Interrogating the human immune response via cutaneous inflammatory challenges to aid drug development

A Thesis Submitted for the degree of Doctor of Philosophy, University College London

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Declaration

‘I, João Joaquim Dias de Matos Oliveira confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Abstract

Clinical research is continuously under pressure to develop new therapeutic entities whilst containing the spiralling costs of research and development. Obtaining critical data regarding target engagement in relevant tissue, and proof of mechanism in early phase clinical trials, allows evaluation of the efficacy of the asset and thus identifies programmes for termination before the significant economic, health and reputational cost of late-phase failure. Skin blisters allow an easy access for investigating inflamed tissue, where type and level of inflammation can be experimentally adjusted with the use of local inflammatory challenges.

Although the technique is in widespread use, the biology behind the inflammatory stimuli driven by cantharidin or suction that results in leukocyte rich blisters is not well understood; nor is the variability of the cellular and soluble components of the resulting blisters both within and between subjects, a crucial element required for the design of well powered in vivo experiments.

This research project aims to identify the role of cantharidin in driving the blister reaction and how this compares to the suction model. By drawing samples shortly after challenge, it highlights the primary effect of TNF secretion by stromal cells in response to cantharidin, prior to the formation of an observable blister, and allows the identification of key mediators of the immune response. It also provides a detailed time course of the cellular and humoral components throughout the blister reaction. Also, by using duplicate and repeat challenges in the same subjects, this project allows the determination of inter- and intra-subject variability estimates for the main blister outputs and identifies best practices for the design of clinical trials using these models.

To demonstrate the potential of blistering models in drug development, this project showed the effects of steroids in the pharmacological modulation of the local cantharidin blister response, by reducing oedema formation and cell recruitment and highlighted differences in cellular responses to topical and systemic doses of similar compounds in both tissue and circulating leukocytes.

Impact statement

The work described in this thesis was designed to address gaps in the use of human challenge models in clinical research, but is also directly relevant to the investigation of the biology underlying tissue inflammation and subsequent resolution.

Past research, both clinical and academic, has predominantly used one of two different methodologies to produce skin blisters to access inflamed tissue. By characterising the immunological processes that follow suction and cantharidin application, describing the technical differences between these, and comparing the cells and soluble mediators elicited by these two approaches side-by-side, the results on this thesis permit selection of the most appropriate model for different applications based on the biomarker profiles here described.

In cataloguing the sequence of immunological events that follow each challenge in parallel for the first time, this thesis allows for the determination of the optimal time to intervene in the blistering process to modulate the inflammatory response with pharmacological agents and informs when to collect samples for specific biomarkers.

By not only analysing a wide variety of blister outputs from a cohort of healthy participants, but also undertaking repeated measurements in the same individuals using concurrent, parallel challenges over a period of two weeks, the data in this report accurately describes baseline variation within a demographically homogenous group as well as within- and between-participant variability. Intra-individual responses were seen to be strikingly more consistent than inter-individual. The variability estimates that can be obtained from these data will inform power calculations which determine the number of participants necessary for future experimental medicine studies.

Data from this report further show that using a known immunomodulator (corticosteroids) in a study design where blister tissue is sampled pre- and post-dosing (within-subject), pharmacological effects can be determined in small cohorts of participants, whereas previous studies were unable to identify differences in larger

cohorts using parallel groups due to the high variability of the immune response observed between individuals. This observation is extremely pertinent for the design of future early phase clinical trials or experimental medicine studies that seek to determine proof-of-mechanism and establish pharmacokinetic-pharmacodynamic relationships in vivo.

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Chapter 1: Introduction

1.1 Current challenges in pharmaceutical development

Technological advances, especially in computing power have been used in drug discovery for identifying new medicines and targets. With advances in genetics and different 'omics' platforms providing massive datasets and the use of machine learning and artificial intelligence to mine these resources, the result has been the identification of multiple novel targets for pharmacological intervention.

Given these technological advances and huge gains in scientific knowledge, it is striking that the global output of pharmaceutical R&D has not delivered a similar increase in novel medicines. The number of novel therapies reaching the market was flat throughout the 1970s to 2010 with only around 23 new drugs per year (Ward, Martino et al. 2013). This trend has recently showed a small increase, fuelled by an increase in approvals for rare diseases with a heavy genetic link, but is likely not to be sustained (Ringel, Scannell et al. 2020).

High throughput screens have been used since the early 2000s, employing 'brute force' strategies to screen hundreds of thousands of compounds to identify promising candidates that modify function of these novel targets, with a large number of compounds emerging as promising new drug substances. But the path to final approval and clinical use is a long and vastly expensive one.

Despite advances, the number of new drugs launched per billion dollars of R&D spending has fallen by around 80-fold since 1950 (Scannell, Blanckley et al. 2012). Current estimates place the price to take a new drug to market somewhere between $314 million to $2.8 billion, depending on therapeutic area and study design, with this number increasing year upon year (Wouters, McKee and Luyten 2020). Although difficult to calculate, more than half of that value is spent in clinical trials (DiMasi, Grabowski and Hansen 2016), with costs escalating over the progression of the drug development.

Only a small minority, currently around 7% (Dowden and Munro 2019) of assets that initiate clinical trials will ever achieve approval. Most of these assets will fail at the initial hurdle due to safety signals when first tested in healthy participants. The major cost-wastage in clinical development, however, come from assets that are deemed safe and continue to more expensive later stages of evaluation, but fail to demonstrate clinical efficacy (Hwang, Carpenter et al. 2016). As clinical development costs escalate

from early to late stage, data-based decision-making should ensure that only likely efficacious drugs are progressed when safety is observed, and those data could be obtained from well-designed phase I clinical trials.

The huge cost of drug development directly impacts patients in multiple ways. Not only does it inflate the cost of medicines when it is passed on to the final consumer, as pharmaceutical companies seek to recoup their losses through approved drugs, but it limits the resources available for other programmes, often deterring companies from putting efforts towards developing treatments for complex diseases such as Alzheimer's, which are less likely to be successful.

Many experts argue that the prevailing economic models are unsustainable in the long term and internal lessons must be translated into changes to 'standard' processes. Productivity must be enhanced, and the key points in the clinical journey that can catalyse this have been clearly identified as increases in probability of success during Phase II and Phase III. To achieve this, money and talent need to be re-focussed onto the preceding Phases, namely discovery, research and early translational medicine (Paul, Mytelka et al. 2010).

1.2 Focus on early proof of mechanism in target tissue – Lessons from pharma companies

In a critical review of the fate of their pipelines, pharmaceutical companies have identified clear attributes that candidate drugs must possess to have higher chances of progressing to market and avoiding costly late phase attrition: 1. Exposure of drug at target site over the desired amount of time; 2. Target occupancy and 3. Pharmacological activity. Pfizer noted that when the presence of the drug at the site of action or an adequate tissue surrogate was demonstrated, binding of the drug to the target in the relevant tissue was observed, and modulation of downstream pathways was established, 60% of their Phase II programs progressed, compared with 7% of those where at least one of these goals was not observed (Morgan, Van Der Graaf et al. 2012).

AstraZeneca also refocused its strategy in the early 2010s and identified a set of principles to guide their pipeline (Cook, Brown et al. 2014). The 5 Rs – right target, the

right patient, the right tissue, the right safety and the right commercial potential – were implemented as a framework. By reducing the number of clinical trials allowed to proceed without substantial evidence of target engagement in tissue and clinical utility, this approach increased the success rate for new drug substances proceeding from Phase I to Phase III from 4% (2005-2010) to 19% (2012-2016) (Morgan, Brown et al. 2018).

Also looking back on exceptional successes and many failures, the Merck group published their Translational Medicine Guide. This is an attempt to design a framework of processes that ensure that the correct questions are asked at the right point in time, to provide the right evidence to: 'Trust the target, trust the therapeutic window and trust the patient population'. These three translational aspirations are tested in each of 4 milestones at different stages in the development process with pre-clinical (model) and clinical (human) 'Proof of Principle' (demonstrating biomarkers of drug effect in disease biology) and 'Proof of Concept' (demonstrating modulation in disease models) studies (Dolgos, Trusheim et al. 2016).

In a slightly different approach, Eli Lilly has deferred development of some of their assets and tested them in the 'Chorus' model. Here, programmes with a low initial probability of success are subjected to 'lean to proof of concept', where estimations on the probability of success of a project are determined rapidly and at a low cost in the early clinical development stage. Central to this is the attempt to establish proof of mechanism in Phase I clinical trials, where a successful asset demonstrates target engagement with an acceptable therapeutic index, a practical dose regimen and a manageable degree of PK/PD variability. Programmes going through Chorus show a higher level of attrition in Phase I, but a much lower capitalised development cost to launch due to reduction in number of ineffective Phase II trials (Owens, Raddad et al. 2015).

Table 1: Summary of lessons from pipeline analysis from big pharmaceutical companies

Pfizer (2012) 3 pillars of survival	• exposure at the target site of action • binding to the pharmacological target • expression of pharmacology	Three fundamental PK and PD principles that collectively determine the likelihood of testing the mechanism of action and influencing the likelihood of candidate survival in Phase II
AstraZeneca (2014) The five 'R's	• the right target • the right patient • the right tissue • the right safety • the right commercial potential	Focus on building confidence in each component of the 5R framework – to identify gaps in knowledge and understanding, and to work to fill those gaps as quickly as possible during the clinical development phase.
Merck (2016) Translational Medicine Guide	3 aspirations – Build trust in: • Target • Therapeutic window • Patient population At critical milestones: • Preclinical PoP • Preclinical PoC • Clinical PoP • Clinical PoC	Proof of principle: demonstrate the beneficial therapeutic effect on the targeted disease process or pathophysiology Proof of concept: demonstrate that modified disease biology translates into a beneficial therapeutic effect on clinical outcomes
Eli Lilly (2015) The Chorus Model	Lean to Proof of Concept – Quick win, fast fail model focussed on reaching PoC efficiently, faster and with lower cost	An alternative approach designed for large scale organisations aimed to take advantage of a large portfolio. 'Killer experiments' are designed to address the major uncertainties of many assets as quickly as possible and investment in downstream activities are deferred until obtaining supporting data, resulting in fewer, but more promising candidates.

1.3 Utility of animal models of disease

Translational science aims to convert biological discoveries in the laboratory into therapies that improve human health, a bench-to-bedside process that starts with studying drugs and drug targets at a molecular level and will progress into cells, organs and organisms in both healthy and disease states. Although the first stages can be achieved in vitro, there is the need to progress findings into animals where efficacy is tested in well-defined animal disease models.

The considerable communality between mammals, both genetical and physiological, has allowed for a long and successful history of animal use in many scientific fields. Analogous conditions to human diseases such as type 1 diabetes, cancer, and hypertension can be observed in various animal species. and most veterinary drugs are identical or very similar to human medicines (Nelson, Tipney et al. 2015), indicating similarities in the disease mechanisms. The use of insulin as treatment for diabetes was originally established in the dog by Banting and MacLeod, leading to a Nobel prize (Diem, Ducluzeau and Scheen 2022), the development of anti-Tumor Necrosis Factor (TNF) for rheumatoid arthritis and inflammatory bowel disease was achieved in mouse models of the disease (Piguet, Grau et al. 1992, Powrie, Leach et al. 1994) and more recently, tissue regeneration using stem cells was first demonstrated in mice (Klug, Soonpaa et al. 1996). Animal use in translational research is also mandated by regulatory agencies. Before any human trials can commence, safety and toxicity studies must be performed in at least 2 separate species, one being non-rodent.

Nonetheless, the use of animals in clinical research has also been criticised. Animal protection and welfare are frequently disregarded in science, but a policy of 3R's (replacement, reduction and refinement) (Russell, Burch and Hume 1959) ensures that only relevant experiments are done with the minimum number of animals and considerations to the welfare of the animals are in place.

The use of animal models relies on a key understanding of differences in fundamental inter-species biology, appropriate study design and - crucially - an appreciation of what features they are and are not able to recapitulate of human disease. A review of papers reporting successful therapies in animal models from the seven highest impact journals that had accrued more than 500 citations, found that only a fraction proved effective in human trials (Hackam and Redelmeier 2006). Large genetic distances

between species, the homogeneity of inbred mice and the inability to accurately recapitulate the complexity of human diseases, especially chronic conditions associated with multi-morbidity may explain the lack of observed translation.

