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Abstract:

Since 1996, JP-8 has been the primary jet fuel used by the military with secondary usage in heaters, generators, stoves, and other military vehicles as well a coolant in engines and other aircraft components. It is estimated that worldwide over 2 million people are exposed to roughly 60 billion gallons of military and/or commercial jet fuel annually. Chemically, JP-8 is similar to the commercial fuel Jet-A but compared to its predecessor JP-4, JP-8 has a lower volatility and flammability. However, this change in character resulted in increased duration of topical exposure. JP-8 has been found to adversely affect numerous tissues including lung, and immune; however, the most common complaint following JP-8 exposure is "skin problems" or irritant dermatitis. Continuous or repeated exposure to JP-8 causes skin barrier damage, inflammation, and increased production of inflammatory mediators known as cytokines, such as Tumor necrosis factor alpha and particularly interleukin 6 (IL-6).

Since IL-6 is well known to be involved in inflammation as well as skin repair, and JP-8 is known to alter IL-6 expression in skin, it was hypothesized that the modulation of this cytokine contributes to the severity of jet fuel dermatitis. To investigate this, mice were obtained that that made no IL-6 (IL-6KO) or made more than normal (transgenic). Acetone (control), jet fuel, or a common irritant benzalkonium chloride was applied to there skin to assess how increasing or decreasing this cytokine would affect dermatitis. It was initially thought that, based on its pro-inflammatory properties, increased IL-6 production would worsen skin inflammation while deficiency would lessen it. However, quite the opposite was found. Increased IL-6 in the skin of JP-8 exposed mice resulted in decreased dermatitis, characterized by fewer inflammatory cells, and decreased expression of inflammatory cytokines and chemokines. It was further found that this effect might be related to the infiltration of so-called "anti-inflammatory" macrophages (aka. M2), that are associated with healing, which has not previously been associated with IL-6.

Occupational contact dermatitis ranks second most prevalent over all occupational diseases. Aside from aviation, this pathology can affect workers in essentially every industry. For instance Healthcare workers are commonly exposed to detergents from hand washing or cleaning activities, and irritants from antibacterial gels, and those in the manufacturing field can be exposed to petroleum-based lubricants. Results from this research may lead to identification of a so-called "biomarker" (i.e. IL-6) that can be used to predict the susceptibility of individuals to skin irritants, as well as suggest possible protective or treatment methods. For instance, a test could be developed that would assess a workers ability to produce IL-6 in skin. The results of this test would help industrial hygienists to determine the appropriate protective equipment for an individual worker, leading to reduced cost, and increased productivity from reduced injury.

Section I.

Significant Findings:

Specific aim one: Determine if low interleukin 6 expression affects severity of jet fuel induced irritant dermatitis.

1. *IL-6 deficient mice have worse skin inflammation than normal mice.* Mice were exposed to a small amount of acetone (control), JP8 jet fuel, the well known irritant benzalkonium chloride disinfectant (BKC) on their skin for up to 7 days. Mice that produced no interleukin 6 (IL-6KO) in their skin showed the worst dermatitis as a result of exposure to chemicals.
2. *Inflammatory mediator expression seemed to be higher in IL-6 deficient mice for key cytokines, and this depended on the irritant.* IL-6KO mouse skin showed increased production of certain inflammatory molecules known as cytokines, which act as immune hormones, and can help or inhibit inflammation. The cytokines TNFα, interleukin 1 beta and CCL3 which increase inflammation, were induced in IL-6KO skin as compared to normal mice, following BKC exposure for seven days. However, JP8 exposure seemed to increase different cytokines, CCL3 and CCL20, in IL-6KO mouse skin, which may explain varied response these mice had to the two irritants.
3. *IL-6 deficient mice had much decreased anti-inflammatory macrophages in their skin.* Following treatment with either BKC or JP8, IL-6KO mice showed profoundly lower ratios of so-called anti-inflammatory (healing) M2 macrophages in skin as compared to normal mice. This lower level of these type of inhibitory immune cells may cause inflammation to be worse in IL-6KO skin. It could be that modulation of cytokines caused this alteration in cell populations, since these molecules communicate to inflammatory cells, telling them to move into an area of damage or infection.

Specific Aim two: Determine if increased IL-6 expression or treatment modulates the severity of jet fuel induced dermatitis.

