Title Page

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Title: The Role of IL-6 in Irritant dermatitis (1R01OH010241-01A1)

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Final Report Abstract:

Of reported occupational injury associated with workman's compensation, contact dermatitis ranks second most prevalent over all, and irritant contact dermatitis (ICD) is the most common. If specific immune mediators can be associated with irritancy, this will be of significant predictive value when judging the response of an individual to irritant exposure. One such mediator, interleukin 6 (IL-6) is closely associated with skin maintanence. Thus, the hypothesis was proposed that modulation of skin IL-6 levels contributes to severity of dermatitis. Genetic analysis from a database of ~600 volunteers exposed to irritants, indeed showed that certain changes in IL-6R and related genes, were associated with ICD severity. Using mouse models, the protective effects of IL-6 are associated with altered barrier and/or inflammatory mediator expression were investigated. Using three genetically altered strains of mice; one that lacks IL-6, one that lacks the receptor for IL-6 in skin, and one that lacks the receptor in inflammatory cells, it was found that irritant dermatitis was worse in all three strains of mice using two chemically distinct irritants. Further, while each mouse strain had a specific inflammatory mediator and cell type signature associated, specific common genes appeared to be associated with severe ICD and may prove to be predictive markers for risk assessment in humans.

Since humans vary from one another by multiple gene changes resulting in wholistic immune propensities or "phenotypes", two mouse strains of known immune phenotypes were also assessed for their response to irritants. The C57BL/6 mouse strain is known to show a propensity towards anti-viral immunity, whereas the Balb/c mouse has an anti-parasitic immune preference. ICD was induced in these mice, and inflammation was evaluated. Indeed, it was found that C57 mice had worse ICD as compared to Balb/c and the skin inflammatory mediators and cell types varied greatly between mouse types, again indicating specific inflammatory signatures that can be associated with ICD severity. One mediator in particular, IL-22, was found to be closely associated with skin thickening, a particularly troublesome result of ICD, throughout the mouse models and may serve as both a biomarker for risk assessment as well as a target for therapy. To investigate these inflammatory signatures in much greater depth, skin dermatitis samples from the IL-6 deficient (genetic), as well as the C57BL/6 and Balb/c (phenotypic) mice were analyzed for very broad gene expression using NextGen RNA sequencing (RNAseq). This analysis showed ~3 million gene expression changes associated with each immune phenotype and ICD severity, indicating differences as well as commonality in expression that should be of great use in human risk assessment. Further, the skin bacterial populations were also assessed by sequencing, and profound changes were found to be associated with both immune phenotype as well by mere IL-6 deficiency, that may contribute to ICD severity. This shows further that ICD is not just a product of the skin, but its associated microbiome. The knowledge gained from these investigations will provide the basis for future intervention research to reduce the incidence of occupationally related irritant contact dermatitis.

Section 1

Significant or Key Findings.

Specific Aim One: Determining the role of IL-6 in protective gene expression in irritant dermatitis. The hypothesis for the first specific aim was that IL-6 was protective during ICD, and this protection could manifest itself through altered barrier and/or inflammatory gene expression.

Key Finding: Specific cytokines are associated with IL-6 function in ICD. Dermatitis lesions and control skin were assessed for analyses 36 cytokines at once. Very specific cytokine gene expression changes occur in association with IL-6 deficiency. Following exposure to the irritant benzalkonium chloride (BKC), IL-6 deficient skin (IL-6KO) displayed reduced expression of the chemokines CCL3, CCL5 and CCL11. As well, following exposure to the irritant JP-8 jet fuel IL-6 deficient skin exhibited reduced expression of CCL7 and CCL11. Interestingly, these cytokines can attract eosinophils into tissues, yet no differences in eosinophils themselves were detected during ICD in either deficient or control mice. Samples from IL-6KO as well as the C57BL/6 and Balb/c (phenotypic) mice were analyzed for very broad gene expression using NextGen RNA sequencing (RNAseq). This analysis showed ~3 million gene expression changes associated with each immune phenotype and ICD severity, indicating differences as well as commonality in expression that should be of great use in human risk assessment, and these results are detailed in an article (Luckett-Chastain, 2018).

Key Finding: Specific inflammatory cells are associated with IL-6 function in ICD. Inflammatory cell types were also assessed in skin via flow cytometric analysis. Following BKC exposure, decreased macrophage, neutrophil, NK cell, and γδ T cell populations were apparent in IL-6 deficient skin as compared to wild-type mice, while JP8 exposure resulted in a greater dendritic cell influx in deficient skin.

Specific aim two: Determine if treatment with IL-6 or IL-6 modulated factors protects against dermatitis. The purpose of this aim was to determine if a treatment could be formulated utilizing IL-6 or associated protective factors. One tactic was to produce AAV vectors that would induce IL-6 expression in mouse skin. This tact was disappointing as while mRNA for IL-6 could be induced in skin, this induction did not result in increased expression of the cytokine protein. Administration of IL-6 was initially attempted but not further pursued as intradermal injection of the cytokine was deemed impractical for translation to human patients based on extreme cost and difficulty (intradermal injection) of administration.

Key Finding: Specific chemokines may mediate ICD skin thickening (acanthosis). This aim also proposed to examine the function of IL-6 induced gene products as possible treatments. Indeed various chemokines are downregulated in IL-6 deficient skin during ICD, indicating IL-6 may act in a protective manner by upregulating these cytokines. It was found that specific chemokines reduce keratinocyte proliferation in culture, and decrease the expression of keratin 1 which is a marker of differentiation, indicating they may be associated with protection against ICD induced skin thickening and targets for therapy development.

Specific aim three: Determine if there is an association between IL-6 polymorphisms and dermatitis susceptibility. The goal of this specific aim was to investigate how ICD could vary between individuals based on IL-6 expression changes.

Key Finding: IL-6R gene polymorphisms are associated with ICD. The first subaim was to examine the genetic data previously collected by our collaborator Dr. Yucesoy, for associations with IL-6 gene mutations and severity of ICD in that study population. Six polymorphisms were found to be significantly associated with ICD. Three were located in introns, and thus their effects are unknown. Two were in the IL-6R and may affect function; rs4845617 or G-208A which affects cleavage of the receptor from the membrane, and rs6427641 the function of which is unknown. SOCS3 can suppress IL-6R function, and on snp was found that can affect its transcription (rs4969168). The next sub aim was to attempt to translate these genetic polymorphisms into an animal model. Based on the uncertainty of reproducing the exact human effects of a specific mutation into an animal, and the fact that the exact function of several of the polymorphisms was unknown, it was decided to utilize genetic models of more certainty. As several snp's were in the IL-6Rα gene, models were chosen that would delineate its role from a skin centric or inflammatory cell centric view.

Key Finding: IL-6R deletion in keratinocytes exacerbates ICD. Mice with tamoxifen inducible (K14-Cre) knockout of IL-6R(KO) were produced and displayed much worse ICD as compared to control in response to both BKC and JP8 in terms of both epidermal hyperplasia as well as inflammatory cell infiltration. Skin inflammatory cell populations from IL-6RKOmice were characterized as having greater populations of inflammatory monocytic cells and fewer of the γδT Cells which play a role in homeostasis and healing.

Inflammatory cytokine expression was altered as well where following BKC exposure transgenic mice had higher IL-1, IL-6, and IL-22 expression, and following JP8 exposure higher IL-1 and IL-18 was apparent. Further, anti-inflammatory cytokine expression (IL-10 and IL-14) was decreased in IL-6R transgenic mice.
Key finding: IL-6R deletion in myeloid cells exacerbates ICD to a lesser extent than epidermal deletion. Mice possessing tamoxifen inducible IL-6R knockout in all myeloid cells (LysM-Cre) were produced and as well displayed worse ICD as compared to control in response to both BKC and JP8 in terms of both epidermal hyperplasia as well as inflammatory cell infiltration, but at a lesser severity than K14-KO. These mice also displayed a greater influx of monocytic cells, and more broadly altered chemokine expression with decreases in CXCL1, 2, 10, CCL3, 5, and 7 as compared to WT.
Key finding: IL-6R modulates IL-22 function in epidermis. Of the cytokines found altered in these transgenic mice, IL-22 is most closely associated with epidermal keratinocyte function and is known to induce epidermal thickening. Epidermal keratinocytes from IL-6 deficient mice were cultured, treated with IL-6 and examined for IL-22 function. Indeed, IL-22R expression was induced by IL-6 treatment, as was IL-22 sensitivity as measured by increased proliferation and keratin 1 expression.
Key Finding: Immune phenotype is associated with specific cytokine and cell type signatures. As IL-6 is classified as a Th2 or anti-parasitic pro-allergy cytokine, it was of interest if more broadly defined Th phenotypic mice showed alterations in ICD severity. This was of significant interest as humans are more likely to be polygenically defined immunologically rather than by an alteration in a single gene. Thus, Th1 phenotype (anti-viral, pro-inflammatory) C57BL/6 mice and Th2 phenotype (anti-parasitic, pro-allergy) were evaluated for ICD, where the Th1 phenotype might be loosely analogous to an IL-6 deficient genotype. Indeed, the Th1 phenotype showed more severe ICD as compared to Th2, and each had very different immune cell and cytokine expression signatures in skin.
Key Finding: Chemical characteristics of irritants produce greatly altered inflammatory signatures. It is well accepted that irritants of differing chemical nature induce different levels of ICD. The comparison of BKC to JP8 defined these differences where the former is a detergent and the later a petroleum mixture. Indeed, BKC is defined by influx of monocytic cells, whereas jet fuel altered $\gamma\delta$T Cells and dendritic cells.
Key Finding: To investigate these inflammatory signatures in much greater depth, skin dermatitis samples from the IL-6 deficient (genetic), as well as the C57BL/6 and Balb/c (phenotypic) mice were analyzed for very broad gene expression using NextGen RNA sequencing (RNAseq). This analysis showed ~3 million gene expression changes associated with each immune phenotype and ICD severity, indicating differences as well as commonality in expression that should be of great use in human risk assessment. Further, the skin bacterial populations were also assessed by sequencing, and profound changes were found to be associated with both immune phenotype as well by mere IL-6 deficiency, that may contribute to ICD severity. This shows further that ICD is not just a product of the skin, but its associated microbiome.
Translation of Findings. Based on the K2A Framework, this discovery work would fit squarely in the research phase, and thus direct application to clinical practice or workplace is as yet beyond the scope of these results. However, interventions could be developed in the future based on these findings. As it is convincingly shown herein that IL-6 or IL-6R deficiency is closely associated with ICD severity, and IL-6 polymorphisms are statistically associated with ICD development in humans, a genetic test could be explored. If workers are found to possess IL-6 associated snp's they may require altered PPE, work duties and/or additional training if irritants are known to be present. Further, it may be found that workers with a Th1 propensity endure worse ICD and thus require altered PPE or training. Although, further research would be necessary before the implications of these immune phenotypes are determined. Two very promising clinical applications may come from the findings that specific eosinophil chemotractants as well as IL-22 are closely associated with ICD acanthosis. Blockade of these mediators may prove to be very promising treatments, especially if small molecule pharmaceuticals could be developed and applied in a topical form.
Research Outcomes/Impact. There are several potential outcomes related to this research. First, as stated above, genetic testing for IL-6 related polymorphisms could inform the worker and clinician of a workers propensity towards developing ICD and its potential severity. From this information, measures could be taken to reduce risk such as specialized PPE, training, and reduced exposure. Immune tests to evaluate immune phenotype (ie. allergy testing) may also inform the worker of their risk.