Significant genetic variation can be observed even within members of the same species, and this can translate into very different outcomes. Commonly used mouse strains challenged with Ebola virus, for example, differ in the response to the disease with phenotypes ranging from complete resistance to full mortality (Rasmussen, Okumura et al. 2014). The same full range of responses is observed in mouse models of sepsis, where genetic background of strain can skew the outcome of endotoxin challenge from resistance to death, which fails to recapitulate the complex pathophysiology observed in human disease (Rittirsch, Hoesel and Ward 2007). This in part reflects the differences in outcomes observed in the human population and demonstrates that no single approach can mimic complex, heterogenous human pathology, and that differences between species or strains must be understood to appreciate the predictive value of a given model system.

Another example of the limitations of animal models in translational science comes from inflammatory challenges. Here, it has been demonstrated that whilst trauma, burns and sepsis induce similar genomic responses in humans, the corresponding mouse models correlate poorly with the human conditions and even between the models (Seok, Warren et al. 2013), indicating that whilst some elements match human pathophysiology, the molecular pathways diverge or are wholly different. Chronic human inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and asthma are often multifactorial, involving complex interactions between genetic, environmental, and lifestyle factors that are difficult to replicate in laboratory animals (Mestas and Hughes 2004).

Still animals are used in clinical research for multiple goals, from early discovery to mandatory safety assessments of new pharmaceuticals. The utility of animal models of disease depends on their ability to approximate the processes that a novel therapy will modify when applied to humans. A useful method may be one which replicates a human disease, when processes are sufficiently overlapping, but also when therapy-relevant pathways/processes are common. Human pathology may be approximated

by a different animal disease model as long as it demonstrates targeting of a critical pathway in the disease of interest.

1.4 Design of Early Phase clinical trials

The corollary to these observations is that more effort should be put into human pre-clinical and very early clinical development. By determining a clear relationship between target and disease using human genetic linkage data (Furdos, Fazekas et al. 2015), and by using adequate clinical translational models and validated biomarkers of efficacy, drugs are much more likely to succeed (Cook, Brown et al. 2014).

It is now clear that well-designed Phase I trials should focus on measuring biomarkers of possible efficacy (pharmacodynamics) rather than just safety and pharmacokinetics, with inclusion of either diseased cohorts or optimised human models of disease to show clear proof-of-mechanism, to allow the critical shifting of attrition to the early stages of clinical development (Paul, Mytelka et al. 2010).

Phase I clinical trials offer higher flexibility for data collection. By designing more effective sample collections, in better characterised participants which can be more thoroughly assessed, data from a small number of individuals can be used to demonstrate the mechanism of action of the investigational product. After reaching a clinically relevant dose, use of novel analytical methods such as single-cell RNA sequencing or spectral flow cytometry can be done in a small number of individuals or well-characterised patients, at multiple times pre- and post- dosing and/or pre- and post- immunological challenge to derive huge datasets exploring the relevant pathways and biological effects of the drug in each participant.

Such studies are not feasible in larger scale, multi-site clinical trials in later stages and enable informed go/no-go decisions to be made following Phase I, which may prevent drugs failing in expensive later phase clinical trials due to lack of efficacy.

1.5 Human models

Some relevant information supporting pre-clinical proof of concept can be obtained from the use of in-vitro human models. 2D cultures of patient samples can provide

basic evidence supporting target engagement and pharmacology, but inevitably poorly replicate the tissue environment in a human body where different cells are spatially connected in a dynamic state. Improvements can be found with the use of bioengineered tissues, an area in active development, that include organoids, microphysiological systems, organs-on-chips and 3D-printed platforms, but these experimental systems are still unable to fully recapitulate the in vivo human biophase, due to lack of complete vascular and immune systems.

When exploring the pharmacology of immunomodulatory drugs, the use of healthy humans or tissues in homeostasis may not be appropriate due to the lack of expression of relevant targets, pathways or processes. In-vivo human challenge models can however be used to transiently recapitulate elements of disease states in healthy participants in a controlled manner. Such models include inoculation with respiratory syncytial virus (DeVincenzo, McClure et al. 2015) and vaccination against Salmonella typhi (Hingorani, Cross et al. 2000), to trigger both acute and chronic inflammation, and recapitulate features of disease. These interventions can be conducted in a highly standardised manner in a selected homogeneous population to obtain data that allow investigation of novel drugs or mechanisms of systemic inflammation in vivo (Bahador and Cross 2007).

As a further example, endotoxin (LPS) can be used to simulate elements and features of different disease states. Intravenous LPS can elicit either an acute systemic inflammatory response, using a high dose (2-4 ng/kg) (Seok, Warren et al. 2013) as well as low-grade inflammation (using a lower dose/longer infusion), as observed in type 2 diabetes (Andreasen, Krabbe et al. 2008). Intradermal LPS elicits local inflammation (Buters, Hameeteman et al. 2022), whereas respiratory models can be accomplished by inhaled (Loh, Vyas et al. 2006) or instilled LPS (van Lier, Geven et al. 2019). A standardised challenge agent, manufactured to GMP grade, can thus be used in a variety of administration routes and dosages to produce controlled inflammation of different degrees and duration in multiple tissues to permit the modulation of discrete pathways in different healthy and patient populations (Hingorani, Cross et al. 2000). Safety and tolerability, as well as inter- and intra-subject variability in inflammatory responses in healthy participants can also be assessed (Janssen, Schaumann et al. 2013). Challenge/re-challenge methodologies may also be possible after a wash out period (typically of at least 5 weeks), allowing for the use

of a model in the same subject at different stages of the clinical trial and intra-subject comparisons (e.g. after placebo vs. after active dose) (Rittig, Thomsen et al. 2015).

If used in early clinical trials, human challenge models are often deployed in a homogeneous population, by defining clear demographic or genetic inclusion criteria, to constrain variability and allow for a clearer observation of signal vs noise. Drug effect can be observed in the presence and absence of challenge, and biomarkers of modulation of disease-like state can be assessed frequently after challenge until return to homeostasis. Large volumes of highly complex data, such as proteomic or single cell transcriptomic, can be obtained from a small cohort of participants in such studies, allowing for a rapid and cost-effective way of ascertaining the ability of the drug candidate to modulate a specific disease pathway in early phase clinical research. This provides the ability to make critical go/no go decision regarding progression to later phases.

1.6 Cutaneous models of inflammation

The study of the human leukocyte component of inflammation has been undertaken predominantly in cells isolated from blood samples. However, cells such as neutrophils and monocytes/macrophages carry out their function in extravascular tissues following the process of recruitment and migration that necessitates and/or induces clear activation profiles. Therefore, use of cells derived from the circulation, often examined outside a tissue matrix, may not accurately reflect the behaviour of extravasated cells contributing to the inflammatory response.

The skin is the most accessible organ of the body in which to both view and access inflammatory processes and assess their pharmacological modulation. It is possible to directly challenge this tissue, visually assess changes to the dermis and associated vasculature, biopsy a full section of the skin, and to analyse the vascular response with non-invasive methods. Further, drug concentration may be quantified and pharmacokinetic (PK) / pharmacodynamic (PD) relationships established.

Delayed-type hypersensitivity (DTH) skin reactions to intradermal injections of specific antigens have been historically used to investigate immune function in different populations. Data on clinical measures of induration and erythema from the use of

neo-antigens like keyhole limpet haemocyanin (KLH), (Palestine, Roberge et al. 1985) or recall antigens such as PPD in individuals previously vaccinated with Bacillus Calmette Guérin (BCG) (Vukmanovic-Stejic, Reed et al. 2006) supports the use of these methods for clinical research (Belson, Schmidt et al. 2016, Saghari, Gal et al. 2021). Recent data from a Phase I study demonstrated proof-of-pharmacology for a novel drug via observing suppression of blood perfusion and redness in response to KLH re-challenge in the presence of an anti-OX40L drug (Saghari, Gal et al. 2022) further underlines their translational potential.

Other stimuli can also be used to elicit a controlled, transient inflammatory response in the skin. Trauma is often used to create a window to the stratum corneum. This has been achieved with a scalpel (Rebuck and Crowley 1955), high-speed drill (Senn, Holland and Banerjee 1969) or sandpaper (Marks, Harbord et al. 2006) to create the lesions that initiate the inflammatory response. Equally, intradermal injection of histamine has been used as a model of allergen induced inflammation (Clough, Bennett and Church 1998), specifically to investigate the pharmacological mechanisms underlying microvascular responses. Application of Imiquimod, a topical drug for the treatment of genital warts, superficial basal cell carcinoma, and actinic keratosis can also create a local inflammatory reaction characterised by erythema and skin perfusion, especially after the removal of the upper layers of skin by tape stripping (van der Kolk, Assil et al. 2018), that has similar effects in a murine model (van der Fits, Mourits et al. 2009).

The most common way of forming aseptic lesions in the skin however is by creating skin blisters. The raising of a blister is appealing as it allows easy access to both inflamed tissue cells and interstitial fluid via harvesting of the exudate in a safe and well tolerated manner. These methods can easily be used in a clinical trial setting to track the pharmacological modulation to the inflammatory response in human tissue. Two methodologies are currently used to create blisters: one via topical application of a chemical agent (cantharidin) (Day, Harbord et al. 2001), the second via use of suction or negative pressure (Holm, Vukmanovic-Stejic et al. 2018). Both allow the sampling of extravasated cells and the assay of soluble mediators in inflamed tissue; they will be discussed in detail in the following chapters.

1.7 Hypothesis

The use of skin blisters in clinical research allows for the exploration of tissue pharmacology in humans to predict likely efficacy of drug in early phase clinical trials.

1.8 Aims

In order to address this hypothesis, I intend to:

1. Elucidate the biological effect of cantharidin in the blister model and its role in the inflammatory response observed.
2. Map the early inflammatory response in both suction and cantharidin blisters to reveal the full cellular and humoral kinetics of both models, allowing for selection of appropriate sampling times for selected biomarkers.
3. Compare and contrast the immune response in the two blister models by investigating leukocyte composition as well as cytokine and chemokine profile in concurrent suction and cantharidin blisters and determine variability estimates for these models within-subject in simultaneous samples, within-subject between temporally-separated samples, and between-subject samples.
4. Prove that detection of pharmaceutical modulation of the inflammatory response can be improved via optimisation of study design.

Chapter 2: General Materials and Methods

2.1 Clinical studies and ethical approval

2.1.1 Healthy volunteer blood samples for cantharidin ex-vivo assays

Whole blood samples for understanding the effect of cantharidin in human primary cells were obtained under the approved UCL ethics application 1309/006: 'Understanding human immune responses to infection and injury' that permits the withdrawal of whole blood from healthy volunteers, male or female, from 18-85 years of age.

2.1.2 Optimisation of cantharidin blister model

Data described in relation to the development of the novel technique for cantharidin application was obtained via compiling output from GlaxoSmithKline (GSK) clinical studies RES112593, EMI114416 and 207654, collected under ethical approval (Research ethics committees: Essex 2 09/H0302/50, Cambridgeshire 2 10/H0308/63, Oxford B 17/SC/0286) and after obtaining appropriate informed consent. Volunteers for all studies were healthy males (age range of 18-55). Exclusion criteria included very fair or very dark skin type (Fitzpatrick scales I and V-VI); presence on either forearm of tattoos, naevi, scars, keloids, hyperpigmentation or any skin abnormalities or history of lymphangitis and/or lymphoedema. All work was conducted at the GSK Clinical Unit Cambridge (Addenbrooke's Hospital) with lab work conducted by myself.

2.1.3 Direct comparison of suction and cantharidin blistering procedures

Data pertaining to the inter- and intra-individual assessment of chemical and suction blistering was collected in project 5060/003: 'Comparison and characterisation of two skin blister models of inflammation', approved by the UCL Research Ethics Committee (Appendix 1). All work with participants was conducted in the Clinical Research Facility in the Rayne Building, Division of Medicine, UCL following the research facility's internal guidelines. Non-smoking, healthy male volunteers aged 18 to 50 years were recruited. Exclusion criteria included taking of regular medication, darker skin tone (Fitzpatrick scale V-VI, due to risk of keloid scarring), tattoos, damaged skin or existing scars at blistering sites. 11 volunteers were recruited (mean age 29.6, range 20-49).

One subject withdrew after visit 1 due to an adverse event not associated with the study. One subject did not attend his last visit due to COVID-19 self-isolation.

2.1.4 Cellular and humoral kinetics of blister contents and impact of known immunomodulators

A second ethics application was submitted to further characterise the time course of the responses to suction and cantharidin challenges and to determine whether these models can effectively be used in clinical trials to show a pharmacological effect: 23833/002 'Utility of in vivo skin blister models to detect inflammatory modulation by pharmacological agents' (Appendix 2). This was approved by the UCL Research Ethics Committee on the 24 May 2023 and work was conducted in the Clinical Research Facility in Rayne Building, Division of Medicine, UCL.