1. *Mice that overproduced IL-6 in their skin seemed to show the lowest level of skin inflammation following exposure to JP8 or BKC.* When treated for seven days mice that overexpress IL-6 seemed to have fewer inflammatory cells in their skin, and less edema as compared to IL-6KO and normal mice.
2. *No significant changes were noted concerning inflammatory mediator expression, in IL-6 overproducing mice.* Despite showing less overt inflammation, mice that overexpressed IL-6 did not seem to have significantly altered inflammatory mediator production. Thus, it seems that IL-6 itself might be causing decreased inflammation.

Translational Findings: The results of this research seems to indicate that a specific inflammatory molecule, IL-6, can alter the response of skin to an irritant. This research is too preliminary to be directly applied to the workplace; however, in the future it could provide a means to determine how a worker might respond to common workplace hazards. For instance, a simple test could be utilized to determine if workers possess the propensity to over or under express IL-6. Protective equipment or hazard avoidance could be recommended based on the results of such a test.

Outcomes/Impact: The purpose of this project was to investigate role of a specific cytokine (interleukin 6) in modulating the severity of irritant dermatitis. Occupational contact dermatitis, ranks second most prevalent over all occupational diseases (1), and can effect workers in essentially every industry designated by the NORA Sector Groups. For instance Healthcare workers are commonly exposed to detergents from hand washing or cleaning activities, and irritants from antibacterial gels. Workers in the manufacturing field can be exposed to petroleum-based lubricants such as cutting fluid, and those in the Transportation and Oil and Gas Extraction industries are commonly exposed to fuels and complex petroleum mixtures. Potentially, results from this study may lead to the utilization of IL-6 as a biomarker to predict the sensitivity of individuals to irritants. This can be applied to the avoidance of known hazards by individuals identified to be low IL-6 producers, as well as development specifically tailored protective equipment. Since this equipment could be individualized, hazards could be minimized and resources used more efficiently reducing worker injury and cost. It may also lead to the development of treatment methods, where perhaps skin creams containing IL-6 agonists could be used to augment the skins barrier function, further reducing injury and time away from work. Additionally, this work provides an

additional model where irritant chemicals can be evaluated. Since IL-6KO mice are particularly sensitive to irritants, these mice could be used to predict reactions in “so-called” sensitive individuals that might not be apparent using standard animal models.

Section II.

Scientific Report:

Background: Irritant dermatitis is a very common occupational disease. In the military and aviation fields, jet fuel exposure is known to commonly cause a profound irritant dermatitis (2). Studies in laboratory animals show that mRNA of interleukin 6, a proinflammatory cytokine, is induced corresponding with repeated daily exposure to JP-8 jet fuel (3, 4). However, protein levels of this cytokine steadily decrease during the exposure period to below control levels. Since IL-6 is known to be essential for skin wound healing (5), low expression could delay skin healing during JP8 dermatitis. The hypothesis of this proposal is that modulation of skin IL-6 levels contributes to the severity of jet fuel induced irritant dermatitis. To test this hypothesis, two specific aims were proposed. The first was essentially to evaluate whether the absence of IL-6 would exacerbate dermatitis, and the second was to evaluate if over expression of the cytokine was protective. Furthermore, these models were in the mouse rather than the rat. Overall, it was felt that the essence of the specific aims was completed, and that the mouse models of IL-6 over and under expression were validated. Data from these studies was utilized in support of an R01 application that was submitted to CDC/NIOSH in June of 2011. Specific progress and accomplishments are described below.

Specific aim one: Determine if low interleukin 6 expression affects severity of jet fuel induced irritant dermatitis. In this specific aim it was proposed to evaluate how irritant dermatitis was modulated in the absence of IL-6 utilizing the IL-6KO mouse. These mice were readily obtained through Jackson Laboratories, and had been used by this laboratory for several other projects in the past.

Specific Aim two: Determine if increased IL-6 expression or treatment modulates the severity of jet fuel induced dermatitis. In this specific aim it was proposed to evaluate how irritant dermatitis was modulated by the overexpression or treatment with exogenous IL-6. The overexpression transgenic IL-6 model (6) required that embryo's from Dr. Elaine Fuch's laboratory be sent to Jackson Labs in September of 09, where they were successfully cryorecovered. Mice were received by the OUHSC Animal Barrier Facility in November of 09 and breeding was initiated in December after an acclimation period. These mice breed exceedingly well, and experiments were initiated utilizing offspring of founder mice in February. To evaluate the data more completely, both mouse models were assessed similarly and data presented together comparing IL-6KO vs. Tg(IL6) vs. respective WT mice.

