinical dermatitis score (A) and ear thickness (B) of the low responding (LR) and the high responding (HR) Tac:SW recipients before and after betamethasone treatment.

recipient group had much higher concentrations of KC/GRO in comparison with donor mice, indicating a potentially poor effect of the betamethasone treatment on this particular cytokine. The concentrations of the Th1 cytokines IFNγ, IL1β and TNFα and the Th2 cytokines IL4 and IL10, in both donor mice and recipients, indicated that the inflammation was in the transition from a Th1 inflammatory response to a Th2 inflammatory response, which is also characteristic for the OXA model.[23]

Originally, we found a strong correlation between GM composition and cytokine concentrations.[21] Therefore, these were applied for selection of donors in our first attempt to transfer a HR and LR phenotype with the gut microbiota, as previously published.[37] However, in that study, CDS and ET phenotype was nicely transferred with the GM, and therefore in the present study HR and LR donor mice were selected using CDS as the primary readout, which afterward was confirmed by the cytokine concentrations. ET and CDS correlated well with one another, and also with the cytokines in the LR recipients. As the LR and HR phenotypes were also nicely transferred in the present study, this shows that CDS and ET are also good selection criteria. This is a faster method than measurement of cytokine concentrations and could, therefore, be used in future studies.

In the present study, the inoculum transferred to recipients contained fecal pellets collected from different days after OXA applications were started. The fact that both LR and HR phenotypes were successfully transferred, could indicate that the bacteria which plays a role in HR and LR phenotypes, are still present. In another study, in which sensitivity to AD phenotypes were transferred with GM, feces was collected before starting OXA challenges. This study showed a highly significant difference between mice with LR and HR phenotypes. These mice were challenged for 3 wk after sensitization,[37] compared with mice used in our study, which were challenged for one week before initiating the betamethasone treatment, which might explain why the difference between our LR and HR recipients was smaller although still significant. Also, the age and strain of the mice should be considered in relation to the weak response of betamethasone. Young Swiss Webster mice, which were the ones available as GF for our study, have not been used as OXA model before, to our knowledge. Because local skin inflammation was successfully induced,our results show that the model can be induced in this strain. However, the treatment effects may be smaller than in BALB/c and C57BL/6 mice, which are more commonly used for this model.

It is probably not realistic to inoculate individual mice before each study in the future, and it would also be problematic

Table 2. Spearman correlation on clinical dermatitis score, ear thickness, histopathologic score and cytokine concentrations after betamethasone treatment of Tac:SW microbiota recipient HR and LR mice with oxazolone induced dermatis on the ear.

	CDS		ET		HPS	
	r	*P* value	r	*P* value	r	*P* value
			LR recipients			
CDS			**0.4978**	0.015		
ET	**0.4978**	0.015				
IL10	**0.4427**	0.0288				
IL1β	**0.4462**	0.0277	**0.4133**	0.0393		
IL2	**0.6685**	0.0009				
IL4	**0.4223**	0.0358	**0.4941**	0.0158		
KC/GRO	**0.6029**	0.0031				
TNFα	**0.6888**	0.0006	**0.43**	0.0331		
IL17A					*-0.3946*	0.0473
IL17F			*-0.4256*	0.0346		
IL22			*-0.4021*	0.0439		
IL31	**0.4383**	0.0303				
IL33	**0.4932**	0.016	**0.4511**	0.0263		
			HR recipients			
IL1β			**0.7912**	0.001		
IL4			**0.5659**	0.0237		
IL5					*-0.6946*	0.0221
KC/GRO	**0.5516**	0.0268	**0.7582**	0.0019		
TNFα			**0.7802**	0.0012		
IL17C			*-0.7785*	0.001		
IL21	*-0.585*	0.0193				

Positive correlations are shown in bold and negative correlations in italics. Abbreviations: HR High responding; LR Low responding; CDS Clinical dermatitis score; ET Ear thickness. HPS Histopathologic score.

to create a large pool of the same microbiota. We know that mice can be inoculated and bred to still maintain colonization rates up to 80% for generations in IVC systems.[20] However, we know from other disease models that the loss of just a single bacteria, such as *Akkermansia muciniphila* in type 1 diabetic NOD mice,[12] and *Faecalibacterium prausnitzii* in colitis-associated cancer mouse models,[16] may have a significant impact on model expression. Future studies should identify bacteria related to the development of atopic dermatitis, before HR colonies can be set up, and monitored currently to ensure their status.

We conclude that this study confirms that the phenotype of the OXA AD mouse model is transferable with the GM and that HR recipients can be treated to a clinical level comparable with the LR recipients. Further studies will be needed to state whether the production of HR recipients for this model can be used to optimize power and group sizes.

Acknowledgments

We thank Helene Farlov, Mette Nelander, Susanne Kronborg, and Joan Frandsen for technical support, and Randi Lundberg, Susanne Knoth Clausen and Pernille Christensen for sharing their experience with this animal model.

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