The same inclusion and exclusion criteria were used as in project 5060/003 and a total of 10 subjects were enrolled over 2 the separate parts of the study.

2.2 Biological samples

The majority of experiments were carried out using samples obtained from healthy participants enrolled in ethically approved clinical studies. Blinding of laboratory operators was not feasible for these studies so laboratory processing of clinical samples was carried out unblinded.

Commercially available cell lines were used for specific ex-vivo experiments.

2.2.1 Human blood samples

Blood samples were collected from the antecubital fossa using a 20g butterfly needle and aseptic non-touch technique, into BD Vacutainer® sodium heparin tubes (17 IU heparin per mL of blood) and either used as whole blood or processed to PBMCs (peripheral blood mononuclear cells).

2.2.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood collected into BD Vacutainer® sodium heparin tubes was diluted with same volume of sterile PBS (Gibco) and layered over 5 mL of density gradient medium (Ficoll-Paque™, GM Healthcare) in a 15 mL Falcon tube. Samples were centrifuged at 800 g for 20 minutes with low acceleration and no break. After centrifugation, the PBMC layer, a whitish cloudy band located in between the plasma layer on top and the gradient (Figure 1), was collected into a new 15 mL centrifuge tube and washed in RPMI 1640 medium (Gibco) by topping up tube and centrifuging at 400 g for 10 minutes. This was followed by a second wash in RPMI 1640 medium, centrifuging at 500 g for 6 minutes. Cells were counted for further use on a haemocytometer (Neubauer improved).

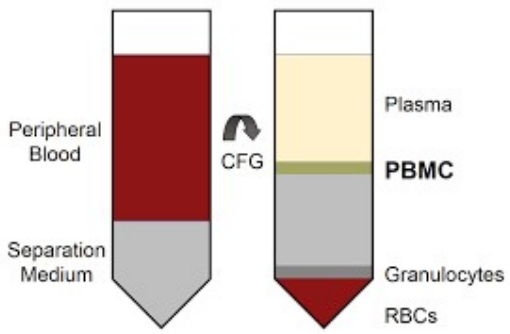


Figure 1: PBMC isolation. Whole blood is layered on top of a gradient solution and after centrifugation a PBMC layer is collected between the plasma and gradient layers.

2.2.3 Suction pressure blister formation

Suction blisters were generated as described by several groups (Davidsson, Bjorkman et al. 2013, Motwani, Flint et al. 2016), using a chamber with a 10 mm diameter aperture connected to a negative pressure device (NP-4, Electronic Diversities, USA, Figure 2A) and secured with tightly fitting straps (Figure 2B). A pre-determined sequence of pressure increments was applied to the skin (2 inHg for 1 min, 3 inHg for 1 min, 4 inHg for 1 minute, 5 inHg for 5 minutes, 6 inHg for 5 minutes, 7 inHg for 10 minutes, 8 inHg for 10 minutes and 9 inHg for 15 minutes) which allowed for a gradual increase in pressure up to a maximum pressure of 10 inHg. Periodic visual observation of the blister chamber was carried to determine the time for first small bleb formation (Figure 2D). At that point, the pressure was reduced by 1 inHg and maintained until

the blister expanded to occupy the full aperture (Figure 2G). The pressure was then gradually decreased at a rate of approximately by 2 inHg per minute until equilibrated with atmospheric pressure.

Visual checks were performed regularly and if at any point the blister was found to be leaking, the pressure was reduced by 25-50% to allow the blister wall to re-seal prior to increasing the pressure to the previous value.

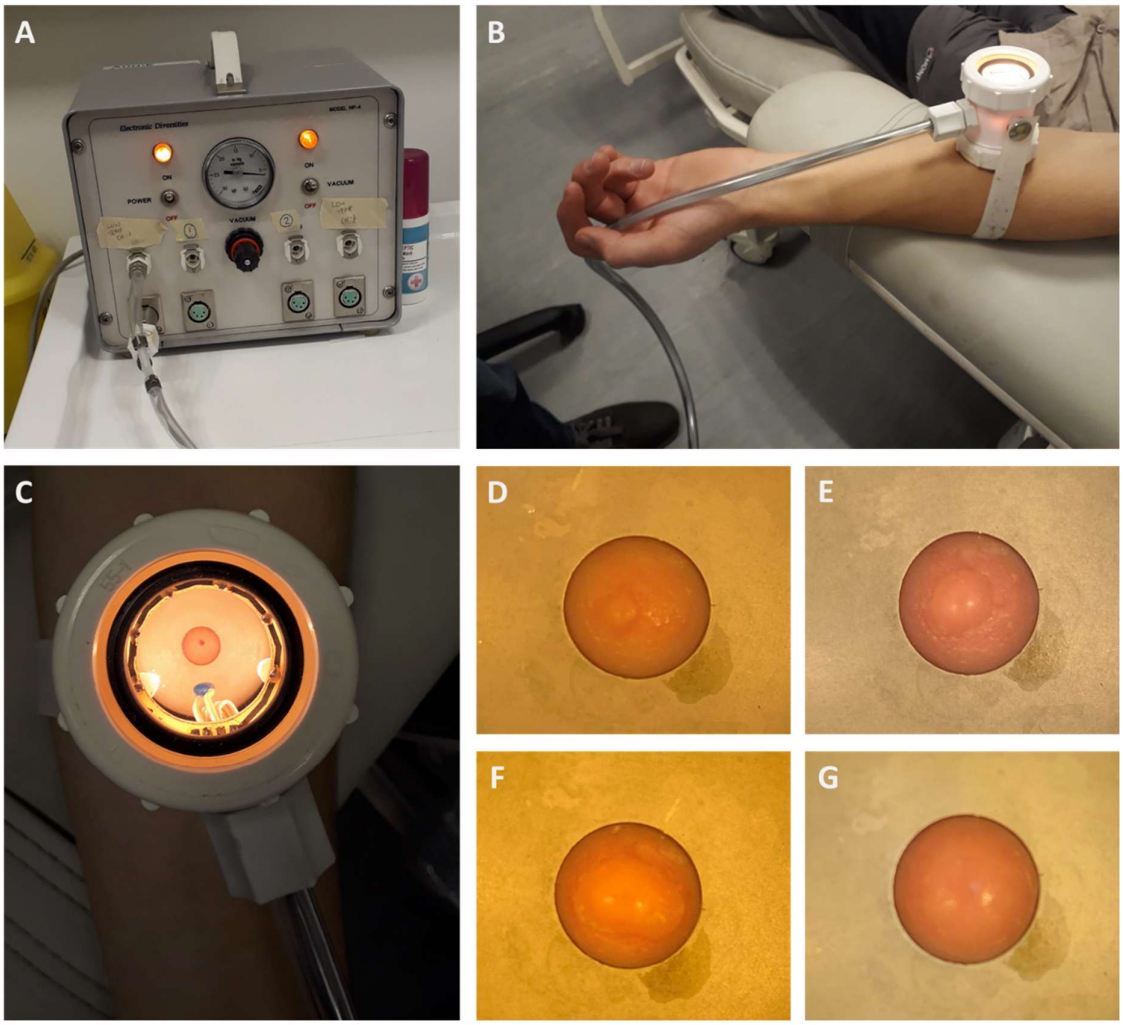


Figure 2: Formation of suction blisters. [A] Negative pressure device; [B] Blister chamber securely fitted to a participant's forearm; [C] Blister formation was followed via the transparent cover of the chamber; [D-G] Different phases of blister formation from initial bleb to complete blister formation.

2.2.4 Cantharidin blister formation

Chemical blister induction was adapted from the primary literature source (Day, Harbord et al. 2001) and was carried out using a dilution of Cantharone® (supplied as

a 0.7 % cantharidin solution) from Dormer laboratories (ONT, Canada). The compound was diluted in acetone (Sigma-Aldrich) to achieve a 0.2% concentration. One preparation was carried out for the whole study, and single-use aliquots were prepared for each daily application and left in a sealed glass vial to reduce the possibility of increasing compound strength due to solvent evaporation. On each pre-selected site on the ventral aspect of the forearm, a containment ring of 10 mm diameter was created by dipping the wide end of a p1000 pipette tip in Vaseline® and applying it to the skin using a twisting motion, to provide a barrier that prevents spreading of the solvent onto skin outside the ring. 5 µL of the diluted cantharidin solution was pipetted to the centre of the ring, allowing the contents to spread evenly inside the ring; solvent was then allowed to evaporate, which typically took less than 30 seconds (Figure 3).

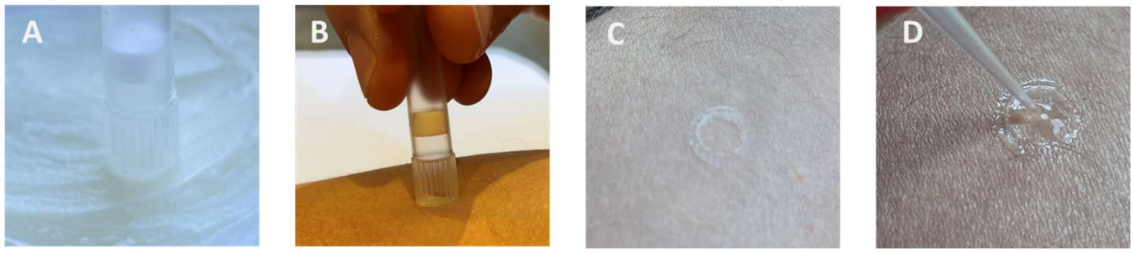


Figure 3: Cantharidin application for blistering procedure. A p1000 pipette tip is dipped in Vaseline®, the circular barrier is produced by rotating this tip onto the participant's skin. Cantharidin is applied to the centre of the ring and allowed to evaporate.

2.2.5 Blister dressing and harvesting

Upon completion of the blister induction procedure, each blister site was surrounded with a padded dressing (Coloplast, Comfeel® Plus Dressing), covered with a 15 mL centrifuge tube lid (Falcon ®, Corning), secured in place with non-allergenic surgical tape, such that the rim of the lid was resting on the padded surface (not digging into the skin), and sites were covered by a soft stretchy bandage (Figure 4). Blisters were allowed to develop (typically for 24 hours); during this time the volunteers were requested to avoid strenuous activity, trauma to the area or washing the arm.

24 ± 2 hours after blister formation, blisters were punctured using a sterile needle and the contents were collected using a p200 pipette whilst gently rolling a pipette tip over the blister roof to push the contents out. The fluid was collected into pre-weighed

protein lo-bind 1.5 mL tubes (Eppendorf) containing 5 µL of EDTA 0.5 M (Sigma-Aldrich). The blister sites were disinfected with an antiseptic spray and plasters were used to cover the wound. The use of Lo-bind tubes and EDTA minimised the risk of adherence to tube walls and clotting that may be observed in the absence of these precautions.

Participants were asked to document healing by submitting regular photos of the forearms to monitor any complications and verify return to baseline.

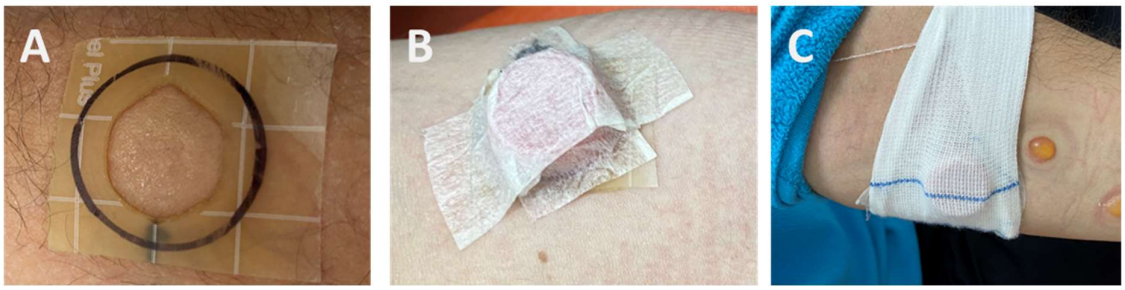


Figure 4: Blister dressing. Blister sites were surrounded by a padded adhesive [A], protected with a hard cap and secured in place with surgical tape [B] and covered with a stretchy bandage [C] to allow for blister development overnight.

2.2.6 Blister sample processing

Blister samples were weighed using an analytical balance with readability up to 0.1 mg, and volume was calculated assuming a density of 1.025 g/mL, the same as for normal plasma (Spector 1956). 5 µL of the sample was transferred to a haemocytometer for determining cell density and the remainder was centrifuged at 500 g for 6 minutes. The blister supernatant was removed carefully, aliquoted, and frozen at -80 °C. The cells were resuspended in a minimum of 100 µL of PBS, to a maximum concentration of 5 x 10^6 cells/mL. 100 µL of this suspension was transferred to a FACS tube in preparation for flow cytometry, excess volume was discarded.