Methodology:

Dermal JP-8 exposure: All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). C57/BL6, IL-6KO (7), CD-1, or Tg(IL6) mice were acquired from Jackson Laboratories (Bar Harbor, ME) or bred in-house were group housed in polycarbonate cages containing hardwood chip bedding at room temperature (21± □C) on a twelve-hour light/dark cycle. Animals (6-8 week old) were allowed to acclimate to the animal facility for at least one week prior to skin irritant exposure. One day prior to exposure, mice were sedated with isofluorane and a ~9cm2 section of fur was clipped on the dorsal surface of the upper back. Treatments were not initiated until at least 24 hours post hair removal, to ensure that no irritation occurred from the hair removal process. Immediately prior to exposure, treatment groups were placed in separate fume hoods to minimize exposure to JP-8 vapor. Filtered *(0.45µm)* JP-8 (lot # UN1863) was provided by the Air Force Research Laboratory (AFRL/HEPB), Wright-Patterson Air Force Base and the Air Force Office for Scientific Research (AFOSR). In order to ensure consistent results, the fuel supplied by the AFOSR (lot # UN1863) to all research labs requesting fuel for study is a homogenous mixture of multiple batches of fuel collected from various Air Force bases around the world. Jet fuel, benzalkonium chloride (positive control), or acetone (negative control) (100 □L) was applied to the upper back of the animals and the groups were kept in fume hoods for one hour before being returned to the animal facility. Mice were treated as described for seven days. 24 hours following final exposure, rats were anesthetized and skin samples were collected. Harvested skin was flash frozen in liquid nitrogen or fixed in 10% buffered formalin for histology.

Real-time quantitative PCR: Total RNA from rat skin was prepared and cDNA was synthesized using random decamer primers essentially as previously described (8). Primers for mouse genes were generated from Genbank sequences and acquired from Invitrogen (Carlsbad, CA). Real-time

quantitative PCR was performed on an ABI PRISM 7000 SDS, utilizing TaqMan Sybr Green Master Mix according to the manufacturer's instructions (Invitrogen/ABI).

Pro-inflammatory cytokine protein expression: Total protein from frozen skin samples was prepared by homogenizing skin samples in PBS containing protease inhibitor cocktail (#P8340 Sigma, St. Louis, MO).

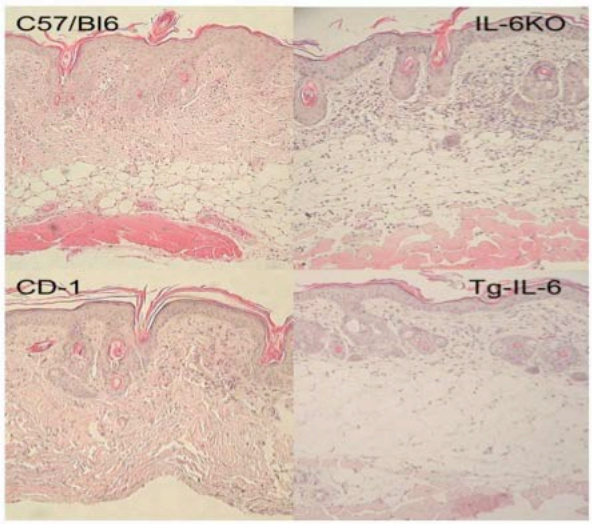

Figure 1. Greater neutrophil infiltration in JP-8 treated IL-6KO animals. Following a 7 day exposure to JP-8, 4mm skin biopsies were obtained and embedded in paraffin for H&E histology. Representative photographs of hematoxylin/eosin stains are presented for c57/bl6 (C57) background control for IL-6KO animals, IL-6 knockout (IL-6KO), CD-1 (CD-1) background control for Tg-IL-6, and transgenetic IL-6 overexpressing (Tg-IL-6) animals.

Statistical Analysis: All experiments were replicated and representative findings are shown. Statistical significance was determined by one-way ANOVA. In all statistical comparisons, a P value of < 0.05 was used to indicate a significant difference.