Section 2

Scientific Report

Specific Aim One: Determining the role of IL-6 in protective gene expression in irritant dermatitis. The hypothesis for the first specific aim was that IL-6 was protective during ICD, and this protection could manifest itself through altered barrier and/or inflammatory gene expression. Several significant findings presented themselves. Overall, it was found that specific cytokine signatures are associated with IL-6 deficiency, and inflammatory cell populations seem to reflect these signatures.

Irritants induce epidermal hyperplasia & cellular infiltration in WT and IL-6 deficient skin

Chemical irritants cause ICD through different inflammatory pathways, and are an extrinsic factor that contributes to the clinical variability associated with ICD. Thus, in order to more broadly investigate the role of IL-6 deficiency on the pathophysiology of ICD, both BKC and JP-8 exposures were evaluated. 3-day exposure duration was utilized to assess the early onset of ICD, which influences the chronic form of the disease that was previously investigated.

Following 3-days of irritant exposure, both WT and IL-6$^{-/-}$ skin exhibited erythema. However, IL-6$^{-/-}$ skin manifested a more severe form of ICD that included pronounced dryness and fissuring (not shown). Histopathology revealed that in response to BKC and JP-8 exposure WT and IL-6$^{-/-}$ mice had significantly elevated cellular infiltration as compared to controls (Figure 1A, B). Significant epidermal hyperplasia was evident in WT and IL-6$^{-/-}$ mice following JP-8 exposure, but not BKC (Figure 1A, C). However, irritant induced cellular infiltration and epidermal hyperplasia, did not appear to significantly differ between WT and IL-6$^{-/-}$ strains overall (Figure 1B, C).

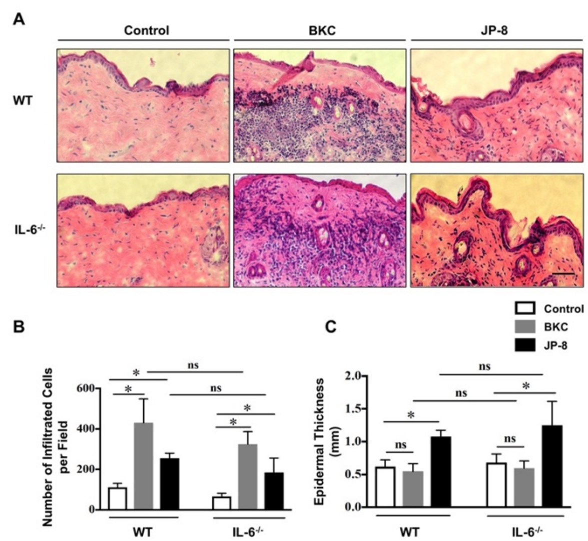


Figure 1. Irritants Induce Epidermal Hyperplasia & Cellular Infiltration in WT and IL-6 Deficient Skin. WT and IL-6$^{-/-}$ mice were exposed to control, 2% BKC, or JP-8 jet fuel for 3 consecutive days at a 2-hour interval each day (A). Twenty-four hours post final exposure; 4-mm biopsies of the exposed skin were collected for histopathology. Cellular infiltration (B) and epidermal thickness (C) were determined on paraffin embedded H&E stained section. Image J (NIH) was utilized to determine cellular infiltration and epidermal thickness. Data presented as mean ± SD (n=15 per treatment per mouse phenotype; *$p \leq 0.05$; ns, not significant), scale bar = 1mm.

IL-6 deficiency alters skin immune cell populations following irritant exposures

Previously, IL-6 has been shown to impact cellular recruitment during peritoneal inflammation; however, this has not yet been extensively evaluated in the context of irritant contact dermatitis. Flow cytometric analysis of cells isolated from dermatitis lesions revealed that in response to BKC exposure, WT skin had significantly greater presence of neutrophils (Ly6G^{+}), macrophages (F4/80^{+}), T cells (CD3^{+}, CD49b^{-}) and NK cells (CD3^{-}, CD49b^{+}) as compared to IL-6$^{-/-}$ skin (Figure 2A, B, D). However, dendritic cell (CD11c^{+}, MHCII^{+}) presence did not vary significantly between WT and IL-6$^{-/-}$ skin (Figure 2C). In contrast to BKC exposure, JP-8 exposure elicited a significantly elevated presence of dendritic cells in IL-6$^{-/-}$ mice compared to WT (Figure 3C). However, neutrophil, macrophage, T cell and NK cell presence did not differ significantly between WT and IL-6$^{-/-}$ mice following JP-8 exposure (Figure 3A, B, D). Eosinophils, basophils, and B cells were not detected in either BKC or JP-8 exposed skin of WT and IL-6$^{-/-}$ mice (data not shown).

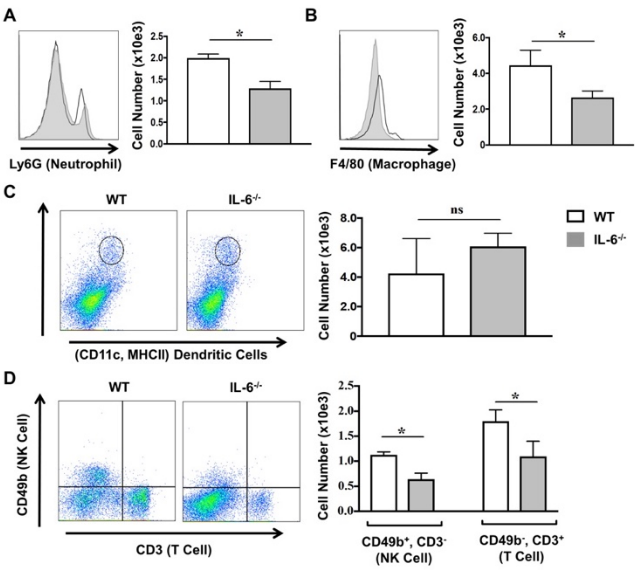


Figure 2. IL-6 Deficiency Alters the Immune Cell Presence Following BKC Exposure. WT and IL-6$^{-/-}$ skin was exposed to BKC for 3 days. Skin samples were processed and immune cells isolated for flow cytometric analysis, where neutrophil (A), macrophage (B), dendritic cell (C), T cell (D), and NK cell (D) populations were determined. Representative histograms and scatter plots for each are shown. Data presented as mean ± SD of percent positive, (n=5 pooled samples; *$p \leq 0.05$; ns, not significant).

Of the aforementioned cells, CD3$^+$ cells represented a substantial percentage of immune cells detected within irritant exposed skin (Figure 2D & 3D). The role of lymphocytes in ICD has not been well defined; however, the skin has been shown to contain resident CD3$^+$ cells capable of modulating the cutaneous immune reaction . Additionally, IL-6 is known to have a multitude of effects on T cell activity]. Therefore, the CD3$^+$ cells present in the exposed skin of WT and IL-6$^{-/-}$ mice were further evaluated to determine their phenotype. Interestingly, it was found that the CD3$^+$ cells also expressed the gamma-delta (γδ) T cell receptor (Figure 4A). Indeed, following BKC exposure WT skin had significantly increased presence of γδ TCR cells compared to IL-6$^{-/-}$ (Figure 4B, C). However, γδ TCR cell presence did not vary between IL-6$^{-/-}$ and WT skin following JP-8 exposure (Figure 4B, C).

IL-6 deficiency decreases cutaneous cytokine expression following irritant exposures

The acute inflammatory response that characterizes ICD is modulated by cytokines, and differential cytokine production is postulated to be responsible for the pathological variability associated with ICD. It is unknown if the protective effects of IL-6 during ICD are the result of an altered cytokine profile. After 3-days of BKC exposure, both WT and IL-6$^{-/-}$ skin had increased expression of IL-1β, IL-22, G-CSF, LIF, TGF-βI, TNF-α, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CXCL1, CXCL5 and CXCL10, but decreased expression of IL-1α and IL-23 (Figure 5A). However, expression of the chemokines, CCL3, CCL5 and CCL11, was decreased in BKC-exposed IL-6$^{-/-}$ skin compared to WT (Figure 5A-B). Interestingly, only CCL11 was increased and IL-23 decreased

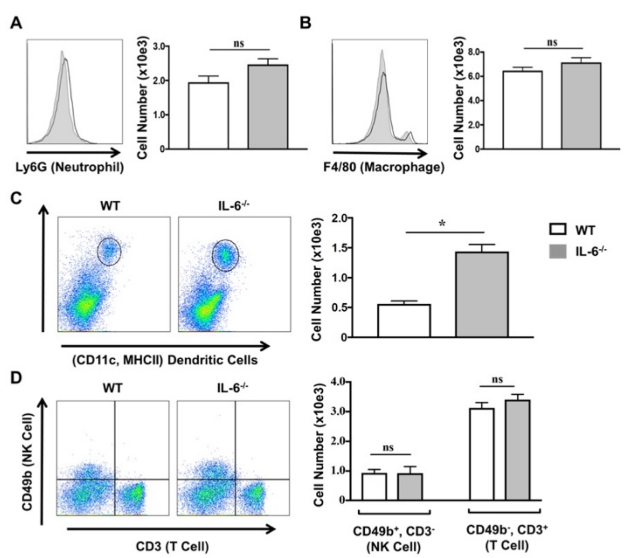


Figure 3. IL-6 Deficiency Alters the Immune Cell Presence Following JP-8 Exposure. WT and IL-6$^{-/-}$ skin was exposed to JP-8 jet fuel for 3 days. Skin samples were processed and immune cells isolated for flow cytometric analysis, where neutrophil (A), macrophage (B), dendritic cell (C), T cell (D), and NK cell (D) populations were determined. Representative histograms and scatter plots for each are shown. Data presented as mean ± SD of percent positive, (n=5 pooled samples; *$p \leq 0.05$; ns, not significant).

following JP-8 exposure in both WT and IL-$6^{-/-}$ skin as compared to control exposures (Figure 5A, Table 1). However, expression of CCL7 and CCL11 was decreased in JP-8 exposed IL-$6^{-/-}$ skin as compared to WT (Figure 5A-B).

Given the effect of IL-6 deficiency on chemokine expression, corresponding receptor mRNA expression was evaluated. BKC exposure resulted in significantly up-regulated expression of CCR1 and CCR3 in WT skin compared to IL-$6^{-/-}$ (Figure 5C), while CCR2 and CCR5 expression did not vary between strains (Figure 5C). JP-8 exposure did not alter CCR1, CCR2 or CCR3 mRNA expression between WT and IL-$6^{-/-}$ skin (Figure 5C).