2.3 Flow cytometry

2.3.1 Selection of cell surface markers and optimisation of panel

The antibody/marker panel was designed on Fluorofinder.com and selected according to the following principles: avoidance of overlapping fluorochromes for markers expressed on the same cell type; use of brighter fluorochromes on markers with low expression levels (e.g. PE for PD-1); dimmer fluorochromes for highly expressed markers (CD45 in APC-cy7); practical considerations such as availability of marker/fluorochrome combinations.

All samples were run on one instrument, a BD LSRFortessa X-20 (SORP), which was calibrated on a daily basis. Voltages were optimised and maintained for the full duration of each of the studies. Once set, the same compensation matrix derived using BD mouse compensation beads was employed throughout the study.

Antibody titrations were carried out for both blood and blister samples. Starting with the manufacturers' recommended volumes per test, serial dilutions of the antibodies were carried out in two separate experiments to define the optimum volumes to be used. This was defined as the lowest volume that provided the best separation between negative and positive cells in a given sample.

An antibody mix (combined panel) stability experiment was additionally carried out, where the mix was left at +4 °C for a week with no significant changes compared to a newly produced mix (ability to discriminate between populations, <10% variation in determination of subset percentages and activation marker fluorescence intensity; data not shown). This allowed all samples obtained within a week to be stained with the same mix, reducing variability between day one and two blood samples for each subject and minimising variability between different subjects carried out over the same period.

2.3.2 Antibody mix

The antibody panel described in Table 2 was added to blister cell suspensions (100 µL at a maximum of $5x10^6$ cells/mL) and whole blood samples (50 µL of whole blood) and samples were incubated for 30 minutes at room temperature, in the dark.

Blood samples were then fixed (BD Phosflow Lyse/fix) for 10 minutes at 37 °C, centrifuged (6 minutes at 500 g) and washed with 2 mL of PBS, before centrifuging again at the same speed. Cells were reconstituted in 300 µL of PBS ready for analysis.

At the end of blister cell incubation, 2 mL of PBS was added, cells were spun at 500 g for 6 minutes and then cells were resuspended in 300 µL ready for analysis.

Table 2: Antibodies selected for use in the leukocyte panel. Table details clone, manufacturer and volume used for each of the markers, as well as reason why it is selected for panel

Antibody	Marker	Clone	Manufact.	Volume (µL)		Antigen/Function
				blister	blood	
CD45	APC cy7	2D1	BD	1.25	1.25	Pan-Leukocyte marker
CD16	PE cy7	3G8	BD	0.25	0.5	FcγRIII; Neutrophil and NK cell marker
CD64	BV510	10.1	BD	2.5	2.5	FcγRI, Monocyte/ neutrophil activation
Siglec-1	AF647	7-239	BD	2.5	2.5	Pathogen identification, viral biomarker
CD14	AF488	MφP9	BD	0.63	1.25	Classical monocyte marker
HLA-DR	BUV395	G46-6	BD	0.63	1.25	Present in monocytes and DC. Infection biomarker
CD206	BV711	19.2,	BD	1.25	2.5	Mannose receptor
CD163	BV421	GHI/61	BD	1.25	2.5	Haemoglobin/ haptoglobin scavenger
CD123	PercP cy5.5	7G3	BD	2.5	2.5	IL-3 receptor, pDC marker
CD11c	BV786	3.9	Biolegend	2.5	2.5	Integrin alpha X, mDC marker
CD3	BUV805	UCHT-1	BD	0.63	0.63	T cell co-receptor; T cell marker
CD19	PECF594	HIB19	BD	0.2	075	Co-receptor for BCR; B cell marker
CD4	AF700	L200	BD	0.2	0.2	MHC class II receptor; Th cell marker
PD-1	PE	MIH4	BD	10	10	Programmed cell death 1, exhaustion marker

2.3.3 Flow cytometry data analysis

All flow cytometry data were analysed with FlowJo X (BD). Leukocyte sub-populations were identified by the gating procedure in Figure 5. Data for each population were exported as percentage of leukocytes and absolute number derived from haemocytometer counts. Geometric means for each activation marker were extracted for the selected cell subsets.

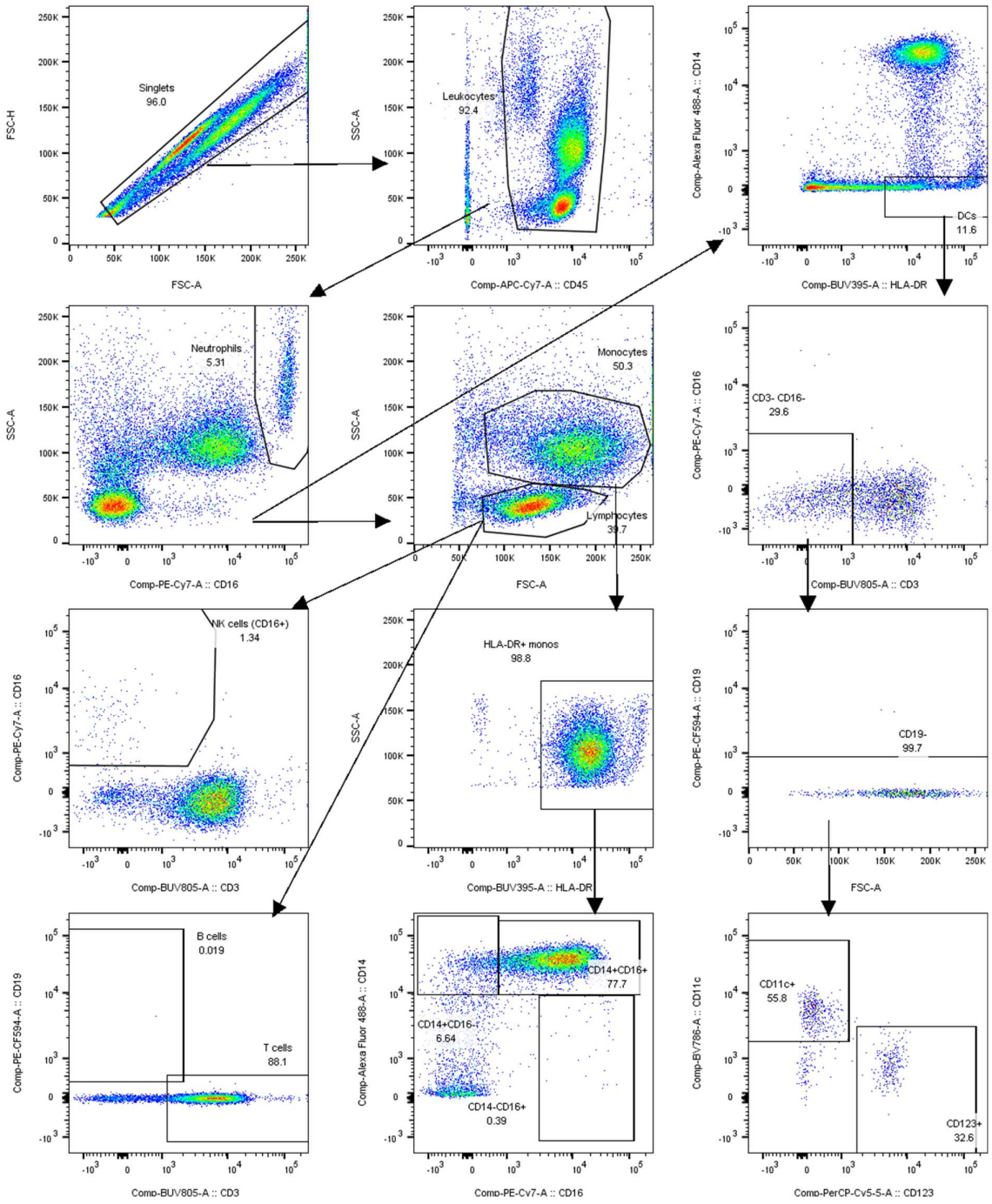


Figure 5: Gating procedure. Single cells were gated on FSC-H vs FSC-A and leukocytes were selected based on CD45. Neutrophils were selected as SSChi (granular) and CD16+ and a non-neutrophil gate was created. From that non-neutrophil gate, DCs were selected as HLA-DR+, CD14-, CD3-, CD16- and CD19-. On those cells, CD123 and CD11c expression defined populations of myeloid (CD11c+) and plasmacytoid (CD123+) DC. Also from the non-neutrophil population, monocytes (larger and slightly more granular) and lymphocytes (smaller and non-granular) were selected based on FSC and SSC profiles. The monocyte population was then further selected based on HLA-DR expression, and CD16 and CD14 expression characterised the 3 main populations of classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical monocytes (CD14-CD16+). In the lymphocyte gate, CD3, CD19 and CD16 were used to define T cells, B cells and NK cells respectively.

2.4 Cytokine and chemokine quantification

Plasma and blister supernatants were kept at -80 °C before analysis. Soluble mediators were analysed by electrochemiluminescence using of-the-shelf kits from MSD (Meso Scale Discovery). 10-plex cytokine (MSD, analytes: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF), 10-plex chemokine (MSD, analytes: Eotaxin, Eotaxin-3, IL-8, IL-8 (HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC) and TNF (MSD) kits were used according to manufacturer's instructions.

After thawing, samples were diluted in the corresponding diluent for each kit as per manufacturer's instructions (diluent 2 for cytokine and TNF assays and Diluent 43 for 10-plex chemokine kits) to optimised concentrations as defined in pilot runs (cytokine assay: blister 1/5, plasma 1/2; chemokine assay: blister 1/25, plasma 1/5; TNF assay: control samples 1/2, LPS stimulated 1/30; data not shown) and plated in preparation for the assay. 7-point standard curves were prepared in the same diluent as samples. Samples from each study were analysed in batches to minimise batch to batch variation within the same cohort.

After plates were washed 3x in assay buffer, 50 µL of samples, standards and blanks were added according to predefined plate maps and samples were incubated for 2h (+/- 20 min) at RT in an orbital shaker.

Plates were washed 3x with wash buffer and 25 µL of a 1x working stock mix of the respective secondary antibodies were added. Plates were incubated for a further 2h (+/- 20 min) at RT in an orbital shaker.

Plates were washed 3x in wash buffer, and 150 µL of 2x read buffer was added to each well before plates were analysed on the MSD reader. Data was exported and analysed on the MSD discovery workbench. Mediator concentrations were interpolated to each standard curve and any data falling outside the lower limits of quantification was imputed with half that lower limit.

2.5 Data analysis

Data were analysed using GraphPad Prism 10 (GraphPad Software, Inc.). Data routinely did not follow Gaussian distribution so non-parametric statistical tests were employed.

Unless specified in the specific chapter, the following statistical tests were used throughout the thesis:

For group comparisons, Mann-Whitney test was used when testing independent samples (unpaired comparisons) whereas Wilcoxon signed-rank test was applied to matched (paired) samples. For testing strength and direction of correlation, Spearman's test was used. Data in figures were correspondingly presented as medians with interquartile ranges.

Variability estimates were compared using coefficients of variation (CV), calculated as the standard deviation of a distribution divided by the mean.

The small studies described in this thesis were designed as exploratory and hypothesis-generating, aiming to identify signals, provide descriptive information and generate effect-size estimates rather than confirming a specific hypothesis. As such no explicit testable primary or secondary endpoints were elaborated, all data being treated as exploratory. Consequently, multiplicity control methods, such as Bonferroni correction, or any hierarchical testing plans, were not applied.

Due to resource limitations whereby the same individual (JO) acted as both operator (delivering/undertaking clinical challenge procedures) and laboratory analyst, data analysis was carried out in an unblinded manner, the risk of bias this introduces being acknowledged as a limitation within this thesis.

Chapter 3: Biological effect of cantharidin in healthy tissue

3.1 Introduction

Cantharidin is a toxic defensive compound produced by male blister beetles (order of Coleoptera and family Meloidae), which has a long history of use in traditional medicine. (Moed, Shwayder and Chang 2001). It is highly toxic when ingested, with a lethal dose for studied mammals in the region of 1 mg/kg (Matsuzawa, Graziano and Casida 2002). Human consumption of cantharidin or derivates, either accidentally from ingestion of blister beetles (Lecutier 1954, Al-Binali, Shabana et al. 2010), or most commonly during the dubious use of cantharidin preparations as an aphrodisiac (Karras, Farrell et al. 1996), can be fatal.