Results:

IL-6 expression alters inflammatory cell migration into skin. This laboratory reported previously that a time dependent decrease in rat skin IL-6 protein expression occurred in response to JP-8 exposure. To assess this further, genetically altered mouse models were used. IL-6KO and Tg-IL-6 animal models were treated with 100 □l of JP-8 on the denude dorsal surface for seven consecutive days. Skin samples from animals exposed to irritants for seven days were sectioned and H&E stained for examination. Histology indicated that inflammatory cell migration was greatest in IL-6KO animals and lowest in the Tg-IL-6 animals (Fig 1). Wild type mice (C57 and CD-1) had moderate yet similar inflammatory cell migration.

Modulation of proinflammatory cytokine mRNA expression in JP8 treated mice. TNFα and IL-1 are expressed by most tissues in response to infection or damage and are important initiators of inflammation, with similar if not redundant activities. It was previously shown that JP-8 exposure did not alter TNFα protein expression in rat skin while IL-1β appeared to be somewhat changed (3). Herein, it was shown that neither TNFα nor IL-1β mRNA expression was induced significantly in skin of JP-8 exposed animals (figs 2a and b dark bars). However, exposure to the irritant BKC produced a profound induction of both TNFα (C57 and IL-6KO) and IL-1β (all strains) after 7 days of exposure (fig 2a and b gray bars).

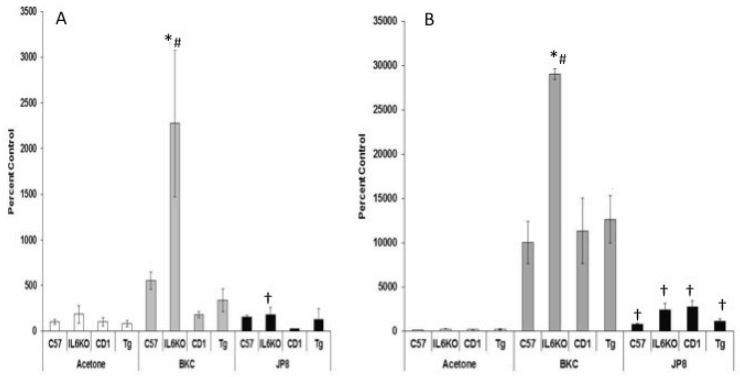

Figure 2. TNFa and IL-1b expression is increased in IL-6KO by BKC but not JP8 exposure. Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression of TNFa (A) or IL-1b (B) was analyzed via Q-PCR. Expression differences were normalized to 28s rRNA expression and presented as a percentage of C57 control animals. Data are presented as the means +/- SE; n = 6. (*p<0.01 vs similarly treated WT control, #p<0.01 vs similarly treated Tg, †p<0.05 vs same strain BKC)

While interpretation of IL-6 mRNA expression in animal models where the gene has been manipulated is difficult, some interesting observations were noted. As expected, Tg(IL6) overexpressing animals greatly induced (60k to 2M fold) IL-6mRNA expression as compared to all other animal groups. However, JP8 exposure induced the greatest expression (>4 fold) in Tg mice (fig 3). Despite the fact that IL-6KO mice do not produce IL-6 protein, mRNA of the cytokine is expressed and its induction

can be evaluated. Interestingly, JP8 exposure also induced mRNA of IL-6 in IL-6KO mice (fig 3, gray bars). Thus, this would be an indication of the activation of the IL-6 promoter, and JP-8 would appear to be a potent inducer of IL-6 mRNA transcription, as this laboratory has previously shown (3).

IL-10 is an important anti-inflammatory cytokine and is upregulated in response to inflammatory stimulus. In this study, IL-10 mRNA expression appeared to be greatest in IL-6KO animals and lowest in Tg-IL6 animals after either BKC or JP8 exposure. Jet fuel in particular induced nearly a 10 fold increase in IL6KO mice, while there was a similar decrease between WT and Tg IL-6 mice. While this seems paradoxical when considering IL-6KO mice had increased inflammation in response to irritants, indeed increased IL-10 mRNA expression would be expected in the animals with the greatest inflammation where the degree of gene expression is reflective of the degree of inflammation.