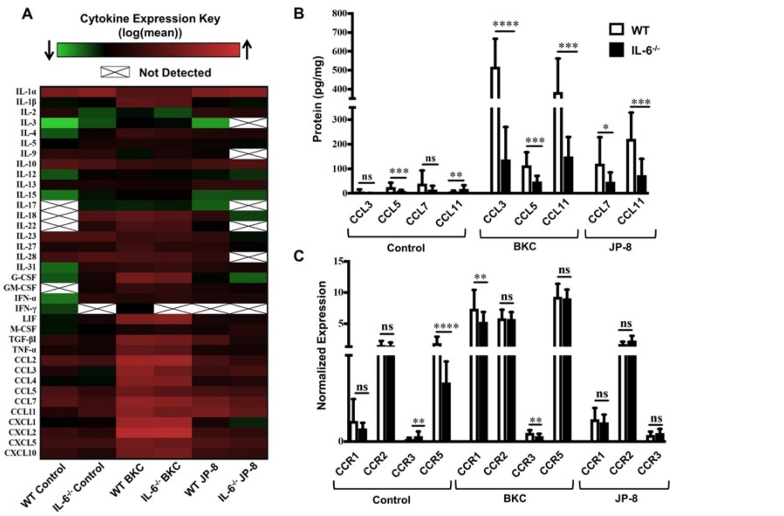


Figure 5. IL-6 Deficiency Alters Cutaneous Cytokine Expression Following Irritant Exposures. WT and IL-$6^{-/-}$ skin was exposed to irritants for 3 consecutive days. Biopsies of irritant exposed skin were obtained and cytokine protein expression (A, B) was quantified by Luminex Multiplex Assay, and chemokine receptor mRNA expression (C) by QuantiGene Plex Assay. Values represent log of the cytokine mean protein expression for heatmap (A), and mean ± SD for graphs (B, C) (n=15 mice per treatment per mouse phenotype; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; ns, not significant).

Specific aim two: Determine if treatment with IL-6 or IL-6 modulated factors protects against dermatitis. The purpose of this aim was to determine if a treatment could be formulated utilizing IL-6 or associated protective factors. One tactic was to produce AAV vectors that would induce IL-6 expression in mouse skin. This tact was disappointing as while mRNA for IL-6 could be greatly induced in skin, this induction did not result in reliably and predictably increased expression of the cytokine protein (not shown). Indeed, irritant exposed skin can display is many fold increases in transcript but little cytokine produced (unpublished observations). This result in and of itself is interesting, as it appears that IL-6 protein production is not directly associated with IL-6 gene transcription, at least in the skin. Thus, many articles that utilize mRNA as an indicator of IL-6 production may very well be mistaken (although that is another story). It was also proposed to treat animals with recombinant IL-6 to determine if this was a viable intervention. Administration of IL-6 was initially attempted but not further pursued as intradermal injection of the cytokine was deemed impractical for translation to human patients based on extreme cost and difficulty (intradermal injection) of administration. However, it was quite probable that IL-6 was not mediating its effects directly. Thus, this aim also proposed to examine the function of IL-6 induced gene products as possible interventions. Indeed, was found that the eotaxin-related chemokines CCL11 and CCL24 are induced by IL-6 during ICD and these mediators may be protective by modulating epidermal keratinocyte function and proliferation.

Effects of CCL24 and CCL26 on Keratinocyte Proliferation and function. Keratinocyte are known to express the chemokines CCL24 (eotaxin-2) and CCL26 (eotaxin-3) and eotaxin-like chemokines appear to be downregulated in IL-6 deficient mice during ICD. To evaluate the function of these chemokines on epidermal cells, human newborn epidermal keratinocytes (HEKn) were subjected to scratch assay to initiate damage analogous to erosive irritants. Total RNA was isolated from keratinocytes 0 hours, 30 minutes, 1 hour, and 2 hours post-scratch, and quantitative PCR was used to measure CCL24 and CCL26 mRNA expression. Expression of CCL26 was significantly up-regulated in keratinocyte cultures 2 hours post-wounding (Figure 6B); however, CCL24 did not differ significantly between wounded keratinocytes and control (Figure 6A). As well, the effect of CCL24 and CCL26 on keratinocyte proliferation and function was evaluated. Treatment with either CCL24 or CCL26 at concentrations of 20ng/mL, 50ng/mL and 100ng/mL significantly reduced keratinocyte proliferation (Figure 6C, D). However, treatment with either CCL24 or CCL26 did not significantly alter keratinocyte function as determined by migration (Figure 7E, F). Thus, treatment with eotaxin receptor agonists or modulation of the expression of these chemokines may be an attractive intervention for ICD induced acanthosis.

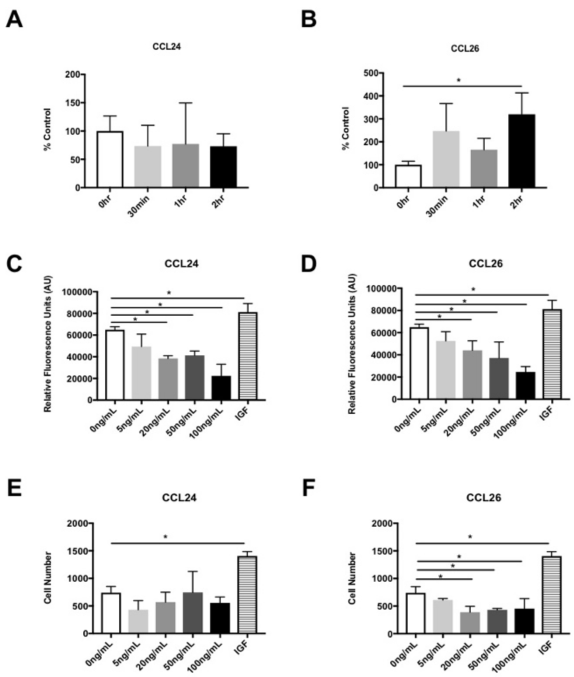


Figure 6: Effect of CCL24 & CCL26 on Keratinocyte Proliferation and Migration. Cells (HEKn) were grown to confluency and wounded. Total cellular RNA was prepared as previously described, and expression of CCL24 (A) and CCL26 (B) mRNA was determined by real-time polymerase chain reaction. For evaluation of proliferation (C, D) and migration (E, F) keratinocytes were treated with CCL24, CCL26, or IGF as positive control, for 16hours. Cells were subjected to PrestoBlue for determination of proliferation (C, D) or were labeled with calcein-AM to determine cell migration from images using ImageJ. Bar graphs are expressed as mean $\pm$ SD, n=3 replicate experiments, * $p < 0.05$ versus control (0hr (A-D) or untreated (C- F)).

Specific aim three: Determine if there is an association between IL-6 polymorphisms and dermatitis susceptibility. The goal of this specific aim was to investigate how ICD could vary between individuals based on IL-6 expression changes. The first subaim of this aim was to examine the genetic data, that our collaborator Dr. Yucesoy previously collected (Yucesoy, et al., 2016, PMID:27206134) for associations with IL-6 gene mutations and severity of ICD in that study population. The study population was from 200-600 volunteers exposed to irritants via patch test for 48 hours, and evaluated for signs of contact dermatitis. Upon statistical analysis, six polymorphisms were found to be significantly ($p \leq 0.05$) associated with ICD (Table 1). Three were located in introns, and thus their effects are unknown. Two were in the IL-6R and may affect function; rs4845617 or G-208A which affects cleavage of the receptor from the membrane, and rs6427641 the function of which is unknown. SOCS3 can suppress IL-6R function, and one snp was found that can affect its transcription (rs4969168). While at this point the exact function of these mutations in skin is not known, the fact that there is a significant association with ICD incidence supports the hypothesis of this project and lends translational validity to our observations. A manuscript describing the methods and analysis of these genetic data is currently be prepared by Dr. Yucesoy.

Table 1. Human Gene Polymorphisms Associated with ICD Development from Patch Test.

Gene	Location	Mutation	snp ref#	n	p value
IL6R	**5'-UTR**	**G/A**	**rs4845617**	**587**	**0.0309**
IL6R	**5'-UTR**	**G/A**	**rs6427641**	**587**	**0.0287**
IL6R	**intron**	**A/C**	**rs7518199**	**197**	**0.0311**
Socs3	**3'-UTR**	**A/G**	**rs4969168**	**588**	**0.0285**
STAT3	**intron**	**T/C**	**rs12949918**	**591**	**0.0199**
STAT3	**intron**	**T/C**	**rs4796791**	**601**	**0.0307**

Unfortunately, the lack of functional information about these snp's also complicated the next subaim, which proposed to examine analogous mutations in mouse skin. As there was significant uncertainty of reproducing the exact human effects of a specific mutation in an animal, and the fact that the function of several of the polymorphisms was unknown, it was decided to utilize genetic models of more certainty. As several snp's were in the IL-6Rα gene, models were chosen that would delineate its role from a skin centric or inflammatory cell centric view. To accomplish this, mice were acquired from Jackson Labs that contained a floxed IL-6Ra gene. These mice were mated with a strain containing either a tamoxifen inducible (K14-Cre) gene for specific keratinocyte KO or containing the LysM gene which would target myeloid cells. Sibling matings were performed to produce a homozygous strains with cell specific inducible IL-6RαKO.

$Il6ra^{\Delta ker}$ mice show increased epidermal hyperplasia and immune cell infiltration following irritant exposure. To investigate how IL-6Rα influences epidermal hyperplasia and leukocyte infiltration during ICD, mice with a keratinocyte-specific knockout of the IL-6Rα ($Il6ra^{\Delta ker}$) and littermate control (WT) mice were exposed to benzalkonium chloride (BKC) and Jet Propellant 8 (JP-8) fuel, two well-characterized occupational irritants for a period of three (3) consecutive days. Acetone was applied as control. Lesional skin was harvested from irritant-exposed mice and used for histological analysis.

Quantitative image analysis of Hematoxylin and eosin (H&E) stained lesional skin from mice revealed that $Il6ra^{\Delta ker}$ mice presented with an exaggerated response to BKC and JP-8 fuel relative to WT (Fig.1A) characterized by increased epidermal hyperplasia (Fig. 7 A,B). Also, both JP-8 and BKC induced a significant infiltration of leukocytes cells into lesional skin (Fig. 7C). This occurrence was more pronounced in BKC treated skin relative to JP-8 and control treatments (Fig. 7B, C red versus blue bars).