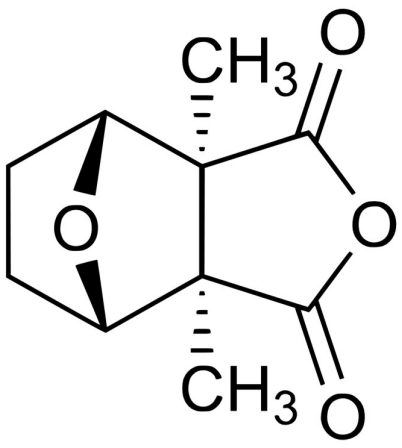


Figure 6: Cantharidin structure

Cantharidin, or exo,exo-2,3-dimethyl-7-oxabicyclo [2.2.1] heptane-2,3-dicarboxylic acid anhydride (Gadamer 2006), is a monoterpene found in the insects' blood and haemolymph. It is a potent inhibitor of protein phosphatases types 1 and 2A (Honkanen 1993). Phosphorylation of proteins on Serine, Threonine and Tyrosine amino acids residues is a well-known mechanism for regulation of many cellular processes as diverse as glycogen synthesis, cell division, gene expression, neurotransmission or muscle contraction (Sheppeck, Gauss and Chamberlin 1997). By interfering with the reversibility of these phosphorylation reactions, cantharidin will ultimately cause cell death. However, the potent biological properties have been proposed as potentially therapeutic and there have been efforts to develop more selective and bioactive cantharidin derivatives with lower toxicological profiles (Puerto Galvis, Vargas Mendez and Kouznetsov 2013).

Cantharidin has been long recognised as an acantholytic agent. Blister beetles use cantharidin as part of a defence mechanism, and when in contact with human skin, it causes dermatosis (Nicholls, Christmas and Greig 1990). Dried blister beetles, commonly known as mylabris, have been used in China for hundreds of years to treat warts, furuncles and piles (Moed, Shwayder and Chang 2001). Likewise, compounds containing cantharidin are used as a treatment for warts and molluscum contagiosum in western countries. Cantharone® is a topical drug containing 0.7% cantharidin, in a

film-forming vehicle containing acetone, collodion, castor oil and camphor, manufactured by Dormer Laboratories Inc. in Canada; it is used by physicians for removal of common warts, molluscum contagiosum and periungal warts.

3.1.1 Study of cantharidin and derivates as cancer treatment

As previously described, most of cantharidin's biological effects originate from the strong inhibition of protein phosphatases types 1 and 2A (Honkanen 1993) and the dysregulation of many pathways dependant on this reversible reaction. This switch mechanism plays a crucial role in signalling pathways controlling cell proliferation and carcinogenesis, and a small molecule which can inhibit these processes may help to study or even to treat conditions such as cancer (McCluskey and Sakoff 2001, Remmerie and Janssens 2019). Transition to clinic has been blocked by the heavy toxicity observed in this compound, especially nephrotoxicity, and led to the development of analogues such as norcantharidin and other similar compounds with better safety profiles (Tarleton, Gilbert et al. 2012).

Cantharidin and its derivatives have demonstrated in vitro efficacy in patient derived tumour cells or cell lines which model different cancers including leukaemia, oesophageal, cholangiocarcinoma, nasopharyngeal carcinoma, lung cancer, rectal cancer, breast cancer, and liver cancer (Zhou, Ren et al. 2020). Cantharidin appears to exert its anti-tumour effect via the inhibition of cell proliferation, either by regulating transcription through CDK3 (Zhang, Zhang et al. 2019), arresting G2/M cell cycle transition by downregulating CDK1 and inducing p21 (an inhibitor of CDK1) (Gong, Wu et al. 2015), or inhibition of PP5 (a modulator of cell proliferation) (Chen, Hung et al. 2017). Cantharidin may also have a direct impact in apoptosis of tumour cells by affecting the expression of members of the caspase family (Wu, Chiou et al. 2018) and impacting mitochondrial integrity (Lin, Chen et al. 2017).

3.1.2 Non-deleterious effects of cantharidin in normal tissue

Inhibition of phosphatase activity by cantharidin has been studied in healthy cells. Regulation of phosphorylation of myocardial proteins is thought to be a major mechanism in the maintenance of cardiac function and cantharidin has been shown

to increase the force of contraction in myocardial and vascular smooth muscle cells (Knapp, Bokník et al. 1998, Knapp, Boknik et al. 1998), although due to strong toxicity, this compound is not suitable for clinical use in complications such as septic shock.

There are few published reports on the effect of cantharidin on immune cells, but cantharidin and norcantharidin have been shown to inhibit cell proliferation and maturation of primary monocytes to dendritic cells (DC) (Hsieh, Liao et al. 2011). Also, the DCs that mature in the presence of cantharidin have different morphology, exhibit signs of necrosis, and lack surface receptors that are usually present on DCs (Hsieh, Huang et al. 2011). In fish, incubation of immune cells with cantharidin resulted in apoptosis and a dose dependant decrease of phagocytic activities (Campos-Sanchez, Guardiola and Esteban 2022). Its effect on the functional capacity of human leukocytes is currently unknown, which this thesis seeks to address..

3.1.3 Biological effect of cantharidin on skin tissue

Although cantharidin is not an approved medicine, it has been included in the FDA's "Bulk Drug Substances", a compound list that allows certain drug products to be compounded and used topically by providers in a clinical setting. Similarly, cantharidin is on Health Canada's restricted substance list as a natural health product. It is part of the armamentarium for treating conditions such as warts and molluscum contagiosum (Vakharia, Chopra et al. 2018) and has also been proposed in the treatment for cutaneous Leishmaniasis. Cantharidin was shown to inhibit the growth of both promastigotes and amastigotes and impact their viability in vitro. When used in vivo, applied to lesions in infected BALB/c mice, daily application of a 0.1 % cantharidin ointment cured the lesions without the formation of the characteristic blisters, and when used in solution (0.1 %) and applied via a paper disc, blisters were formed on top of the lesions and complete cure after blister healing was observed in 75% of the animals (Ghaffarifar 2010).

Most of these clinical effects come from the acantholytic properties of the cantharidin application. Due to its lipophilic nature, the compound is readily absorbed by the lipid layers of the epidermal cell membranes. The acantholytic effect of cantharidin is exerted indirectly by promoting the release of neutral serine proteases from epidermal cells, which result in progressive degeneration of the desmosomal plaque (Bertaux,

Prost et al. 1988). Acantholysis, loss of coherence between epidermal cells, is initiated in the suprabasal region, followed by extensive acantholysis of the stratum spinosum, resulting in the formation of an intraepidermal blister on top of the basal layer, the weakest junction on the epidermis (Piérard-Franchimont and Piérard 1988). This causes clinical inflammation, with *in vivo* leukocyte extravasation and cytokine release. The lesion resolves over time and typically does not leave a scar (Maglio, Nightingale and Nicolau 2003). Interestingly, cantharidin has been reported as not affecting keratinocyte viability in situ (Bertaux, Prost et al. 1988).

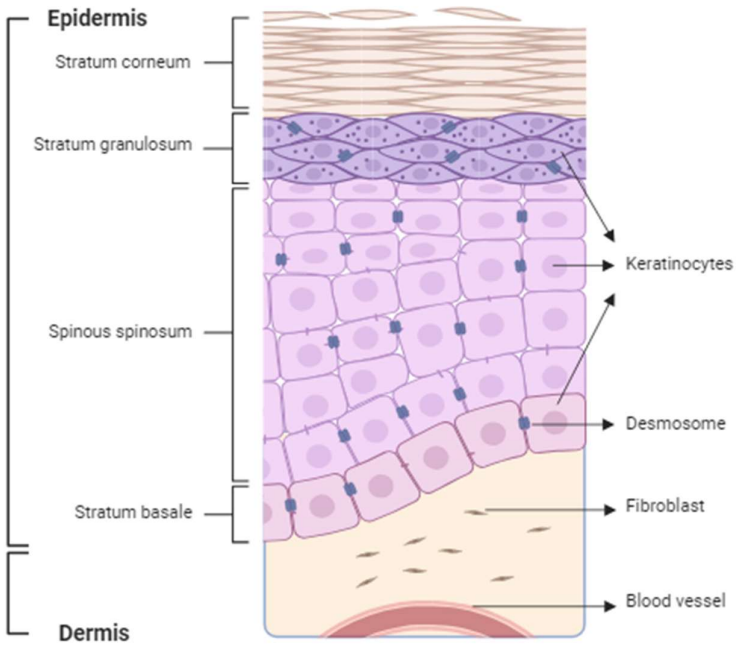


Figure 7: Diagram of skin anatomy affected by the application of cantharidin. Cantharidin acts on desmosomes, causing lack of cohesion between keratinocytes and forming an intra-epidermal blister on top of the basal layer.

3.1.4 Current understanding of the nature of cantharidin acantholysis

Mechanistically, after absorption of the chemical into the skin, cantharidin enters epidermal cells, promoting the release of neutral serine proteases, which result in the degradation of the desmosomes (Bertaux, Prost et al. 1988), which anchor epithelial cells together, leading to the loss of cohesion in the epidermis and the formation of a intra-epidermal blister on top of the basal layer (Piérard-Franchimont and Piérard 1988).

The study of the effect of cantharidin in skin relies on a small number of publications, mostly from more than 30 years ago, done on excised fresh skin. Stoughton and Bagatell, were the first to describe the formation of blisters using cantharidin in fresh human skin (Stoughton and Bagatell 1959). Their work showed cantharidin creating blisters when used in fresh skin maintained at 37 °C and in a humid Petri dish, but not in colder conditions both refrigerated (+4 °C) and at room temperature (20-25 °C), and also on skin pre-treated with freezing, fixation, alcohol and physical damage. Stoughton and Bagatell also showed that many chemical entities that were injected to the skin prior to treatment with cantharidin also prevented blistering: Compounds such as corticosteroids (hydrocortisone, prednisolone, Medrol), some metal salts (Hg, Ag, Cu) and sulfhydryl binding agents (including diisopropylfluorophosphate) stopped blistering. As a corollary to their work, the authors were the first to suggest that cantharidin is a blistering agent which exerts its effect through a secondary enzymatic factor that is generated in the skin itself, but did not provide a clarification of the mechanism.

The characterisation of the mechanism continued with work on fresh mouse skin, where authors continued to find the source of this secondary acantholytic agent, also using possible inhibitor compounds, and demonstrated the effect of this enzymatic factor, extracted from primary cantharidin application, into a second skin sample, in causing acantholysis (Weakley and Einbinder 1962).

A later study, which included a detailed investigation on structural changes after cantharidin application was able to identify the mechanisms of the acantholysis and the nature of the enzymatic factor. Bertaux et al. (Bertaux, Prost et al. 1988) used fresh human skin from plastic surgery for their studies. Using both bright field and electron microscopy, the authors were able to characterise the stages of cantharidin acantholysis. Between 1h and 3h of tissue treatment, the initial signs start to become apparent in the desmosomes with a loss of intercellular bridges. Using electron microscopy, the dissolution of the dense plaque was described, leading to detachment of tonofilaments. Signs of desmosomal degradation started to be evident at this early stage, but with keratinocytes still anchored to each other. After 3h, intercellular vacuoles started to appear, followed by big clefts and loss of adherence between skin cells becomes apparent. Keratinocytes were present as isolated cells within the blister cavity. All changes were observed in the basal, squamous and granular layers, with

the stratum corneum, the topmost layer of the skin, composed of keratin filled cells covered by lipid matrix, being unaffected by the treatment. Importantly, the authors compared cell viability between cantharidin treated and untreated epithelial cells, extracted from these tissue samples using trypsin, and showed no difference between viability of both, although total viability was only in the order of 50% for all samples tested.

The other important finding from this study relates to the type of enzymatic effect that triggers the acantholysis. The neutral serine protease inhibitors diisopropylfluorophosphate (DFP) and N-a-p-tosyl-L-lysine chloromethyl ketone, completely inhibited acantholysis. DFP had already been shown to be an inhibitor in the first Stoughton paper (Stoughton and Bagatell 1959). L-i-tosylamidc-2-phenylethylchlornmethyl ketone (TPCK), an inhibitor of chymotrypsin, but not trypsin, did not inhibit blister formation. These data allowed the authors to suggest the current understanding which is that cantharidin promotes the release of neutral serine proteases, which act on the dense desmosomal plaque, leading to the detachment of tonofilaments from desmosomes and subsequent loss of cohesion between epithelial cells, formation of extracellular clefts and final blister formation.