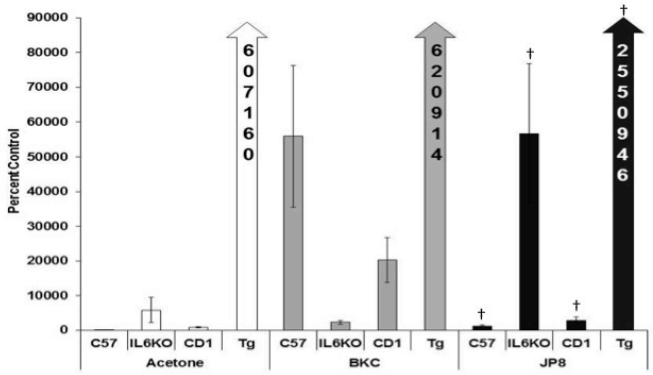

Figure 3. IL-6 mRNA expression is differentially modulated respective to strain and irritant. Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression analyzed via Q-PCR. Expression differences were normalized to 28s rRNA expression and presented as a percentage of C57 control animals. Data are presented as the means +/- SE; n = 6. († p<0.01 vs same strain BKC)

Modulation of chemokine mRNA expression in skin of JP treated mice. Chemokine expression promotes leukocyte migration into areas of inflammation. CCL3 (aka. MIP-1α) is involved in the recruitment and activation of macrophages and T cells during inflammation. IL-6 deficiency resulted in increased expression following either BKC or JP8 exposure (4 and 2 fold respectively, fig 5a). Another member of the CC chemokine family, CCL11 (aka. eotaxin-1) selectively recruits eosinophils, B and Th2 T Cells, but has minimal effects on macrophages or PMN. **T**his laboratory previously reported elevated CCL11 mRNA expression in JP-8 treated rats (3). In the present experiment, CCL11 mRNA expression was significantly increased in JP-8 treated IL-6KO and Tg animals when compared to respective controls, but was not significantly modulated by BKC.

A further member of the CC chemokine family, CCL20 (aka. Mip3a) primarily recruits lymphocytes such as memory T cells, B cells and immature dendritic cells. Mouse strain dependent differential CCL20 mRNA expression was observed following JP8 but not BKC exposure. IL-6 deficiency resulted in increased CCL20 mRNA expression (3 fold), while over expression resulted in a significant reduction (7 fold) as compared to IL-6KO mice (fig 6).

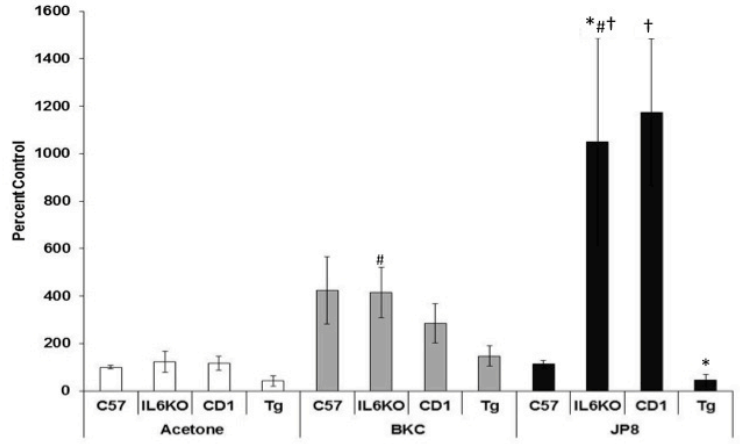

Figure 4. Greater IL-10 mRNA expression in JP-8 treated IL-6KO animals. Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression analyzed via Q-PCR. Expression differences were normalized to 28s rRNA expression and presented as a percentage of c57 control animals. Data are presented as the means +/- SE; n = 6. * p<0.001 vs similarly treated WT control. # p<0.03 vs similarly treated Tg, †p<0.05 vs same strain BKC.

Modulation of infiltrating macrophage populations. CD86 is expressed on antigen-presenting cells, including all macrophages. The CD206 surface marker is expressed primarily macrophages of the M2 or anti-inflammatory/wound healing lineage (9). To assess the composition of macrophages infiltrating into skin exposed to irritants, the mRNA expression of CD86 and CD206 macrophage cell surface markers was analyzed. When examined as a ratio of CD206:CD86 (ie. M2:M1), BKC treated WT control animals showed the highest infiltration of CD206 expressing cells (relative to CD86)

where both IL-6 deficiency and overexpression resulted in significantly decreased CD206 populations (fig 7 gray bars). Following JP8 exposure, only IL-6 deficiency resulted in a significant decrease in CD206 expression (fig 7 dark bars) where expression in Tg and its WT control skin were not different.