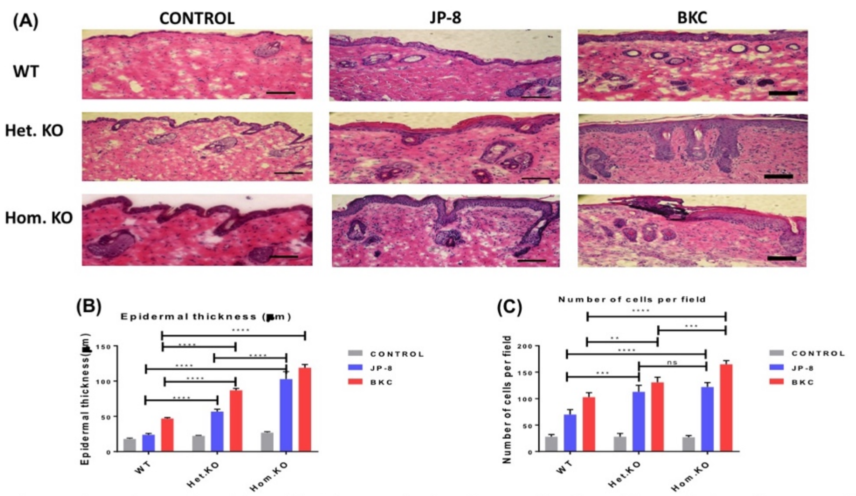


Figure 7: loss of IL-6Rα in epidermal keratinocytes leads to increased epidermal hyperplasia and immune cell infiltration during ICD.
WT and $Il6ra^{\Delta ker}$ mice were exposed to BKC, JP-8 or control for three (3) consecutive days to induce ICD. 24 hours after irritant exposure, 4-mm biopsies of lesional skin were collected and embedded in O.C.T compound for histological analysis. Skin samples were cross-sectioned, and then Hematoxylin and eosin (H&E) stained. Representative H&E stains from $Il6ra^{\Delta ker}$ and WT are shown (A).Quantification of epidermal thickness (B) or cellular infiltration (C) as determined by Image J (NIH) is presented. Data is presented as mean + SD (n=15 mice per treatment per genotype). Statistical test: 2 way ANOVA with Tukey's multiple comparison. **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.ns, not statistically significant. Scale bar = 20µm

Loss of IL-6Rα in epidermal keratinocytes alters immune cell infiltration into skin during ICD. Multiple immune cells including macrophages, neutrophils, dendritic cells, NK cells etc. have been shown to migrate into lesional skin during ICD. How IL-6Rα function in epidermal keratinocytes influences immune cell infiltration during ICD is unknown. Flow cytometric analysis of single cell suspensions from lesional skin revealed that $Il6ra^{\Delta ker}$ mice had higher numbers of infiltrating leukocytes ($CD11b^{+}$ $CD45^{+}$ cells) relative to WT mice after both BKC and JP-8 exposures (Fig 8 A, B). Furthermore, $Il6ra^{\Delta ker}$ mice skin had significantly higher numbers of $CD11b^{+}CD45^{high}$ cells relative to WT after BKC and JP-8 exposures (Fig 8C). Whereas higher numbers of $CD11b^{+}CD45^{low}$ cells were observed in lesional skin from $Il6ra^{\Delta ker}$ mice compared to WT after BKC exposure, there was no difference between $Il6ra^{\Delta ker}$ mice and WT for this cell population after JP-8 exposure (Fig 8 D red bars).

To further characterize the phenotype of these infiltrating leukocytes, immunohistochemistry and flow cytometric analysis of cells was employed. Results from flow cytometry revealed significantly lower numbers of γδ T cells ($CD3^{+}$ γδ TCR^{+}) in $Il6ra^{\Delta ker}$ mice relative to WT after both BKC and JP-8 exposure (Fig.9). After JP-8 exposure, lower numbers of both $CD3^{+}$ γδ TCR^{high} and $CD3^{+}$ γδ TCR^{low} cells were observed (Fig 9) in lesional skin from $Il6ra^{\Delta ker}$ mice. Immunohistochemistry revealed higher numbers of macrophages (F4/80) in both BKC and JP-8 exposed skin (Fig 10.

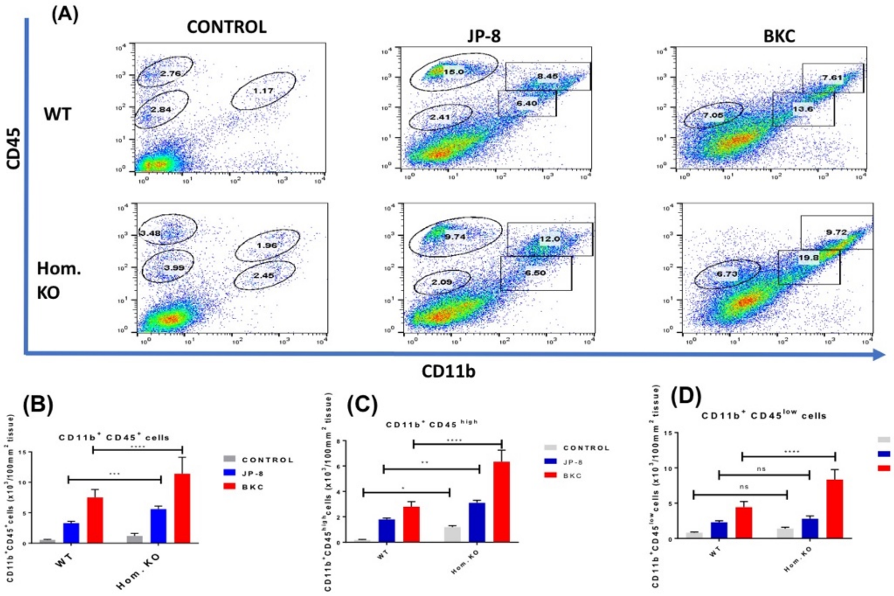


Figure 8: IL-6Rα deficiency in epidermal keratinocytes alters infiltrating leukocyte composition during ICD. ICD was established in WT and Il6ra$^{\Delta ker}$ mice using irritants as described. Lesional skin biopsies were obtained and the number of infiltrating leukocytes (CD11b^{+} CD45^{+}) cells were determined with flow cytometry. Representative scatter plots of three independent experiments are shown. Absolute cell counts of the different populations are shown (n= 4 mice per treatment per genotype). Data presented as mean ± SD of percent positive;. *p≤0.05, ***p≤ 0.001, ****p≤0.0001.ns, not significant.

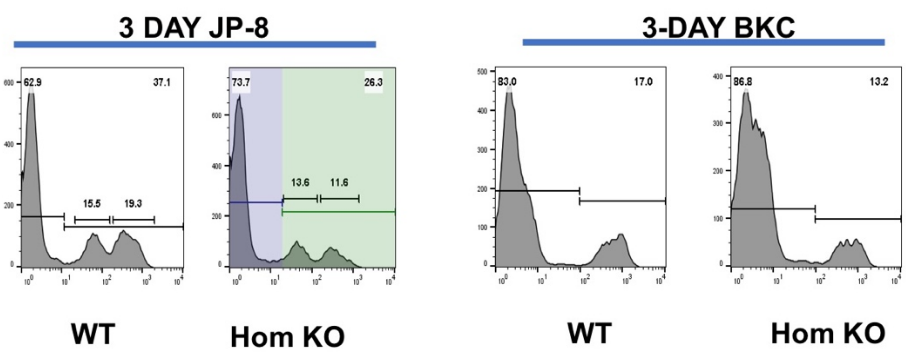


Figure 9. IL-6Rα deficiency in epidermal keratinocytes modifies Gamma Delta (γδ) T cell expression in irritant exposed skin. WT and Il6ra$^{\Delta ker}$ mice were exposed to BKC, JP-8 or control for three (3) consecutive days to induce ICD. Lesional skin biopsies were collected from mice 24 hours post irritant-exposure and the number of infiltrating γδ T cells were enumerated with flow cytometry. Representative histogram plots of three independent experiments are shown. Absolute cell counts of the different populations are shown (n= 4 mice per treatment per genotype).

Irritants induce increased inflammatory cytokine and chemokine expression in Il6ra$^{\Delta ker}$ mouse skin. ICD is characterized by inflammatory cytokine/chemokine expression primarily by keratinocytes and other skin cells. Therefore, cytokine and chemokine multiplex assays were performed to determine what role IL-6Rα plays in influencing the inflammatory milieu. Protein expression data from BKC-exposed skin revealed higher expression of pro-inflammatory cytokines IL-1β, IL-1α, IL-6, and IL-22 in Il6ra$^{\Delta ker}$ mice relative to WT (Fig. 11 A-D red bars). In JP-8 treated skin, the pro-inflammatory cytokines IL-1α and IL-18 (Fig. 121 B blue bars, supplementary Fig.1A) were significantly increased in Il6ra$^{\Delta ker}$ mice relative to WT. The expression of chemokine proteins IP-10/CXCL10, MIP-1α/CCL3, MIP-1β/CCL4, and MIP-2/CXCL2 were significantly higher in lesional skin from Il6ra$^{\Delta ker}$ mice relative to WT in BKC-treated skin (Fig. 12 red versus grey bars). Lesional skin from Il6ra$^{\Delta ker}$ mice showed a marked reduction in the expression of the anti-inflammatory cytokines, IL-10 and IL-4. This was consistent in both JP-8 and BKC exposed skin (Fig. 12 red and blue bars).

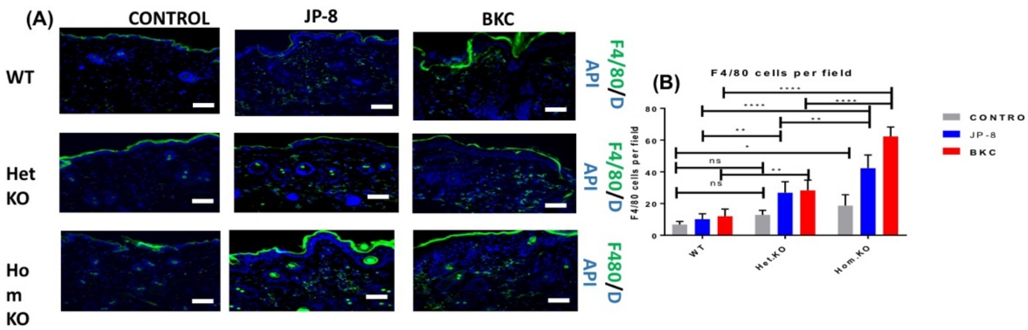


Figure 10. Immunohistochemical analysis of macrophage expression in irritant-exposed skin. Il6ra$^{\Delta ker}$ and WT mice were exposed to BKC, JP-8 or control for 3 consecutive days. Sections of lesional skin from irritant-exposed mice were stained for the expression of F4/80 (green), a marker of macrophages by fluorescent immunohistochemistry. Sections were counterstained with dapi. Representative fluorescent images are shown (A). Quantification of number of F4/80 expressing cells be field was determined by ImageJ (B).(n= 15 mice per treatment per genotype) Magnification= 20x. Scale bars =20µm. Data is presented as mean± SD .Statistical test: 2 way ANOVA with Tukey's multiple comparison. *p≤0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤0.0001

