A peer-reviewed version of this preprint was published in PeerJ on 10 July 2017.

View the peer-reviewed version (peerj.com/articles/3555), which is the preferred citable publication unless you specifically need to cite this preprint.

Early M, Schroeder WG, Unnithan R, Gilchrist JM, Muller WA, Schenkel A. 2017. Differential effect of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) on leukocyte infiltration during contact hypersensitivity responses. PeerJ 5:e3555 https://doi.org/10.7717/peerj.3555
Differential effect of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) on leukocyte infiltration during contact hypersensitivity responses

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BACKGROUND: 2’-4’ Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an established model of contact sensitivity and leukocyte migration. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) deficient mice were used to examine the role of PECAM-1 in the migration capacity of several different leukocyte populations after primary and secondary application. RESULTS: γδ T lymphocytes, granulocytes, and Natural Killer cells were most affected by PECAM-1 deficiency at the primary site of application. γδ T lymphocytes, granulocytes, DX5+ Natural Killer cells, and, interestingly, effector CD4+ T lymphocytes were most affected by the loss of PECAM-1 at the secondary site of application. CONCLUSIONS: PECAM-1 is used by many leukocyte populations for migration, but there are clearly differential effects on the usage by each subset. Further, the overall kinetics of each population varied between primary and secondary application, with large relative increases in γδ T lymphocytes during the secondary response.
Differential effect of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) on leukocyte infiltration during contact hypersensitivity responses.

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Abbreviations:

DNFB 2’-4’ Dinitrofluorobenzene
PECAM-1 Platelet Endothelial Cell Adhesion Molecule-1
CD Cluster of Differentiation
NK Natural Killer Lymphocyte
Abstract

BACKGROUND: 2’-4’ Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an established model of contact sensitivity and leukocyte migration. Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) deficient mice were used to examine the role of PECAM-1 in the migration capacity of several different leukocyte populations after primary and secondary application.

RESULTS: γδ T lymphocytes, granulocytes, and Natural Killer cells were most affected by PECAM-1 deficiency at the primary site of application. γδ T lymphocytes, granulocytes, DX5+ Natural Killer cells, and, interestingly, effector CD4+ T lymphocytes were most affected by the loss of PECAM-1 at the secondary site of application.

CONCLUSIONS: PECAM-1 is used by many leukocyte populations for migration, but there are clearly differential effects on the usage by each subset. Further, the overall kinetics of each population varied between primary and secondary application, with large relative increases in γδ T lymphocytes during the secondary response.
Introduction

2'-4' Dinitrofluorobenzene (DNFB) induced contact hypersensitivity is an established model of inflammation. Haptenylation of proteins by DNFB induces a type IV delayed hypersensitivity [1]. One of the most important recent advances in the field of Natural Killer (NK) lymphocyte immunology was the discovery that not only do NK cells contribute to this response, but also that they have a memory-recall response which resides in a liver CXCR6+ population [2, 3].

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1, CD31) is expressed on platelets, endothelial cells and all leukocytes, while it is shed on activated B and T lymphocytes [4-6]. PECAM-1 is expressed at endothelial cell junctions and PECAM-1 homophilic interactions are important as leukocytes undergo extravasation into inflamed tissues [7].

The role of PECAM-1 had not previously been studied in the context of DNFB-induced contact hypersensitivity nor on γδ T and NK lymphocytes. In this study, we tested both the primary and secondary responses of γδ T and NK lymphocytes in wild type and PECAM-1 deficient mice. There are relatively few papers studying the effects of DNFB on γδ T cells [8-10] but this population plays an important role in skin contact hypersensitivity reactions.

Materials & Methods
Ethics Statement: Colorado State University Institutional Animal Care and Compliance committee approved all protocols related to this project (Approval ID 05-153).

Animals

Age (2-4 months) and gender matched male and female wild type and PECAM-1 deficient mice in the FVB/n strain were used for all experiments. Due to the spontaneous pulmonary fibrosis in the PECAM-1 deficient mice in this strain [11], any animals showing disease were excluded after screening by blood oxygen saturation [12]. For all experiments, 5-6 wild type or PECAM-1 deficient mice were used in each group. Each experiment (primary or secondary exposure) was repeated 3 times.

Primary DNFB exposure

25ul of 0.5% DNFB in olive oil was applied on ~1cm² unshaved flank skin by directly pipetting onto the surface. We used unshaven skin because sometimes shaving caused skin irritation. Skin was harvested 48 hour later for histologic and flow cytometric analysis. For flow cytometry, skin was trimmed to 3cm × 5 cm sections before digestion and cell counts.