3.1.5 Chapter aims:

- Investigate the *in-vitro* effect of cantharidin on the function and viability of human primary cells involved in the blistering process
- Establish the role played by cantharidin in the inflammatory response observed in the blistering process
- Study the *in-vivo* effect of topically applied cantharidin as part of the optimisation of the current established blister model

3.2 Additional methods:

3.2.1 Whole blood and PBMC preparation

Whole blood samples were obtained under ethics application 1309/006: Understanding human immune responses to infection and injury and processed to

PBMCs as described in the general methods section. After counting, cells were resuspended in RPMI 1640 medium at a density of 2 x 10^6 cells/mL.

3.2.2 Preparation of skin cell cultures

Pooled primary human skin cells were purchased from commercial sources, and processed according to manufacturer's instructions to optimum density for use in further work:

Human dermal fibroblasts (106-05A – Sigma-Aldrich) were cultured in Fibroblast Growth Medium (116-500 – Sigma-Aldrich) were seeded at 20,000 cells/well in a 24 well-plate and left to grow for overnight incubation (37 °C, 5% CO_2) to be used in cantharidin *in-vitro* experiments.

Primary Normal Human Epidermal Keratinocytes (C-12006 – Sigma Aldrich) are isolated from epidermis of adult skin from pooled donors. Cells were thawed and cultured in Keratinocyte Growth Medium 2 (C-20111 – Sigma-Aldrich) seeded at 20,000 cells/well in a 24 well-plate and left to grow for overnight incubation (37 °C, 5% CO_2) before used in further experiments.

3.2.3 Cantharidin in-vitro incubations

Cantharidin (C7632 – Sigma-Aldrich) used for all *in-vitro* work was prepared from a dry powder by diluting in sterile DMSO to a final concentration of 100 mM and aliquots were stored at -20 °C for daily use. For each experimental day, serial dilutions of the stocks were prepared in RPMI 1640 medium (100X working stock range: 100 mM – 10 µM).

As a reference, the drug Cantharone 0.7 % corresponds to 36 mM and the Cantharone dilution used in participants to create blisters in the work carried out in this thesis (0.2 %) corresponds approximately to 10 mM.

300 µL of freshly prepared WB and PBMC samples in RPMI 1640 or plated samples of keratinocyte and fibroblast cell suspensions as above were used in experiments. To these, 3 µL of the cantharidin working stock were added, contents mixed by pipetting and samples left for 24 h ± 2 h at 37 °C 5% CO_2.

After overnight incubation, 200 µL of cell culture supernatant was collected for further analysis and cells were harvested into FACS Tubes.

For adherent cells (keratinocytes and fibroblasts), after removing all volume to a FACS Tube, 200 µL of PBS were added to wells and samples mixed before collected to same FACS tubes. 200 µL of pre-warmed (37 °C) trypsin/EDTA (0.025% trypsin and 0.01% EDTA in PBS) was added and samples incubated for 5 minutes for dislodging cells from plasticware. 200 µL of RPMI medium were then added to samples and harvested to corresponding FACS tubes.

3.2.4 Conditioned medium experiments

Fibroblast and keratinocyte culture supernatant samples obtained from cantharidin *in-vitro* 24h incubations were used for stimulation of PBMCs.

Cell supernatants and PBMC cell suspensions were mixed 1:1 and incubated for a further 24 h ± 2 h in 37 °C, 5 % CO_2 incubator. After this incubation, cells were collected for further analysis as described below.

3.2.5 Assessing cell viability

Cell viability and apoptosis were determined using commercially available staining reagents. Annexin V (Biolegend) and apotracker green (Biolegend) are dyes that bind to phosphatidylserine in the cell surface, an event found during apoptosis whereas propidium iodide (PI - Biolegend) and DRAQ7 (Biolegend) are membrane impermeable dyes that bind to DNA, indicating loss of membrane integrity.

For experiments using PI and Annexin V, cell suspensions were washed in PBS followed by Annexin V binding buffer before being resuspended in 100 µL of annexin V binding buffer. Annexin V and PI were added for 10 minutes, before adding an antibody mix with surface binding antibodies for a further 20 minutes. Samples were incubated at room temperature in the dark before 1x fix/lyse (BD) added and samples incubated for 10 minutes at 37 °C. Cells were washed in 2 mL PBS before being resuspended in 300 µL and analysed in the LSR Fortessa (BD) flow cytometer.

For experiments using Apotracker and DRAQ7, cell suspensions were washed in PBS before incubating with these reagents in parallel with other surface markers. Samples were stained for 30 minutes at room temperature, in dark, before a final wash and analysis in LSR Fortessa (BD) flow cytometer.

3.2.6 Reactive Oxygen Species (ROS) generation assay

PBMCs and WB samples (after incubation with cantharidin or fibroblast/keratinocyte + cantharidin conditioned media) were studied for determination of neutrophil and monocyte respiratory burst following phorbol myristate acetate (PMA) stimulation using the Neutrophil/Monocyte Respiratory Burst Assay Kit (601130, Cayman Chemicals) following the manufacturer's instructions.

After isolation, 100 µL of cell suspension were resuspended in 100 µL of assay buffer. 10 µL of dihydrorhodamine (DHR) 123 were added along with surface antibodies against CD14, CD16 and CD45 and samples were incubated for 15 minutes at room temperature in the dark after which 25 µL of PMA 1 µM (or PBS for control samples) were added and samples incubated at 37 °C, in the dark for 45 minutes.

2 mL of RBC lysis buffer was added, and the samples incubated for 10 - 20 minutes to achieve complete lysis. Samples were centrifuged and resuspended in 300 µL of assay buffer before analysis in LSR Fortessa flow cytometer.

3.2.7 Chemotaxis

PBMC samples were used in an assay based on the Boyden chamber principle, carried out using the QCM Chemotaxis Cell Migration Assay (Merck, ECM506) in a 24-well format with a 5 µM semi-permeable membrane.

150 µL of fibroblast culture supernatants after 24h cantharidin incubation were diluted with 350 µL RPMI 1640 in the lower reservoir of the plate. RPMI 1640 medium and a chemokine mix were used as negative and positive controls, respectively.

250 µL of freshly prepared PBMC cell suspension (500,000 cells) were added to the top reservoir before incubating the plate for 3 hours at 37 °C, 5% CO_2.

After 3 hours, the contents in the lower reservoir were analysed. Images were collected for each well and cells were harvested and counted using a Neubauer haemocytometer.

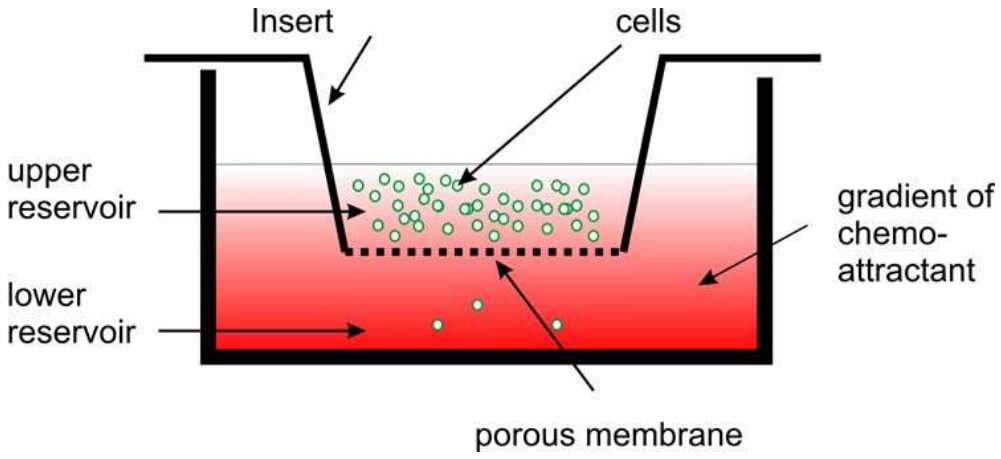


Figure 8: Schematic for assay to determine cell migration towards chemoattractants. PBMCs were placed over media collected from 24h cell incubations with cantharidin and cellular migration was analysed after 3 hours of incubation by observing the contents of the lower reservoir

3.2.8 Cantharidin blister formation: Paper disc

Cantharidin application was carried out as described by Day et al. (Day, Harbord et al. 2001). Paper discs with 1 cm in diameter (Whatman® Grade 1 filter paper), were placed on the volar aspect of the forearm and impregnated with 25 µL of 0.1% cantharidin solution in acetone (v/v) freshly prepared from Cantharone® (Dormer Labs, ONT, Canada). Paper discs were then covered with an occlusive blister dressing. Blisters were harvested using the same technique as those formed by suction at the same timepoint.

3.2.9 Clinical work for optimisation of the cantharidin blister technique

Study schematics are described in Figure 9. The use of the cantharidin challenge using Day et al. methodology (Day, Harbord et al. 2001), was carried out in an evaluation cohort of 26 subjects (mean age 32.4, range 19-52). Four cantharidin

blisters were raised via application of paper disc (PD), with replicate blisters (two per timepoint) harvested at 24 and 48 hours post challenge.

To ensure safety and efficacy of a new method of direct application, a cantharidin dose escalation study was conducted to identify a dose that elicited a robust response (oedema of at least 200 µL). 11 subjects were recruited (mean age 40.0, range 30-48) and each participant was challenged with up to 3 increasing doses of cantharidin. Cantharidin dilutions studied included 0.025%, 0.05%, 0.1%, 0.2%, 0.35% and 0.5%.

The new direct application method was then used in two separate studies that serve here as validation cohorts. 28 volunteers (mean age 39.0, range 25-55) had replicate blisters (two per timepoint) harvested at both 24- and 48-hour collections. The method was further used in a second cohort of 12 volunteers (mean age 32.2, range 20-44) who had replicate blisters (two per timepoint) collected at the same timepoints.

Data analysed from these studies is limited to volume and cellularity of the blister samples, as described below.

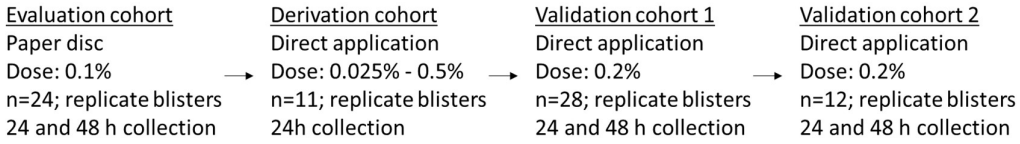


Figure 9: Study schematics. Work carried out to optimise new application method for cantharidin. An evaluation cohort used the established paper disc method, a derivation cohort tested the direct application method and established a new dose, and two validation cohorts used the optimised method.

3.3 Results

3.3.1 High concentrations of cantharidin impact viability and function of human leukocytes

Ex-vivo PBMC and whole blood samples were incubated with cantharidin to observe the effect of this compound on normal human leukocytes (n=8 different donor samples). Concentrations of cantharidin evaluated ranged from 100 nM to 1 mM in 24-hour cultures, for both PBMC and whole blood.

Leukocyte viability was the first endpoint analysed; it appeared to be retained up to the highest concentrations of cantharidin studied in both culture systems. Loss of

viability was observed via changes in cellular structure as seen in a reduction of FSC and SSC in flow cytometry for all leukocytes (Figure 10C) and confirmed by the use of Annexin V and PI that detect apoptosis and necrosis.

Different viability baselines were noted for PBMC and whole blood experiments and between different leukocyte subsets, but concentrations of 10 μM and above had a significant impact on leukocyte viability regardless of preparation or cell type (Figure 10 A,B). Monocyte viability was lower compared to lymphocytes, and viability of PBMC samples started from a lower baseline when compared to whole blood experiments, the extreme being monocyte viability in PBMC samples which started from a low baseline in the control samples without cantharidin (40% viability) but was still maintained at those levels up to the highest concentrations of cantharidin used.

Viable and functional monocytes and neutrophils produce reactive oxygen species (ROS) in response to stimulation with Phorbol myristate acetate (PMA). Prior incubation of WB and PBMC samples with cantharidin at the highest concentrations (10 μM and above) for 24h ablated ROS production in both neutrophils and monocytes after PMA stimuli, whereas lower concentrations (<10 μM) did not have a significant effect on ROS production (Figure 10F).

Potential effects of ex-vivo cantharidin on leukocyte polarisation or activation was assessed by analysing cell surface markers in monocytes (CD163, CD64, CD206 and siglec-1) after WB and PBMCs incubation with cantharidin. In PBMC experiments, cantharidin did not cause changes to baseline levels of monocyte activation markers, whereas for whole blood, baseline levels of these markers were maintained at the low concentrations of cantharidin, but concentrations higher than 5μM significantly reduced the signal observed for these activation markers in a dose-dependent manner. The highest dose of cantharidin led to cell death (Figure 10 D,E).