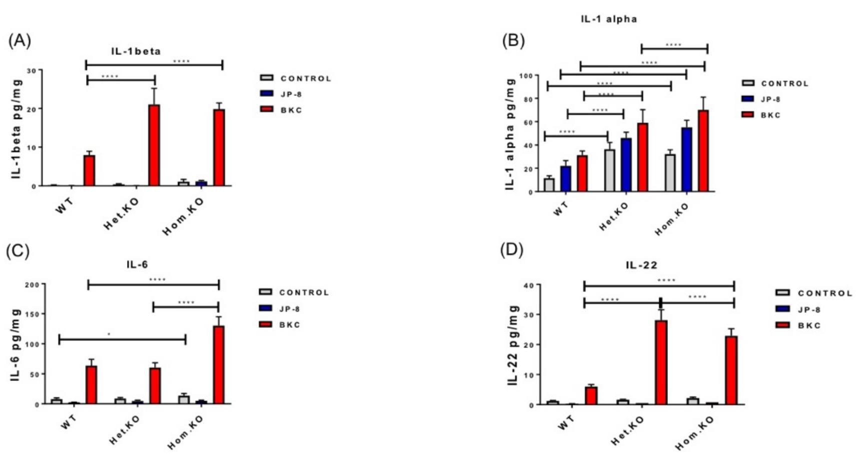


Figure 11: Increased inflammatory cytokine expression in Il6ra$^{\Delta ker}$ mouse skin. (A-D)Lesional skin from Il6ra$^{\Delta ker}$ mice show increased expression of pro-inflammatory cytokines. Il6ra$^{\Delta ker}$ and WT mice were exposed to BKC, JP-8 or control for 3 consecutive days. Lesional skin was harvested from each genotype and inflammatory cytokine protein expression was determined by multiplex immunoassay. Each experiment included cohorts of 15 mice per genotype. Data is presented as mean± SD .Statistical test: 2 way ANOVA with Tukey's multiple comparison. *p≤0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤0.0001

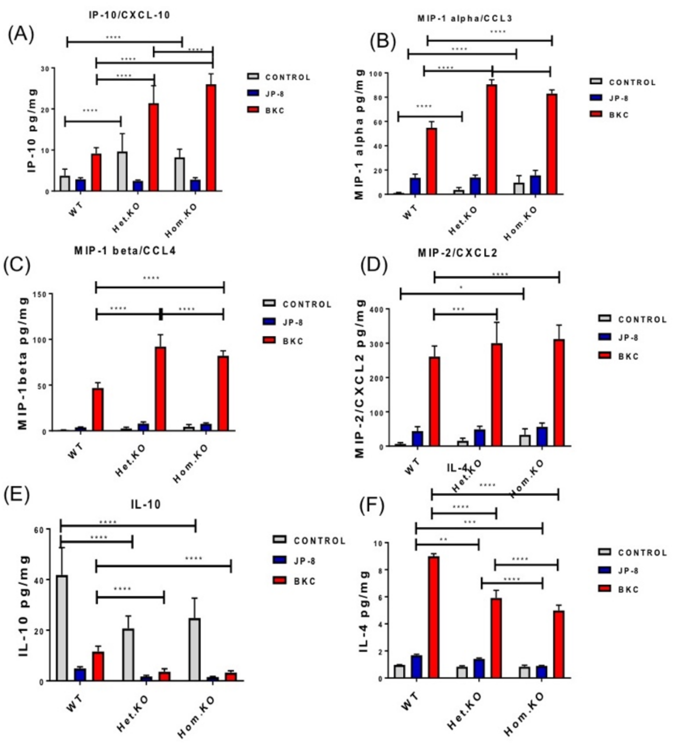


Figure 12: Increased inflammatory cytokine and chemokine expression in Il6ra$^{\Delta ker}$ mouse skin. (A-D)Lesional skin from Il6ra$^{\Delta ker}$ mice show increased expression of pro-inflammatory chemokines**.** (E,F) Lesional skin from Il6ra$^{\Delta ker}$ mice show reduced expression of anti-inflammatory cytokines. Il6ra$^{\Delta ker}$ and WT mice were exposed to BKC, JP-8 or control for 3 consecutive days. Lesional skin was harvested from each genotype and inflammatory cytokine protein expression was determined by multiplex immunoassay. Each experiment included cohorts of 15 mice per genotype. Data is presented as mean± SD .Statistical test: 2 way ANOVA with Tukey's multiple comparison. *p≤0.05, **p≤ 0.01, ***p≤ 0.001, ****p≤0.0001

Loss of IL-6Rα in myeloid lineage cells alters immune cell infiltration into skin during ICD. Mice possessing the tamoxifen inducible IL-6R knockout in all myeloid cells (LysM-Cre) as well displayed worse ICD as compared to control in response to both BKC and JP8 in terms of both epidermal hyperplasia as well as inflammatory cell infiltration (Fig 13), but at a lesser severity than K14-KO. These mice also displayed a greater influx of monocytic cells (Fig 14), and more broadly altered chemokine expression with decreases in CXCL1, 2, 10, CCL3, 5, and 7 as compared to WT (not shown, analysis proceeding).

Of the cytokines found altered in the keratinocyte directed IL-6RKO transgenic mice, IL-22 is most closely associated with epidermal keratinocyte function and is known to induce epidermal thickening. This cytokine has been implicated in the pathogenesis of allergic contact dermatitis, thus it could be that IL-22 is a key mediator of acanthosis during ICD as well.

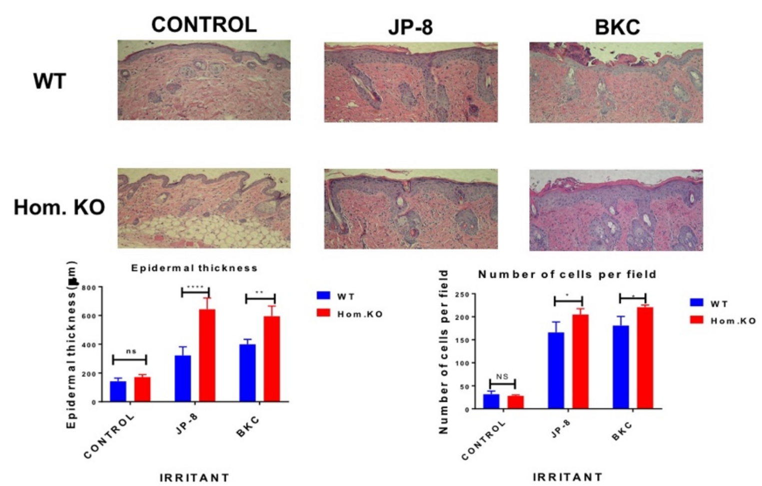


Figure 13. loss of IL-6Rα in myeloid lineage cells leads to increased epidermal hyperplasia and immune cell infiltration during ICD.
WT and Il6ra$^{\Delta LysM}$ mice were exposed to BKC, JP-8 or control for three (3) consecutive days to induce ICD. 24 hours after irritant exposure, 4-mm biopsies of lesional skin were collected and embedded in O.C.T compound for histological analysis. Skin samples were cross-sectioned, and then Hematoxylin and eosin (H&E) stained. Representative H&E stains from Il6ra$^{\Delta ker}$ and WT are shown (A).Quantification of epidermal thickness (B) or cellular infiltration (C) as determined by Image J (NIH) is presented. Data is presented as mean + SD (n=15 mice per treatment per genotype). Statistical test: 2 way ANOVA with Tukey's multiple comparison. **p≤ 0.01, ***p≤ 0.001, ****p≤0.0001.ns, not statistically significant. Scale bar = 20µm

<u>IL-6 negatively regulates IL-22Rα expression on epidermal keratinocytes.</u> The functional IL-22Rα complex consists of the membrane bound IL-22Rα and IL-10Rβ. It has been shown that epidermal keratinocytes express both IL-22Rα and IL-10Rβ. To delineate the effect of IL-6 on the expression of the IL-22Rα by keratinocytes, freshly isolated epidermal keratinocytes from IL-6KO mice were incubated with recombinant mouse IL-6 (rmIL-6) in a dose dependent manner. The expression of IL-22Rα mRNA was assessed by RT-PCR. Results from RT-PCR revealed that exposing keratinocytes to rmIL-6 led to a reduction in the expression of the IL-22Rα mRNA (Fig 15A). The highest expression of IL-22Rα was observed after 0ng/ml and 5ng/ml rmIL-6 exposure. In contrast, higher doses of rmIL-6 (10-50ng/ml) led to decreased expression of the IL-22Rα on epidermal keratinocytes. To confirm these results at the protein level, immunohistochemistry was performed. Results from immunohistochemistry correlated with the results seen at the mRNA level. Exposing epidermal keratinocytes to recombinant IL-6 led to a clear reduction in the expression of IL-22Rα protein (Fig 15B).

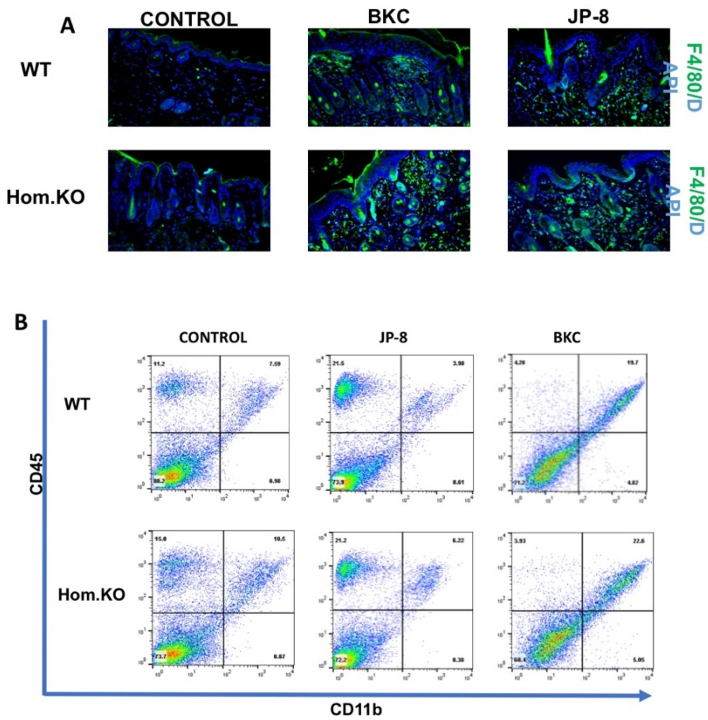


Figure 14. Immunohistochemical and flow cytometric analysis of macrophage expression in irritant-exposed skin. $Il6ra^{\Delta LysM}$ and WT mice were exposed to BKC, JP-8 or control for 3 consecutive days. (A) Sections of lesional skin from irritant-exposed mice were stained for the expression of F4/80 (green), and dapi (blue). Representative images are shown (A). Quantification of number of F4/80 expressing cells be field was determined by ImageJ (B).(n= 15 mice per treatment per genotype) Magnification= 20x. Scale bars =20μm. (B) Lesional skin biopsies were obtained and the number of infiltrating leukocytes ($CD11b^+$ $CD45^+$) cells were determined with flow cytometry. Representative scatter plots of three independent experiments are shown.