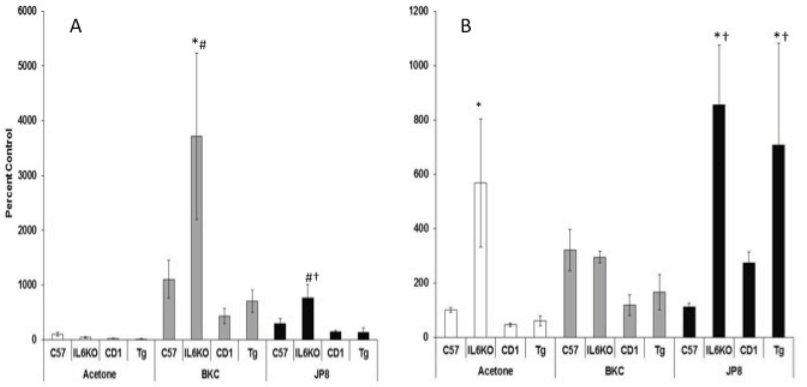

Figure 5. CCL3 and CCL11 expression varies with treatment and strain. Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression analyzed via Q-PCR for CCL3 (A), and CCL11 (B). Expression differences were normalized to 28s rRNA expression and presented as a percentage of c57 control animals. Data are presented as the means +/- SE; n = 6. * p<0.05 vs similarly treated WT control, # p<0.03 vs similarly treated Tg, †p<0.05 vs same strain BKC.

Discussion/Conclusions:

When viewed together, this data seems to indicate that IL-6 acts in an anti-inflammatory manner in skin during irritant dermatitis, which is counter-intuitive given the dogma that this cytokine is pro-inflammatory in nature. This is most apparent when examining irritant dermatitis lesions microscopically, where IL-6 deficient animals display less inflammatory cell infiltration (fig 1). Examination of macrophage subpopulations further supports these observations where macrophage infiltrate into IL-6KO skin significantly lacked the anti-inflammatory M2 type of macrophage (fig 7). That being said, the cytokine profiles associated with these irritants in these specific mouse models are quite complex and poses interesting options for interpretation. Indeed the cytokine response appears to change based on irritant, which is not necessarily surprising. For instance, TNFα and IL-1, both proinflammatory cytokines, are robustly induced by BKC in the absence of IL-6 (fig 2), but alterations of these cytokines are less apparent following jet fuel exposure. Alternately, the anti-inflammatory cytokine IL-10 seems to be robustly induced by IL-6 deficiency and robustly suppressed by IL-6 overexpression (fig 4). Incidentally, while this seems counter-intuitive since IL-10 is anti-inflammatory, this cytokine is well known to be induced concurrently with inflammatory cytokines such as TNFα in an apparent attempt to inhibit a robust active inflammatory response. Thus, the mRNA expression of this cytokine acts as a marker of active inflammation, and herein seems to be indicative of more severe inflammation.

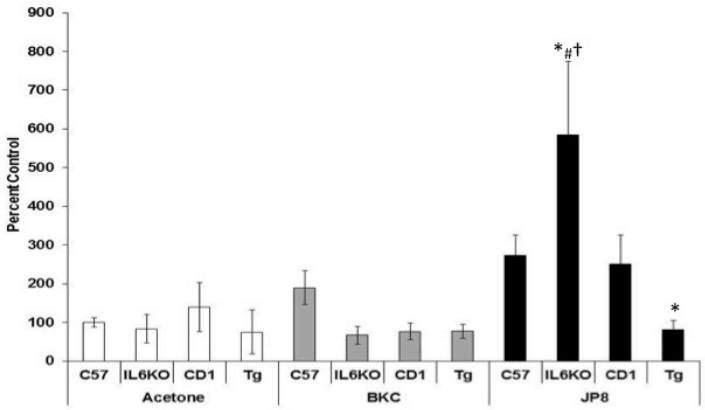

Figure 6. Greater CCL20 mRNA expression in JP-8 treated IL-6KO animals. Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression analyzed via Q-PCR. Expression differences were normalized to 28s rRNA expression and presented as a percentage of c57 control animals. Data are presented as the means +/- SE; n = 6. * p<0.01 vs similarly treated WT control, # p<0.05 vs similarly treated Tg, †p<0.05 vs same strain BKC.