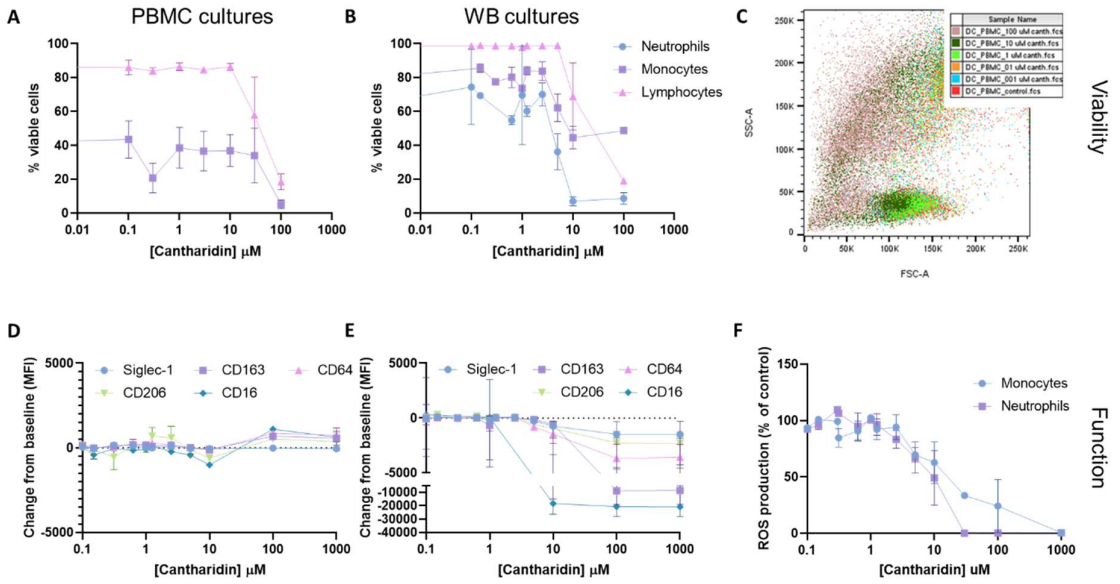


Figure 10: Effect of cantharidin on normal leukocytes in whole blood and PBMC samples cultured for 24h with a range of cantharidin concentrations. Effect of cantharidin exposure on viability of cells in A: PBMC cultures (3 separate experiments, 2 individual wells pooled per condition) and B: WB cultures (4 separate experiments, 2 individual wells pooled per condition) as determined by PI incorporation by flow cytometry; C: representative dot plots showing impact of high doses of cantharidin in cell size and granularity of PBMC samples; Effect of cantharidin exposure on monocyte activation/polarisation in D: PBMC culture (n=4 different participant samples, 2 pooled wells per condition) and E: WB culture (n=6 different participant samples, 2 pooled wells per condition); F: ROS production after PMA stimuli in whole blood samples incubated for 24h in the presence of cantharidin

3.3.2 Effect of ex-vivo cantharidin on stromal viability

Fibroblast and keratinocyte primary cell cultures were stimulated with a range of cantharidin concentrations (0.1 µM – 1 mM) and incubated for 24 h at 37 °C, 5% CO_2 prior to analysis by microscopy. Cantharidin had a clear effect on the shape and size of the cells. After 24h incubation, cantharidin concentrations of 3 µM appeared to impact cell structure. In fibroblast and keratinocyte cultures, cells started to lose their normal elongated profile, becoming more circular. At concentrations at 10 µM and above all cells became smaller and rounded (Figure 11 A, B).

Viability was also determined by flow cytometry, with similar viability trends emerging from the flow data. Viability was maintained until 3 µM for fibroblast and 10 µM keratinocyte cultures and decreased at higher concentrations (Figure 11C). These data do not completely align with the effect of cantharidin concentrations observed by microscopy, where viability appears to impact all cells at high (≥30 µM) concentrations of cantharidin. When running samples in the flow cytometer, it was observed that the

total number of cellular events obtained was reduced on the samples with the highest cantharidin doses.

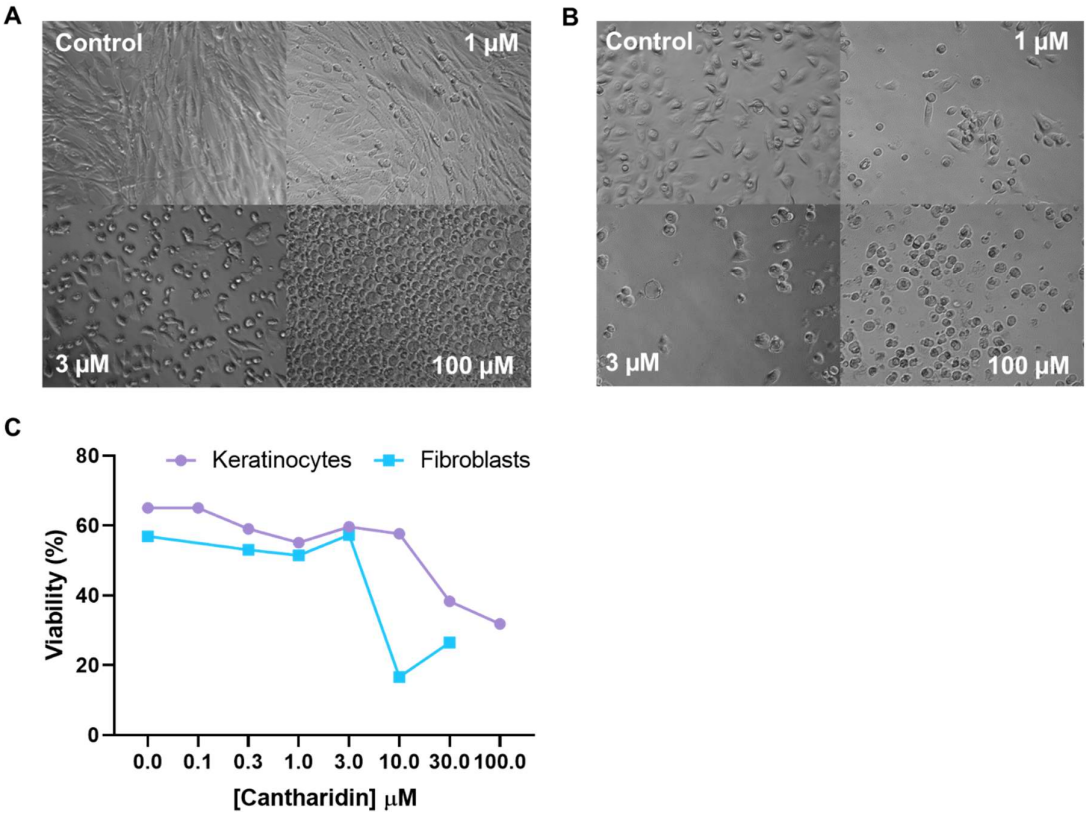


Figure 11: Effect of cantharidin 24h incubation on stromal cells. Selected representative images of A: fibroblasts and B: keratinocyte cultures after 24h at different cantharidin concentrations. C: Viability of fibroblasts and keratinocytes as determined by viability dye incorporation (PI). Results were obtained from single experiments for each cell type with each point being pooled from 3 separate wells for analysis.

3.3.3 Ex-vivo cantharidin elicits different patterns of cytokine production in blood derived leukocytes compared to stromal cells

The effect of 24h cantharidin exposure in cultures of normal leukocytes and primary cells present in skin was also assessed for cytokine production. Supernatants were taken from normal whole blood and PBMC, as well as fibroblast and keratinocyte cultures, with a range of cantharidin concentrations, and analysed by MSD, an electrochemiluminescence immunoassay, that allowed the simultaneous detection of 10 pro-inflammatory cytokines. Whole blood and PBMC data were obtained from 2 different participants and for fibroblast and keratinocyte cultures, data were derived

from 2 independent experiments, each starting with an aliquot of the same type of primary cells.

These data suggest that for stromal cell lines, levels of some pro-inflammatory cytokines were moderately raised in response to cantharidin ex-vivo stimulation. Although absolute changes observed are not of same magnitude as observed in inflammatory triggers, relative changes from baseline were identified, notably a 6-fold increase in IFN-γ, 5-fold increase for TNF and 2.5-fold increase for IL-6 that were observed in fibroblasts at a concentration of 1 µM (below the threshold where cell death is observed). Keratinocytes appeared to be the major producers of IL-1β in response to cantharidin. In general, concentrations of 0.1-10 µM cantharidin led to moderately increased levels of cytokine production, especially in fibroblast cultures, whereas for whole blood and PBMC cultures, cantharidin appeared to only have a toxic effect.

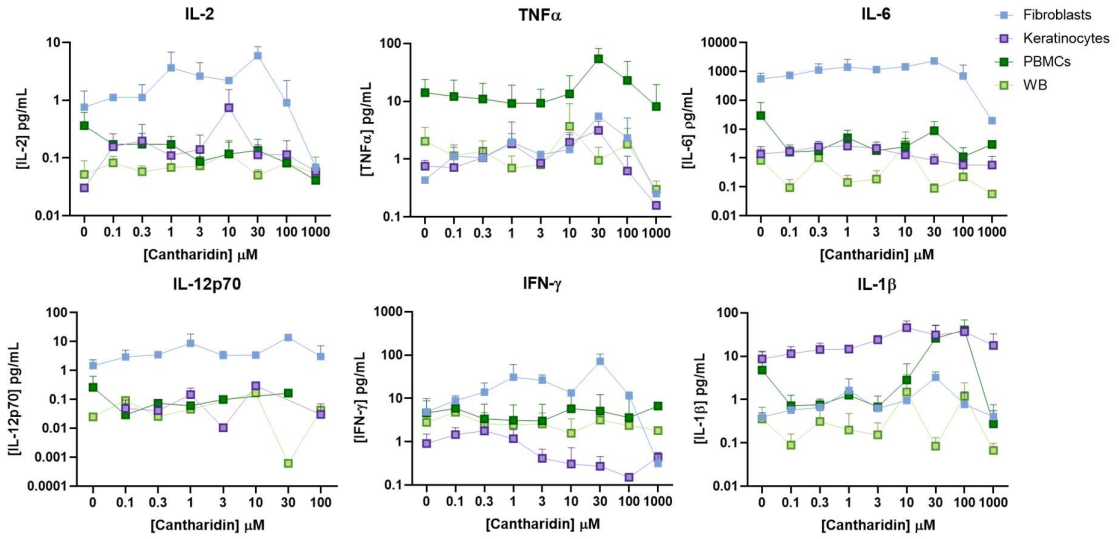


Figure 12: Cytokine release in leukocyte and stromal cell cultures with cantharidin. Absolute levels of cytokine present in culture medium after 24h incubation with cantharidin. Blood samples from 4 participants were used for PBMC and WB experiments. Supernatants from 2 separate cultures of fibroblasts and keratinocytes were used (3 wells per concentration were prepared for each keratinocyte experiment, 2 wells for each fibroblast experiment).

3.3.4 Conditioned medium from stromal cells cultured in the presence of cantharidin activates monocytes

Fibroblasts and keratinocytes were incubated in presence of different concentrations of cantharidin (suspensions at 100,000 cells/mL and with 0-30 µM cantharidin) for 24h. Culture supernatants were collected and added to freshly prepared PBMC samples at a ratio of 1:1 (100 µL of PBMCs at $2x10^6$/mL + 100 µL of culture supernatant). PBMC samples were incubated overnight in the fibroblast/keratinocyte conditioned media or in control RPMI 1640 medium with cantharidin at the same concentrations as in the conditioned media samples (control). After 24h in the conditioned medium, the PBMCs were analysed by flow cytometry, and mean fluorescence intensity of selected monocyte markers was analysed (Figure 13). CD16 and CD163 expression was increased from baseline expression in fibroblast medium compared to keratinocyte and control medium. Fibroblast conditioned medium in the presence of low concentrations of cantharidin (0.3 - 3 µM) increased the background activation for CD163 and CD16, whereas the same concentrations of cantharidin in standard medium did not affect activation, suggesting a priming effect. The levels of these markers were reduced to equivalent or below baseline levels for the two highest concentrations studied (10 – 30 µM).