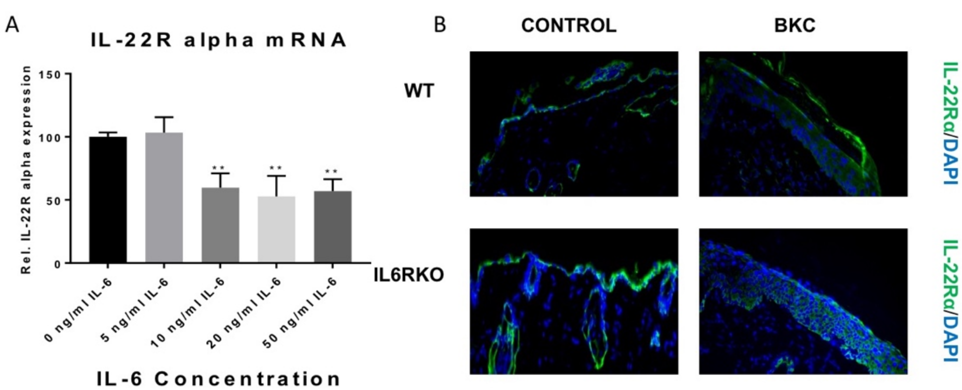
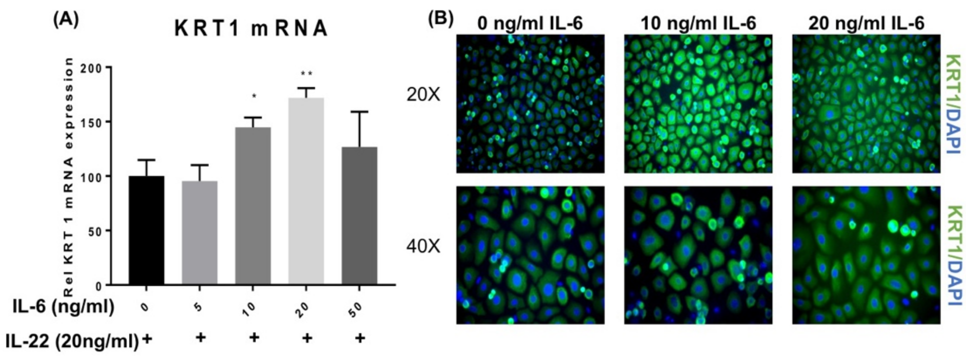


FIGURE 15. IL-6 negatively regulates IL-22Rα expression on epidermal keratinocytes
(A) Primary keratinocytes from IL-6KO mice were treated with rmIL-6 for 4 hours or at the indicated concentrations. The expression of IL-22Rα mRNA was analyzed and normalized to 18 S ribosomal RNA as control. Data is presented as mean ± standard deviation. (B) IL-22Rα (green) expression in keratinocytes was analyzed with fluorescent immunohistochemistry. Sections were counterstained with dapi. Representative fluorescent images are shown.

<u>**IL-6 reduces the effect of IL-22 on keratinocyte differentiation and proliferation**</u>

IL-22 has multiple effects on keratinocytes function including promoting proliferation and inhibiting differentiation. Having determined that IL-6 negatively regulates the expression of IL-22Rα on epidermal keratinocytes, the next goal was to determine whether IL-6 will have an influence on the functional effect of IL-22 on epidermal keratinocytes. Primary IL-6KO keratinocytes were exposed to rmIL-6 in a dose dependent manner for 24 hours. This was followed by exposure to rmIL-22 for 4 hours. Next RT-PCR was performed to determine the mRNA expression of keratin 1 (KRT1), a marker of keratinocyte differentiation. Higher expression of KRT1 mRNA was observed when keratinocytes were exposed to rmIL-6 prior to rmIL-22 exposure (Fig 16A). To further characterize this effect at the protein level, keratinocytes were pretreated with rmIL-6 for 24 hours followed by treatment with rmIL-22. After 24 hours, keratinocytes were stained with KRT1 and immunofluorescence image analysis was performed. Immunofluorescence image analysis revealed that pretreatment with rmIL-6 increased the expression of KRT1 on keratinocytes further confirming the results obtained from RT-PCR (Fig 16B). In the same manner, the effect of IL-6 on IL-22 induced keratinocyte proliferation was analyzed via Ki67 expression. Immunohistochemical analysis of treated IL-6 deficient keratinocytes revealed that in the presence of rmIL-6, the effect of IL-22 on keratinocyte proliferation was diminished as indicated by reduced Ki67 staining (Fig 16C).



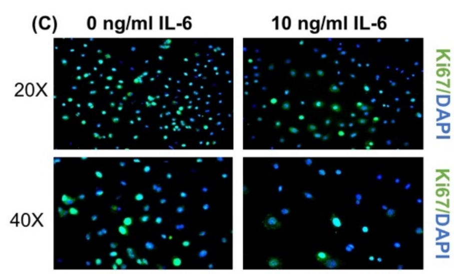


FIGURE 16. IL-6 reduces the effect of IL-22 on keratinocyte differentiation and proliferation
Primary keratinocytes were pretreated with rmIL-6 for 24 hours followed by exposure to rmIL-22 for 4 (A) or 16 hours (B,C). KRT1, Ki67 mRNA expression was analyzed by RT PCR. Data is presented as mean ± standard deviation. The expression of KRT1 (B) or Ki67 (C) (green) protein in primary keratinocytes was analyzed with fluorescent immunohistochemistry. Sections were counterstained with dapi. Representative fluorescent images are shown (B, C).

Immune response is a complex network of immune cells interacting with each other, host tissue, commensal as well as pathogenic organisms. Thus, defining a single immune gene function may be of interest, it does not come close to describing immunity. Further, as there are different "arms" of the immune system that broadly defined, tend to surveil particular classes of pathogens, the heterogeneity of humans lends itself to individuals having a particular "arm" of the immune system more or less capable compared to other individuals. This can be defined as a particular immune phenotype. Indeed, it is known that as one ages, the anti-viral portion of immunity wanes, and the allergic/anti-parasitic portion dominates. One can define these phenotypes by the T helper cells tasked with assisting the anti-pathogen response, where for instance, Th1 is anti-viral/proinflammatory, Th2 is anti-parasitic/allergic, and Treg as mucosal/anti-inflammatory. As ICD also varies between individuals, it seemed a logical assumption that immune phenotype as well might affect ICD. Further, as IL-6 is classified as a Th2 or anti-parasitic pro-allergy cytokine, it was of interest if more broadly defined Th2 phenotypic mice showed alterations in ICD severity compared to Th1. Thus, Th1 phenotype (anti-viral, pro-inflammatory) C57BL/6 mice and Th2 phenotype (anti-parasitic, pro-allergy) were evaluated for ICD, where the Th1 phenotype might be loosely analogous to an IL-6 deficient genotype.

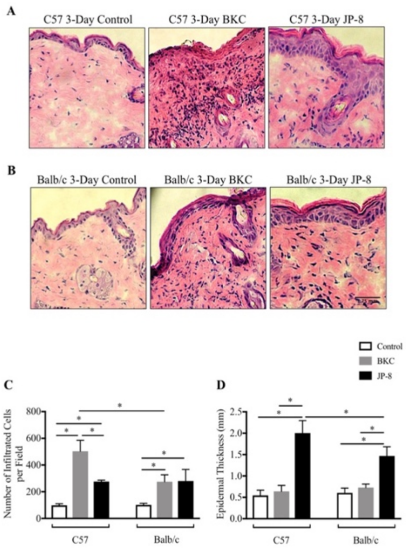


Figure 17. C57BL/6J (A) and Balb/c (B) skin was exposed to acetone (control), benzalkonium chloride (BKC) or JP-8 jet fuel for two hours on three consecutive days. Following collection, exposed skin samples were embedded in paraffin, hematoxylin and eosin (H&E) stained, and digital images were obtained to evaluate skin histopathology. Image J analysis of level of cell infiltration (C) and epidermal thickness (D). Data are mean +/-SD, *significantly different, (p<0.05, n=15).

Irritant exposures differentially alter the epidermal structure and cellular infiltration in C57BL/6 and Balb/c skin. To evaluate the pathology of BKC and jet fuel induced ICD, dermatitis lesions from C57BL/6 and Balb/c mice were examined. After 3-days of irritant exposure, C57BL/6 and Balb/c mice exhibited increased cellular infiltration in response to BKC and JP-8 exposure when compared to controls (Figure 17A-C). JP-8 induced acanthosis in both C57BL/6 and Balb/c after 3-days of exposure, whereas neither mouse strain exhibited acanthosis after 3-days of BKC exposure (Figure 17A-B, D). Following 7-days of exposure to either irritant, C57BL/6 and Balb/c mice had increased cellular infiltration and acanthosis when compared to controls (Figure 18A-D).

Neutrophil and monocyte skin populations during ICD are dependent upon mouse strain and irritant type. As shown in Figures 1 and 2, irritant exposure promotes immune cell infiltration. Of circulating leukocytes, neutrophil and monocytes are quickly recruited to the site of damage to assist with clearance of damaged tissue and have also been shown to contribute to the pathogenesis of inflammation (Wilgus *et al.*, 2013) (Wrigley *et al.*, 2011). Therefore, flow cytometric analysis was utilized to characterize these important cells during ICD.

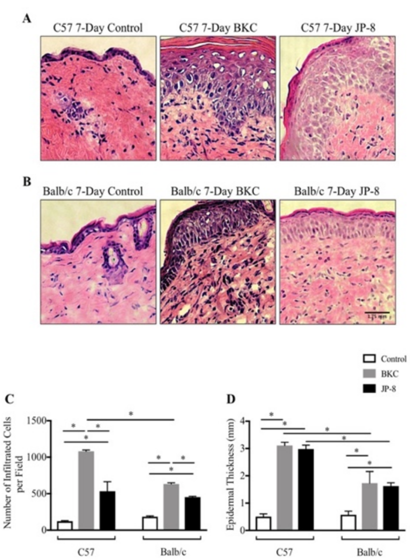


Figure 18. Skin inflammation varies relative to irritant and strain. C57BL/6J (A) and Balb/c (B) skin was exposed to acetone (control), benzalkonium chloride (BKC) or JP-8 jet fuel for two hours on seven consecutive days. Paraffin embedded, H&E stained sections were digitally imaged and evaluated for skin histopathology. Cell infiltration (C) and epidermal thickness (D) was determined via Image J. Data are means +/-SD, *significantly different, (p<0.05, n=15).

3-Day Irritant Exposures. Flow cytometric analysis cells isolated from skin samples revealed that in response to BKC exposure, C57BL/6 skin had a significantly greater population of neutrophils as compared to Balb/c skin (Figure 19A, D). However, monocyte presence did not vary between BKC exposed C57BL/6 and Balb/c skin (Figure 19 A, C). Following JP-8 exposure, Balb/c skin had a significantly greater presence of monocytes and neutrophils as compared to C57BL/6 skin (Figure 19 A, D). When comparing irritants, neutrophil presence was significantly greater following BKC exposure as compared to JP-8 exposure in both mouse strains (Figure 3 A, D). Additionally, Balb/c skin exposed to BKC displayed a significantly reduced monocyte presence in comparison to JP-8 exposure (Figure 19 A, C).