Secondary DNFB exposure

Five days after primary exposure, 10ul of 0.15% DNFB in olive oil was applied to one ear; the opposite ear was also treated with olive oil to account for grooming of the skin. 48 hours later ears were harvested and fixed in 10% buffered formalin for histology or digested in collagenase for flow cytometry.
Ears or skin were incubated in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing collagenase IV (0.7 mg/ml) for 30 minutes, then RPMI containing 10% fetal bovine serum was added to stop digestion. The entire mixture was filtered through 40um filters to collect cells by centrifugation. Cold phosphate buffered saline was used for all further staining steps. Cells were pre-blocked in 1% normal rat serum and Fc-blocking antibodies before staining with the following antibodies or isotype-matched color controls.

All antibodies (Table 1) and isotype color controls were purchased from eBioscience (San Diego, CA) except for CD206 (Biolegend, San Diego, CA). Clones indicated in parentheses. Flow cytometry was performed on a Dako Cyan (Carpinteria, CA) and analyzed using FlowJo (Ashland, OR) and JMP statistical (SAS, Cary, NC) software.

Gating scheme: Live/dead exclusion was not used because some cells might be missed (dying neutrophils) and few dead cells were found outside of the typical debris or doublet gates. CD3+ CD4+ or CD3+ CD8+ T cells were analyzed for CD44 and CD62L markers of activation status. CD19+ CD3- B cells were analyzed for CD5 and IgD. NK cells were defined as CD3-, γδ TCR -, NKG2D+, DX5/CD49b+. CD3+ γδ TCR+ T cells were also defined by CD8 high or low. Neutrophils were defined by GR1+ CD11b+ CD11c<sub>low/neg</sub>. CD115-. Dendritic cells were defined as CD11c+ CD11b<sub>low</sub>. Monocytes and macrophages were defined as CD11b+ CD11c+ CD115+ [13] and
personal communication with Dr. Gwendalyn Randolph (Washington University, St. Louis, MO).

Statistics

Samples from primary and secondary exposures to DNFB were done a minimum of three times with 4-6 mice in each group (PECAM-1 deficient mice and wild type mice) after testing for power. JMP statistical software (SAS, Cary, NC) was used to analyze differences between groups using Tukey-Kramer Honestly Significant Different or One Way ANOVA tests.

Results

The immune response to primary DNFB exposure in PECAM-1 deficient mice has not previously been reported. Upon dissection of the flank skin at the site of a single DNFB exposure, we found that the blood vessels were more dilated around the major vessels in PECAM-1 deficient mice than in wild type mice (Figure 1).

Somewhat paradoxically to the gross appearance, the flank skin layer had a larger inflammatory infiltrate of cells in wild type mice, primarily mononuclear cells with relatively few neutrophils (Figure 2A, 2C) compared to PECAM-1 deficient mice (Figure 2B, 2D). After collagenase digestion of flank skin tissue and collection of cells, we found significantly fewer total cells in PECAM-1 deficient mouse skin (Figure 3A) despite using equal sized (3cm X 5cm) sections from both mice. Of the cells that were positive for the leukocyte panels we used, most leukocytes in the dermis were monocyte/macrophage
phenotype, with differential effects in the two major populations $CD11b^{low}/CD11c^{high}$

“resident” macrophages not as affected as $CD11b^{high}/CD11c^{low}$ monocyte/small inflammatory macrophages (Figure 3b). $\gamma\delta$ TCR+ lymphocytes and $CD11b^{high}/CD11c^{high}$ dendritic cells were significantly lower in PECAM-1 deficient mice. Using two markers, NKG2D and DX5, for Natural Killer cells, we found they were significantly lower in PECAM-1 deficient mice as well. $CD8^{bright}$ CD3+ cells were found at low levels and not significantly lowered by PECAM deficiency although numbers were lower overall. Upon the secondary challenge five days later in the ear, ear redness and swelling were not significantly different between wild type and PECAM-1 deficient mice (Fig 4).

However, the observed number of inflammatory cells was significantly lower in PECAM-1 deficient mice (Figure 5). $\gamma\delta$ TCR+ cells (Figure 6A), neutrophils (Figure 6B), dendritic cells, DX5+/NKG2D+ cells, and classical $CD4^{+}$ and $CD8^{+}$ cells (Figure 6C) were much lower overall. “Resident” macrophages (Figure 6D) appeared to be mostly unchanged and were by far the largest population. PECAM-1 deficiency significantly reduced the influx of some populations (effector $CD4^{+}CD62L^{-}/CD44^{+}$ T cells, NK cells, neutrophils, and $\gamma\delta$ TCR+ cells) but not others (effector $CD8^{+}CD62L^{-}/CD44^{+}$ T cells, and dendritic cells). It was most striking that the cells most mobilized by the secondary inflammatory response (neutrophils and $\gamma\delta$ TCR+ cells) were also most affected by PECAM-1 deficiency.