Analysis of chemokine expression further hints at an even more complex story. The CCL chemokines can act as chemotractants for multiple cell types depending on the cytokine (see above). In the context of jet fuel, it seems that IL-6 would decrease macrophage (CCL3, fig 5a) and lymphocyte (CCL20, fig 6) infiltration. However, IL-6 expression in the context of BKC would result primarily in the inhibition of macrophage (CCL3) chemotaxis. Interestingly, after jet fuel exposure CCL11 can be induced by both over and under-expression of IL-6 (fig 5b). Since CCL11 primarily affects eosinophil and Th2 chemotaxis (both associated with allergic responses), the implications of this are not clear since jet fuel is not known to be associated with allergic dermatitis.

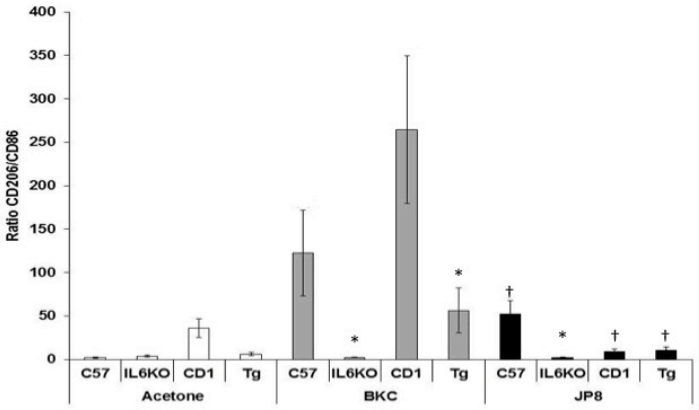

Figure 7. **Decreased ratio of CD206:CD86 mRNA expression in** JP-8 treated IL-6KO animals Following a 7 day exposure to either acetone, benzalkonium chloride, or JP-8, 4mm skin biopsies were obtained for both RNA and protein analysis. Total RNA was isolated, cDNA synthesized, and mRNA expression analyzed via Q-PCR. Expression differences were normalized to 28s rRNA expression and presented as a percentage of C57 control animals. Data are presented as the means +/- SE; n = 6. * p<0.05 vs similarly treated WT control, †p<0.05 vs same strain BKC.

One very interesting aspect of these studies is the regulation of IL-6 itself as a function of irritant. For instance, BKC induced IL-6 expression in WT mice, but not in either IL-6KO or Tg mice (fig 3). This may indicate that following BKC exposure, IL-6 is necessary to upregulate its own expression in response to this type of irritant. Contrary to this, jet fuel did not significantly induce IL-6 expression in WT mice (fig 3) which is somewhat different from the rat response (3). However, IL-6 mRNA was robustly induced in the KO animal. If one assumes that IL-6 is a protective cytokine in skin, its induction would be to counter damage being produced by this complex petroleum irritant. Since it cannot be produced in an the KO mouse, its overexpression would be an attempt to compensate. This discrepancy may also be associated with nature of the inflammation associated with the irritant. The inflammatory response from BKC tends to focus in the epidermis/stratum corneum, as opposed to jet fuel which shows a profound dermal inflammation associated with epidermal hyperplasia (fig 1). However, it is uncertain what the implications of this are, since IL-6 itself seems to act in an anti-inflammatory manner following exposure to either irritant.

When taken in total, these data directly support our hypothesis that IL-6 deficit leads to exacerbation of dermato-pathology. This research will add to the understanding of the overall mechanisms of occupational dermatitis, the basic function of IL-6 in skin inflammation, as well as provide the basis for the development of tools to aid in worker protection and risk assessment.

Publications.

Journal Articles:

One in preparation.

Proceedings:

Lee EG, Mickle BM, and Gallucci RM: [2011] Interleukin 6 modulates irritant dermatitis severity. Proc of the Society of Toxicology, 50th Annual Meeting, Washington DC, p171, March 6-10.

Dissertation/Thesis

Lee EG:[2011] The role of interleukin 6 in inflammatory pathologies, Ph.D. Thesis, University of Oklahoma, Health Sciences Center.

Inclusion of gender and minority study subjects:

Not applicable.

Inclusion of children:

Not applicable.

Materials for Other Investigators:

The Tg(IL6) overexpressing mouse model will be maintained at the OUHSC vivarium. Investigators may obtain this model by contacting the PI and subsequently obtaining permission from Dr. Elaine Fuchs at Rockefeller University.

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