7-Day Irritant Exposures. Following prolonged BKC exposure, C57BL/6 skin displayed a significantly greater infiltration of neutrophils compared to Balb/c skin (Figure 19B, D), but monocyte infiltration did not significantly differ between strains (Figure 19B, C). Conversely, in response to JP-8 exposure, neutrophil and monocyte presence was significantly higher in Balb/c skin when compared to C57BL/6 skin (Figure 19 B-D). When comparing irritants, C57BL/6 skin exposed to BKC had a greater infiltration of monocytes and neutrophils as compared to JP-8 exposure (Figure 19 B-D) whereas neutrophil presence was significantly lower following BKC exposure in Balb/c skin as compared to JP-8 (Figure 19B, D).

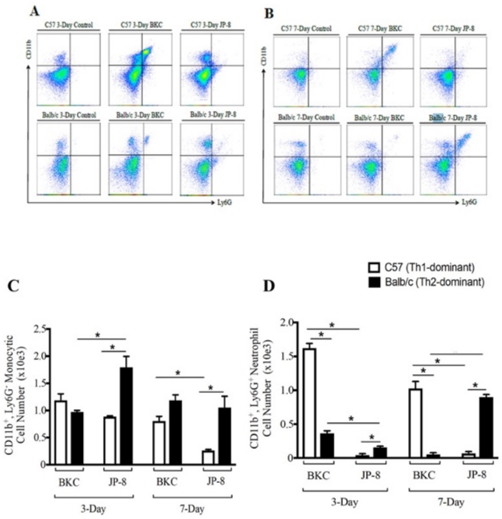


Figure 19. Inflammatory cell populations in skin differ relative to irritant and strain. Skin samples were collected from C56BL6/J and Balb/c after 3- (A) and 7- (B) days of control, BKC, and JP-8 exposures. Cells were isolated and analyzed for cell populations via flow cytometry. CD11b+, Ly6G- monocytic cell (C) or CD11b+, Ly6G+ neutrophil (D), data are mean +/- SD, *significantly different, (p<0.05, n=4).

Dendritic cell and γδ TCR cell skin populations during ICD are dependent upon mouse strain and irritant type

As seen in Figure 19, neutrophils and monocytes represent a fraction of the immune cells present in dermatitis lesions and are known to interact with other cells, such as dendritic cells and γδ T-cells to facilitate an immune response. However, little is known concerning the effect of different irritants on dendritic cell and γδ T-cell populations in skin. Therefore, flow cytometric analysis was utilized to evaluate the presence of these cells during BKC and jet fuel induced dermatitis.

3-Day Irritant Exposures. Flow cytometric analysis revealed that in response to BKC and JP-8 exposure, Balb/c skin had a significantly higher population of dendritic cells as compared to C57BL/6 skin (Figure 20A, C). C57BL/6 skin exposed to JP-8 had a significantly higher presence of γδ T cells compared to Balb/c skin, whereas γδ T cells did not vary between BKC exposed C57BL/6 and Balb/c skin (Figure 21 A, C). When comparing irritants, C57BL/6 skin exposed to BKC had a significantly fewer γδ T cells than JP-8 exposed skin (Figure 21 A, C). Likewise, dendritic cell number was significantly lower in Balb/c skin following BKC exposure as compared to JP-8 exposure (Figure 21A, C).

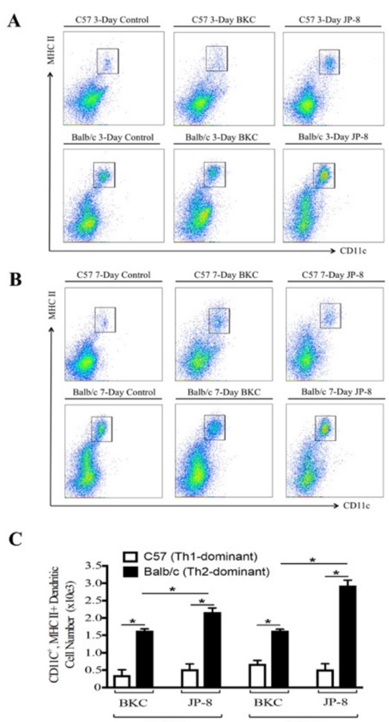


Figure 4. CD11c+, MHCII+ dendritic cell populations in skin differ relative to irritant and strain. Skin samples were taken from C56BL6/J and Balb/c after 3- (A) and 7- (B) days of control, BKC, and JP-8 exposures. Cells were isolated and analyzed via flow cytometry. (C) Data are mean +/- SD, *significantly different (p<0.05, n=4).

7-Day Irritant Exposures. Following both BKC and JP-8 exposure, Balb/c skin displayed a significantly greater infiltration of dendritic cells as compared to C57BL/6 skin (Figure 20 B, C). However, the γδ T cell population was significantly greater in C57BL/6 skin compared to Balb/c skin following JP-8 exposure, while the opposite we apparent following BKC (Figure 21 B, C). When comparing irritants, C57BL/6 skin exposed to BKC had a significantly reduced population of γδ T cells as compared to JP-8 exposure (Figure 21 B, C). Dendritic cell presence was significantly lower and γδ T cell presence was significantly higher in Balb/c skin exposed to BKC as compared to JP-8 (Figures 20-21 B, C).

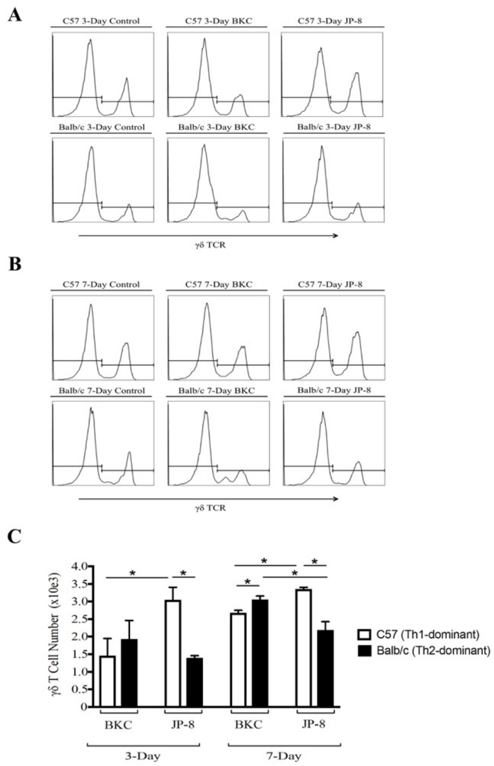


Figure 21. γδ T-cell populations differ relative to irritant and strain. Skin samples were collected from C56BL6/J and Balb/c after 3- (A) and 7- (B) days of irritant or control exposure. Cells were isolated and analyzed via flow cytometry. (C) Data are mean +/- SD, *significantly different, (p<0.05, n=4).

Irritant exposures induce diverse cytokine and chemokine patterns in C57BL/6 & Balb/c skin

Variations in cytokine expression within the skin are postulated to be a major factor leading to pathological differences of ICD between individuals and irritants as the cytokine milieu influences immune cell infiltration and function. To further investigate the variations in cytokine signature following irritant exposure, total soluble protein was extracted from skin samples and multiplex assays were utilized to examine the differences in cytokine and chemokine relative to irritant exposures in C57BL/6 versus Balb/c mice.

Overall 3-day Irritant Exposures of C57BL/6 versus Balb/c. Following acute BKC exposure, C57BL/6 skin exhibited significantly greater expression of 15 cytokines and 4 chemokines in comparison to Balb/c skin (Figure 22, Table S4). However, IL-9 and IL-1α, were significantly lower in BKC exposed C57BL/6 skin as compared to Balb/c skin (Figure 22, Table S4). After acute JP-8 exposure, C57BL/6 skin had significantly elevated expression of M-CSF and GM-CSF as compared to Balb/c skin (Figure 22) Conversely, expression of 15 cytokines and 4 chemokines was significantly reduced in JP-8 exposed C57BL/6 skin as compared to Balb/c (Figure 22).

3-Day C57BL/6 Exposure to BKC and JP-8. C57BL/6 skin revealed significantly elevated expression of 19 diverse cytokines and 10 chemokines following 3 days of BKC exposure (Figure 6 & Table S1). However, the expression of IL-1α, IL-5, IL-10, and IL-23 significantly decreased in BKC dermatitis lesions when compared to control (Figure 22). The cytokine response to JP-8 was quite different where after 3-days of exposure, the expression of 4 cytokines and one chemokine (CCL11) were significantly higher in exposed skin (Figure 22). Unlike BKC, only the expression of IL-23 was significantly reduced following 3-days of JP-8 exposure when compared to control (Figure 22).

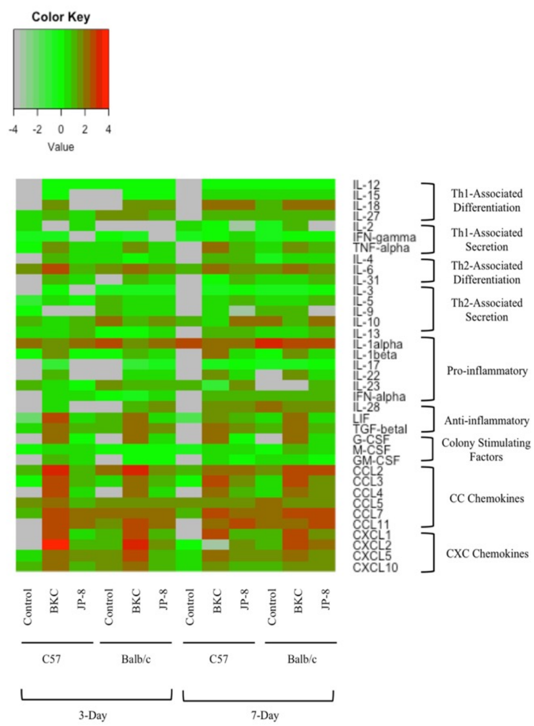


Figure 6. Multiplex analysis of skin cytokine expression. ICD was established in C57BL/6J and Balb/c mice. Biopsies of irritant exposed skin were obtained and cytokine expression was quantified by Luminex Multiplex Assay. Values represent log of the cytokine mean protein expression for the heatmap (n=15/treatment/immune phenotype).

When comparing irritants, 16 cytokines and 10 chemokines had significantly higher expression in BKC exposed skin as compared to JP-8 (Figure 22). However, IL-1α, IL-2, IL-5 and IL-13 were significantly higher following JP-8 exposure as compared to BKC (Figure 22).

3-Day Balb/c Exposure to BKC and JP-8. Comparison of 3-day BKC exposure versus control revealed significantly elevated expression of 9 diverse cytokines and 8 chemokines in Balb/c dermatitis lesions (Figure 22). Conversely, expression of IL-12, IL-27, IFN- γ, IL-9, IL-10, and IL-1α was significantly decreased in these dermatitis lesions when compared to control (Figure 22). After 3-days of JP-8 exposure, 7 cytokines had significantly higher expression in Balb/c skin (Figure 22), and IL-27, IFN- γ, IL-9, IL-10, and IL-1α, were significantly decreased when compared to control (Figure 22).