**Discussion**

This study shows two important results. First, the high participation of $DX5^{+}/NKG2D^{+}$ phenotype cells in the primary immune response to DNFB does not
necessarily correspond to a similarly proportioned response at a secondary challenge. Likewise, $\gamma \delta$ TCR+ cells were relatively minimal (~1% of total) in the primary challenge site but became quite vigorously involved in the secondary response, outnumbering any other single population (other than resident macrophages) by almost 5-fold.

Second, PECAM-1 deficiency manifested itself in differential effects on each population. Most were affected by the deficiency, but it was surprising that CD8+ T effectors were able to overcome the lack of PECAM-1 whereas CD4+ T cell infiltration was clearly altered. We did not look for CD4+ CD3+ T cells at the primary exposure site, as we did not expect to find many in the skin as we thought the primary response would be largely driven at this stage by macrophages and NK cells, and that CD4 responses would be activated later by antigen-presenting cells. Further, it would be fascinating to examine the effects on CD4+ T cells at both the primary skin flank exposure site and the ear as CD4 cells do play a significant helper role in the response [14]. Clearly CD4 help at both sites may potentially drive some activation and differential effects on CD8 and $\gamma \delta$ TCR+ cells at both sites. DX5+/NKG2D+ cells, being innate, may in turn be waning in their responses as the adaptive immune response takes over the inflammatory process.

Another leukocyte population we did not examine is the Innate Lymphoid cells (ILCs), which were largely unknown at the time these studies were performed [15]. NK cells are also part of the Innate Lymphoid cell family, and it makes logical sense to explore the role of ILC1, ILC2, and ILC3 subsets in this response. Additionally, we did not look at cell counts for fibroblasts, epithelial cells, endothelial cells, which may comprise a large number of the total cells collected (Figure 3A). A more holistic
approach to all of the cells involved could be very informative as it was also somewhat
surprising to see the differential effects on the vasculature dilation (Figure 1) in PECAM-1
deficient strain at the site of primary exposure. As PECAM-1 is vital to endothelial cell
permeability [16, 17], this study shows quite dramatically the role of this molecule to the
earliest stages of the inflammatory response in this model as well.

There is considerable evidence that γδ TCR+ cells play a significant role in skin
allergies and hapten-induced contact sensitivity [10]. Many of them also expressed
NKG2D, and NKG2D played a role in the activation cycle [18]. More recently, the
contribution of Vγ4 T cells in the response to DNFB and subsequent neutrophil
infiltration was elegantly mapped out by Jiang et al. [19] Our work largely complements
those studies, though it is important to note that their use of the term “primary exposure"
still is measured after a second exposure to DNFB.

Our studies do pose many questions to answer in possible future studies. In
particular, we would like to pursue a longer lag time between primary and secondary
challenges. For consistency, we used the classical assay used by many other groups
with a total of 7 days between primary challenge and harvest after secondary challenge
[1, 2, 20]. Perhaps the response kinetics would be altered between each population. It
has previously been shown that memory recall response by NK cells in mice can last as
long as four months [3].

We would also would have preferred to use NK1.1 as a marker for confirmation of
the NK phenotype. Unfortunately, we did not use NK1.1 on FVB/n mice as we only
retrospectively discovered that FVB/n mice do indeed express NK1.1 [21]. Finally, it
would be very important to identify which γδ TCR population(s) are mobilized, as there
is tissue specific localization of the γδ TCR subsets. The Vγ3 subset is commonly found in skin [22], however it would be interesting to assess whether any blood Vγ1, Vγ2 or epidermal Vγ5 [23] would be mobilized or differentially affected by PECAM-1 deficiency, similar to the different effects on CD4+ and CD8+ T cells.

Unfortunately, this strain of mice was lost to future studies at this time due to poor breeding and limited lifespan due to spontaneous pulmonary fibrosis [12]. There is a PECAM-1 deficient mouse in the C57BL/6 background. The C57BL/6 surprisingly has a near-normal response in many inflammatory models compared to wild type mice [24], whereas the FVB/n strain exhibited the phenotype we expected with markedly reduced inflammatory responses. This difference mapped to a locus on chromosome 2 [25]. The C57BL/6 strain is also resistant to the pulmonary fibrotic disease [11], and this appears to be related to vascular function [17]. It would be interesting to again compare and contrast these strains and the effects on their populations in future studies. However, we believe any subsequent studies would be best conducted with cell-targeted gene disruption of various subsets like monocytes, natural killer lymphocytes, or γδ TCR+ to also further determine the roles of these subsets in the response to DNFB.

Competing interests: The authors state they have no competing or conflicts of financial or non-financial interest.