Comparison of 3-day BKC versus JP-8 exposure revealed significantly higher expression of 8 cytokines and 8 chemokines respectively in Balb/c skin (Figure 22). The expression of IL-4, IL-10, IL-13 IL-1α, IL-17, and IFN-α was significantly upregulated in JP-8 exposed Balb/c skin, as compared to BKC (Figure 22).

Overall 7-day Irritant Exposures of C57BL/6 versus Balb/c. Following BKC exposure, C57BL/6 skin exhibited significantly greater expression of IL-18, TNF- α, IL-13, IL-1β, CCL4 and CCL5 in comparison to Balb/c skin (Figure 22). However, IL-27, IL-31 and IL-28, were significantly higher in BKC exposed Balb/c skin as compared to C57BL/6 skin (Figure 22). After JP-8 exposure, C57BL/6 skin had significantly elevated expression of IL-4 and LIF as compared to Balb/c skin (Figure 22). Conversely, expression of IL-27, TNF-α, IL-1β, G-CSF and 7 chemokines was significantly higher in JP-8 exposed Balb/c skin as compared to C57BL/6 (Figure 22).

7-Day C57BL/6 Exposure to BKC and JP-8. After 7-days of BKC exposure, the expression of 17 cytokines and 10 chemokines were significantly up-regulated in BKC exposed C57BL/6 skin as compared to control. (Figure 22). Following 7-days of JP-8 exposure, 11 cytokines and 3 chemokines had significantly greater expression as compared to control. (Figure 22).

Comparing irritants after 7-days of exposure, BKC induced significantly higher expression of 11 cytokines and

6 chemokines (Figure 22). However, the expression of 8 cytokines and one chemokine (CCL11) was significantly lower after 7-days of BKC exposure compared to JP-8 (Figure 22).

7-Day Balb/c Exposure to BKC and JP-8. After 7-days of BKC exposure, the expression of 13 cytokines and 8 chemokines was significantly increased in BKC exposed skin as compared to control (Figure 6 and Table S2), while IL-10, IL-1α, IL-28, and CCL5 were significantly down-regulated (Figure 22). Comparatively, following 7-days of JP-8 exposure, 7 cytokines and 3 chemokines, had significantly greater expression as compared to control (Figure 6 and Table S2), while IL-9, IL-1α, and IL-28 expression was significantly decreased (Figure 22).

Comparison of the two irritants after 7-days of exposure showed significantly higher expression of 11 cytokines and 6 chemokines in BKC exposed Balb/c skin as compared to JP-8 (Figure 6 and Table S3). However, 7 cytokines, as well as the chemokines CCL5 and CCL11 were significantly lower in 7-day BKC exposed skin compared to JP-8 (Figure 22).

This study was the most comprehensive comparison of the effects of immunophenotype in skin inflammation to date. It as well offers a very comprehensive comparison of detergent induced ICD versus that produced by a petroleum mixture. A summary slide is presented in Figure 23.

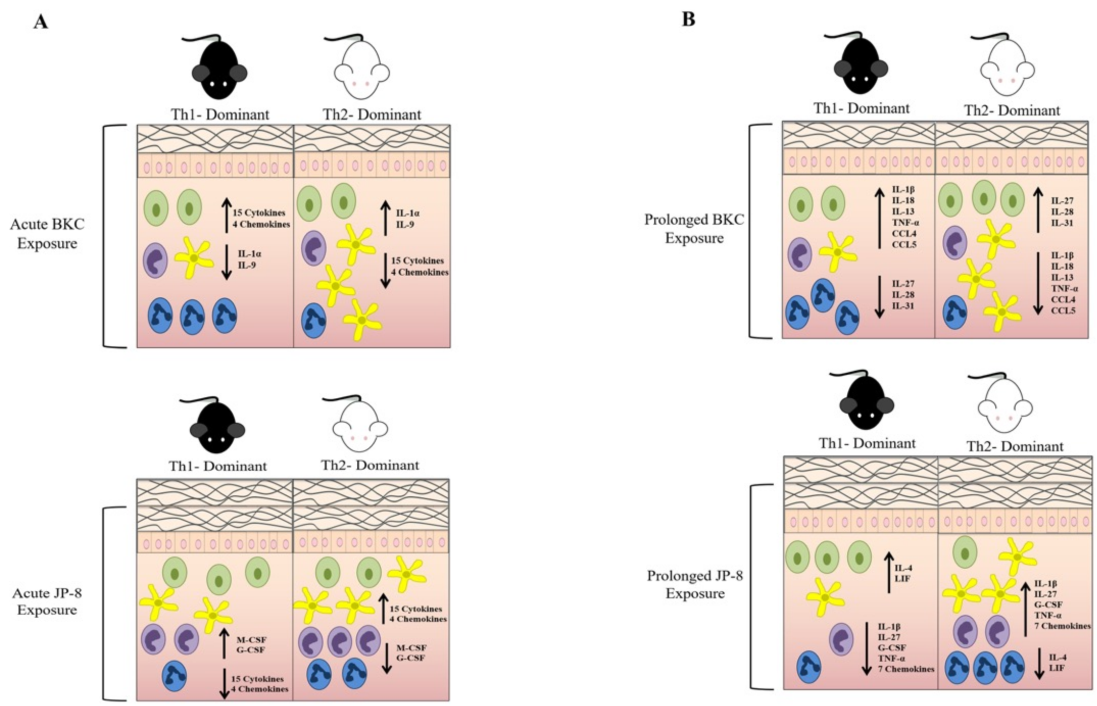


Figure 23. Cytokine profile and immune cell presence following irritant exposures is associated with immune-phenotype. (A) Following acute (3-day) BKC exposure, Th1-dominant (C57) skin displayed increased neutrophil presence and expression of 15 cytokines and 4 chemokines as compared to Th2-dominant (Balb/c) skin, but decreased dendritic cell presence and IL-1α and IL-19 expression After acute JP-8 exposure, Th1-dominant skin exhibited increased presence of γδ T-cell, as well as, M-CSF and G-CSF expression, but reduced presence of monocytic cells, dendritic cells, and neutrophil and expression of 15 cytokines and 4 chemokines. (B) Following prolonged (7-day) BKC exposure, Th1-dominant (C57) skin displayed increased neutrophil presence and expression of IL-1β, IL-18, IL-13, TNF-α, CCL4, and CCL5 as compared to Th2- dominant (Balb/c) skin, but decreased γδ T-cells and dendritic cell presence, and IL-27, IL-28 and IL-31 expression. After prolonged JP-8 exposure, Th1-dominant skin exhibited increased presence of γδ T-cell, as well as, IL-4 and LIF expression, but reduced presence of monocytic cells, dendritic cells, and neutrophil and expression of IL-1β, IL-27, G-CSF, TNF-α, and 7 chemokines.

Publications

Journal Articles:

Calhoun, KN, Luckett-Chastain, LR, Frempah, B, **Gallucci, RM**, Associations between immune phenotype and inflammation in murine models of irritant contact dermatitis, *In Press, Tox Sci,* 2018.

Luckett-Chastain, LR, Gipson, J, Gillaspy, AF, **Gallucci, RM**, Transcriptional profiling of irritant contact dermatitis (ICD) in a mouse model identifies specific patterns of gene expression and immune-regulation, *Toxicology*, 2018 Dec 1;410:1-9. doi: 10.1016/j.tox.2018.08.014. Epub 2018 Aug 29.

Luckett-Chastain, LR, Smith, ML, Mickle, BM, Ihnat, MA, and **Gallucci, RM,** Interleukin (IL)-6 modulates transforming growth factor -β receptor II (TGF-βRII) function in epidermal keratinocytes, *Exp Dermatol*. 2017 Aug;26(8):697-704.

Proceedings:

Frempah, B, Luckett-Chastain, LR, Calhoun, KN, and Gallucci, RM, Keratinocyte-Specific Deletion of IL-6ra Exacerbates the Inflammatory Response During Irritant Contact Dermatitis., *Tox Sci, (suppl.)*, March 14, 2018, Abstract 3058, San Antonio, TX

Calhoun, K, Luckett-Chastain, LR, and Gallucci, RM, Differential Skin Inflammatory Responses in Irritant Contact Dermatitis., *Tox Sci, (suppl.)* 156:1:460, March 14, 2017, Abstract 2952, Baltimore, MD.

Gallucci, RM, Luckett-Chastain, JR, Calhoun, KN, Frempah, B., IL-6 deficiency results in specific cytokine responses in a mouse model of irritant dermatitis, *Proceedings of the International Congress on Immunology*, Abstract #331, ICI 2016, Aug 22-26, 2016. Melbourne, AU.

Calhoun, K, Luckett-Chastain, LR, and Gallucci, RM. Immune phenotype and IL-6 deficiency modulate the inflammatory response in a mouse model of Irritant Contact Dermatitis, *J Immunol* 2016 196:126.34, May 13-17, Seattle, WA.

Luckett-Chastain, L., Calhoun, K., Schartz, T. and Gallucci, R.M., 2016. IL-6 influences the balance between M1 and M2 macrophages in a mouse model of irritant contact dermatitis. *J. Immunol*, *196* (Suppl), pp.196-17, May 13-17, Seattle, WA.

Gallucci RM, Kemp JM, Luckett-Chastain LR, Calhoun KN, American Association of Immunology annual meeting. Skin inflammatory response to complex hydrocarbon mixtures, Abstract 54.14, May 8-12, 2015. New Orleans, LA.

Luckett-Chastain JR, Calhoun KN, Kemp JM, Gallucci RM, Immunological difference between Th1 and Th2 dominant mouse strains in a model of ICD, American Association of Immunology annual meeting. May 8-12, 2015. New Orleans, LA.

LR Luckett-Chastain, JM Kemp, KN Calhoun, RM Gallucci, IL-6 deficiency leads to a specific cytokine profile during irritant contact dermatitis, Society of Toxicology 54^{th} Annual Meeting, San Diego, CA, Abstract #2423, March 25, 2015.

J.M. Kemp, L.R. Luckett-Chastain, W.M. McShan, Randle M. Gallucci; Dermatological Effects of Weathered Petroleum Exposure, *Experimental Biology,* San Diego, CA abstract 844.14, poster B258, April 28, 2014.

Dissertation/Thesis:

Calhoun, KN: [2017] Phenotypic and genetic influences upon the cutaneous inflammatory response, Ph.D. dissertation, University of Oklahoma Health Sciences Center, Oklahoma City, OK.

Inclusion enrollement: N/A

Inclusion of gender and minority study subjects: N/A

Inclusion of children: N/A

Materials available to other investigators:

All data produced by this project has been or will be submitted for publication to publicly accessed scientific journals. Whole genome transcriptional analysis data is available through Genomic Expression Omnibus (GEO https://www.ncbi.nlm.nih.gov/geo/) querying: #GSE95317, and #GSE119202.