Authors’ contributions:

M.E., W.G.S., R.U., J. M. G., M.L., and A.R.S. did the animal care and exposure studies, including tissue harvest, flow and histological preparation, and data analysis.
Mice, funding as principle investigator, and data analysis was supplied by W.A.M.. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to thanks Bianca Junge and Elisa French for expert animal care. Additionally, Jamie S. Schenkel helped proofread the final manuscript.
Figure Legends

Figure 1. Gross anatomy of the skin at the flank site of primary DNFB application 28 hours after application. The dilation of vasculature is much more prominent in the PECAM-1 deficient mice. Lower panels show higher magnification.

Figure 2. Representative sections of inflammation in primary flank skin exposure to DNFB from wild type (A, 2X magnification; C, 20X magnification) and PECAM-1 deficient (B, 2X magnification; D, 20X magnification) mice.

Figure 3. Total (A) and leukocyte subset (B) cell counts from wild type and PECAM-1 deficient flank skin at the treatment sites, 48 hours after DNFB application. * p <0.05, ** p <0.01 Tukey Kramer HSD test. ns = not significant difference.

Figure 4. Right ear swelling by skin caliper measurement upon secondary exposure to DNFB in wild-type and PECAM-1 deficient mice. Left ears were measured as controls.

Figure 5. Representative ear sections after DNFB treatment. A-B: DNFB treated wild type ears. C-E: DNFB treated PECAM-/- ears. F: Untreated wild type control, PECAM-/- were equivalent in examination. 20X magnification.

Figure 6. Cell counts from treated (right) and untreated (left) ears. Larger populations (A, B, and D) are separated out for clarity. A. γδ TCR+ cell counts. B. Granulocytes. C. Other lymphocyte populations. D. Resident macrophages, by far the largest population,
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Table 1 (on next page)

Flow Cytometry Reagents Used
| Color       | CD4+ T                | CD8+ T                | CD19+ B         | NK/γδ T cell     | Myeloid Cells |
|------------|-----------------------|-----------------------|-----------------|------------------|---------------|
| APC CY7    | CD3 (145-2C11)        | CD3 (-)               | CD3             | CD3              |               |
| PE CY5     |                       |                       |                 |                  |               |
| Pacific Blue| CD4 (L3-T4)          | CD8 (53-6.7)          | CD19 (6D5)      | CD8              | CD11c         |
|            | CD44 (IM7)            | CD44                  |                 |                  | CD11b         |
|            | CD62L (MEL-14)        | CD62L                 |                 |                  | GR1           |
| FITC       |                       |                       |                 | NKG2D           | CD206         |
|            | CD44 (IM7)            | CD44                  |                 | 11-26c           | CD206         |
| APC        | CD4 (L3-T4)          | CD8 (53-6.7)          | CD19 (6D5)      | CD8              | CD11b         |
|            | CD44 (IM7)            | CD44                  |                 |                  | GR1           |
|            | CD62L (MEL-14)        | CD62L                 |                 |                  | CD115         |
| PE Cy7     |                       |                       |                 |                  |               |
| PE         | IgD (11-26c)          |                       |                 |                  |               |
|            |                       |                       |                 |                  |               |
Figure 1\textit{(on next page)}

Gross anatomy of the skin at the flank site of primary DNFB application 28 hours after application.

The dilation of vasculature is much more prominent in the PECAM-1 deficient mice. Lower panels show higher magnification.
Figure 2 (on next page)

Inflammation in primary flank skin exposure to DNFB

Representative sections of inflammation in primary flank skin exposure to DNFB from wild type (A, 2X magnification; C, 20X magnification) and PECAM-1 deficient (B, 2X magnification; D, 20X magnification) mice.
Figure 3 (on next page)

Leukocyte counts at primary DNFB exposure flank skin

Total (A) and leukocyte subset (B) cell counts from wild type and PECAM-1 deficient flank skin at the treatment sites, 48 hours after DNFB application. * p <0.05, Tukey Kramer HSD test. ns = not significant difference.
Figure 4 (on next page)

Ear swelling at secondary challenge

Right ear swelling by skin caliper measurement upon secondary exposure to DNFB in wild-type and PECAM-1 KO mice. Left ears were measured as controls for each animal.
Ear Swelling Two Days Post-Secondary
DNFB Exposure

- ● PECAM KO Left Ear
- ■ PECAM KO Right Ear
- ▲ WT Left Ear
- ▼ WT Right Ear

Ear Thickness (mm)

Group
Figure 5 (on next page)

Representative ear sections after DNFB treatment.

A-B: DNFB treated wild type ears. C-E: DNFB treated PECAM KO ears. F: Untreated wild type controls and PECAM KO were equivalent in examination. 20X magnification.
Figure 6 (on next page)

Cell counts from treated (right) and untreated (left) ears.

Larger populations (A, B, and D) are separated out for clarity. A. γδ TCR+ cell counts. B. Granulocytes. C. Other lymphocyte populations. D. Resident macrophages, by far the largest population, were largely unaffected by DNFB treatment. * p <0.05, Tukey Kramer HSD test. ns = not significant difference.