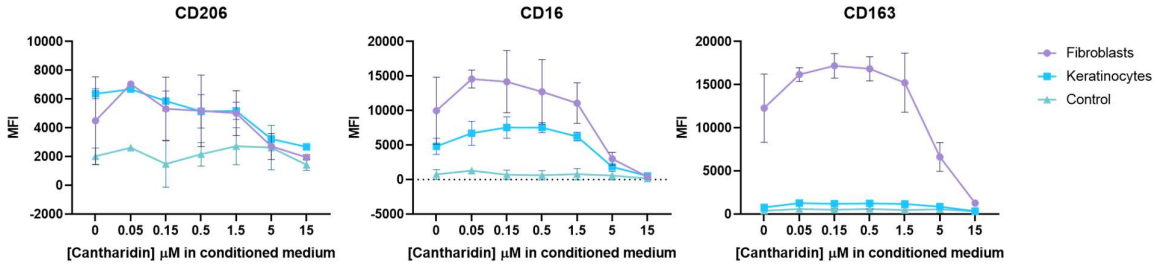


Figure 13: Effect of fibroblast and keratinocyte conditioned medium on PBMCs. Culture supernatants from fibroblasts and keratinocytes exposed to a range of cantharidin concentrations were used for overnight incubation of PBMCs. Media with same concentrations of cantharidin was used as control. Monocyte activation markers were determined by flow cytometry. Supernatants from 2 separate cultures of fibroblasts (2 replicate wells per experiment) and one culture of keratinocytes (3 replicate wells per concentration) were used.

3.3.5 Conditioned medium from cantharidin-treated stromal cells enhances leukocyte chemotaxis

Fibroblast conditioned medium prepared in the presence of cantharidin was used in a chemotaxis assay based on Boyden chamber principle, where cells are left to migrate through a porous membrane to a chemical attractant.

Unstimulated PBMCs, freshly prepared from healthy donors, were suspended in RPMI 1640 medium and positioned in the top chamber of the assay. Media from fibroblasts cultured in cantharidin was placed in bottom chamber. Control medium and a chemokine mix containing IL-8, MCP-1, IP-10, MDC (Macrophage derived chemokine) and TARC (Thymus and activation-regulated chemokine) were used as negative and positive controls respectively. After 3 hours, number of cells migrating to bottom chamber were analysed by microscopy, both by taking representative images of contents in bottom chamber and by counting cells using a haemocytometer.

PBMCs migrated in higher number into the positive control medium containing a mix of chemokines compared with negative control medium containing RPMI 1640 only. PBMCs migrated into supernatants collected from fibroblasts pre-incubated with 1 μM cantharidin at a similar number to the positive control, and at a higher level compared with fibroblasts pre-incubated in the absence of cantharidin (Figure 14) indicating the presence of soluble chemokines induced by cantharidin.

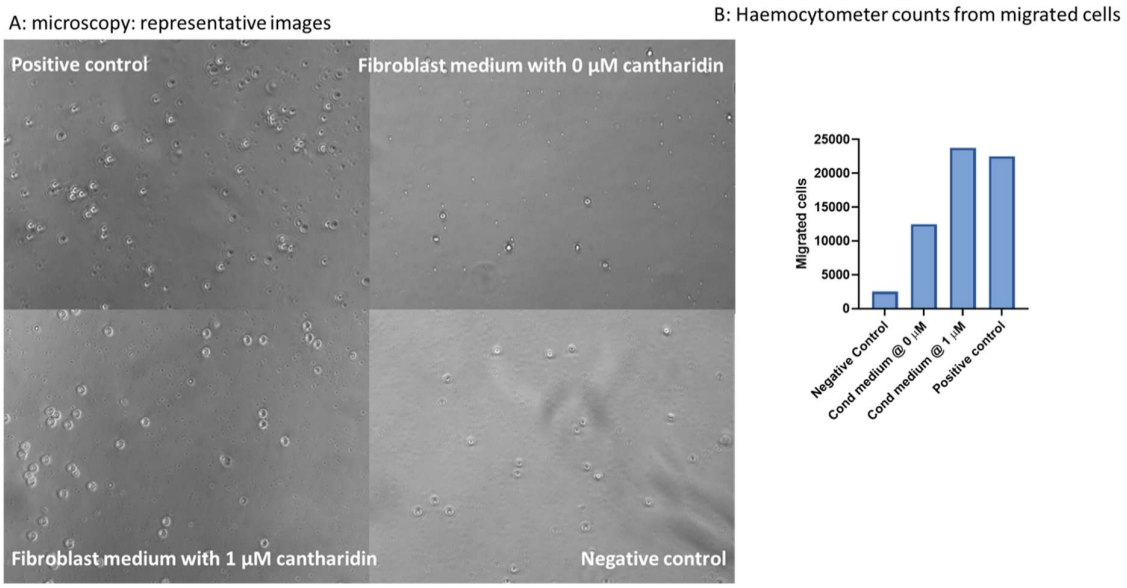


Figure 14: PBMCs preferentially migrate to fibroblast culture supernatant in the presence of cantharidin. A: representative images from each of the conditions tested; B: number of cells migrating through porous membrane determined by manual haemocytometer counts.

3.3.6 Exploration of the in-vivo response to cantharidin

This study was initiated in GSK's Clinical Unit Cambridge to evaluate established methods of human inflammation for use in clinical research. Before using the method in a clinical study, the first approach was to characterise the cantharidin blister response in house, using the published methodology. I was part of the team overseeing the study and the main laboratory operator analysing the blister samples.

3.3.6.1 Evaluation of the human cantharidin blister outputs from established method

In an evaluation cohort, forearm blisters were generated using the established method of impregnating paper discs with cantharidin (Day, Harbord et al. 2001). Each of 26 subjects had 4 blisters induced simultaneously, and pairs of blisters were harvested at 24 and 48 hours. Median volume for the 24-hour blisters was 259.6 µL (range 99.9 - 1098.0) and for 48 hours was 417.9 µL (106.6 - 3059.0 µL). Median cellularity observed was 2.61 $x10^6$ cells/mL (range 0.13 - 9.14 $x10^6$ cells/mL) for 24 hours and 2.248 $x10^6$ cells/mL (range 0.83 - 14.71 $x10^6$ cells/mL) for 48-hour blisters.

The variability observed for 24h blisters was 36.5% for volume and 35.6% for cellularity (in cells/mL) and the method was associated with blister failures and observations of uneven exposure such as paper disc lifting on application and multi-lobed blister formation (see 3.3.6.3 for more specific data). The variability was higher than the internal target of 30% based on a power calculation to enable the method to be used for an upcoming study and a decision was made to test a new application methodology.

3.3.6.2 Direct application of cantharidin with dose escalation

To overcome possible inconsistencies in application, a new method was devised, where cantharidin was directly applied to the skin. This application ensures direct delivery of the complete volume of chemical being administered to the skin. This could result in a stronger response compared with the indirect filter paper disc method using the same concentration of cantharidin. Therefore, a dose discovery cohort was utilised to ensure safety and efficacy of the new methodology, and to identify an optimum concentration of cantharidin, defined as the minimum dose that elicited consistent blister formation with a volume over 200 µL, permitting reliable analysis of the cellular and soluble contents.

Data for dose escalation are summarised in Figure 15. Six subjects were exposed to a starting dose of 0.025% (v/v), which resulted in median blister volumes of 105.8 µL (range 35.1 – 210 µL), below the volume threshold. Same subjects were escalated to 0.05% (median 159.1 µL, range 74.1 – 239.2 µL) and donors who did not reach threshold were further escalated to 0.1% (median 144.5 µL, range 75.3 – 360.9 µL), with both doses still not eliciting a consistent blister response over the pre-set volume of 200 µL. A second cohort of 5 subjects were dosed with a starting 0.2% concentration. The subjects who did not achieve the established target volume with the first challenge, were dose escalated to 0.35% and 0.5%. Concentrations higher than 0.2% did not result in substantially larger blisters. The 0.2 % dose elicited blisters with 165.2 µL median volume (95% CI: 135.2–278.8 µL) and was selected and taken forward to the following parts of the study.

3.3.6.3 Validation of the direct application methodology

The direct application methodology was tested in two separate validation cohorts (n=28 and n=12), with subjects receiving a cantharidin dose of 0.2%.

Cases of burst or leaking blisters were reduced from 10/104 (9.6%) blisters in the original paper disc method, to 4/108 (3.7%) and 0/48 (0%) for the two validation cohorts analysed in this dataset. Better uniformity in shape was observed, with the direct application method universally forming completely circular, non-loculated blisters.

Blisters harvested at 24 hours after paper disc application were larger (Figure 4B – median, 95% CI - 259.6 µL, 185.4–341.3 µL; n=50) when compared with first validation cohort (DA1, 169.9 µL, 123.2–239.9 µL; n=54; p=0.0164) but not significantly different to the second (DA2, 192.1 µL, 100.8–304.0 µL; n=24; p=0.1245). Blisters raised after paper disc application were significantly more cellular than DA2, but not DA1 (Figure 4C – PD: 2.61 $x10^6$ cells/mL, 1.83–3.73 $x10^6$ cells/mL; DA1: 2.01 $x10^6$ cells/mL, 1.80–3.06 $x10^6$ cells/mL p=0.4698; DA2 1.04 $x10^6$ cells/mL, 0.75–1.80 $x10^6$ cells/mL, p=0.0486).

The three studies described above, designed with replicate blisters at all time points, allowed calculation of intrinsic variability of the method by comparing outputs from blisters simultaneously formed in the same subject using %CV. The variability estimates were reduced for the direct application when comparing to paper disc both for volume (PD: 36.5%, DA1: 30.7%, DA2: 14.4%) as well as cells/mL (PD: 35.6%, DA1: 31.7%, DA2: 26.2%). Thus, whilst direct application led to slightly smaller blisters, with lower cell infiltrates, the fewer failures, more consistent morphology and lower variability produced was preferred for translational applications.

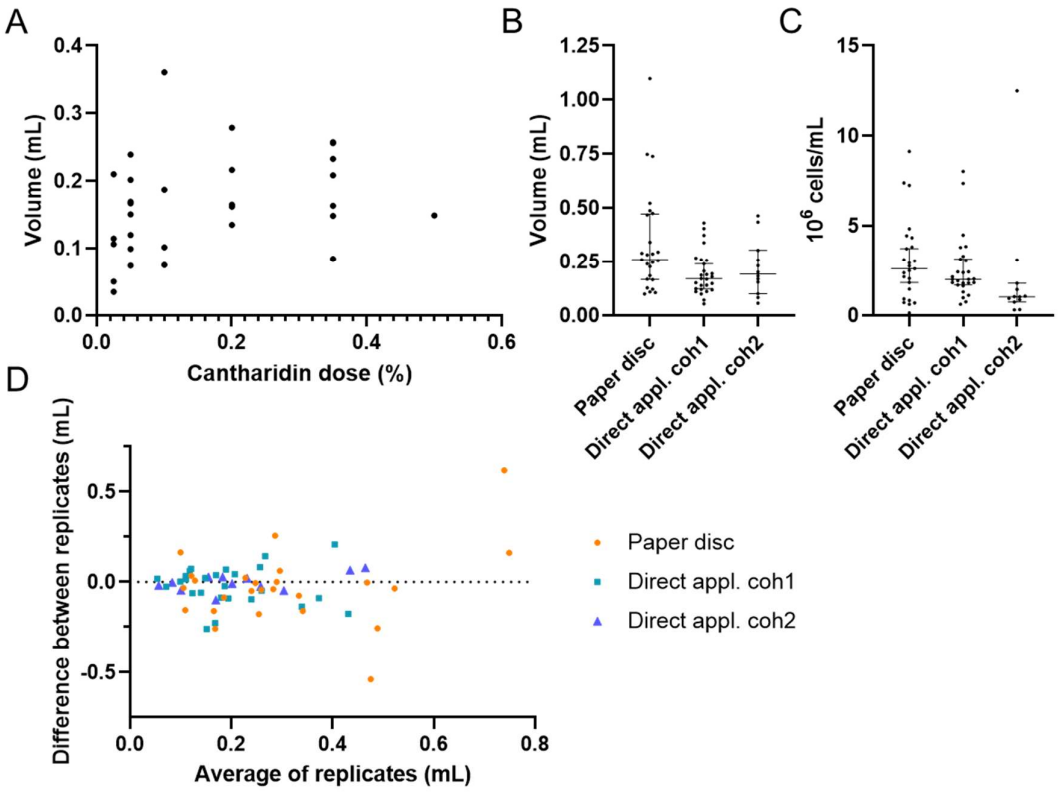


Figure 15: Direct application of cantharidin results in more consistent blisters. [A] average blister volume obtained from duplicate blisters for different doses of cantharidin (n=11 subjects, each with 3 visits); [B] Volume and [C] cellularity of blisters created via paper disc (n=50) or direct application methods (coh1: n=54, coh2: n=24) (median with 95% CI); [D] average (x) and difference (y) between duplicate blisters obtained from different methodologies (PD: Paper Disc method, DA: Direct application method)

3.4 Discussion

3.4.1 Direct effect of cantharidin in blister inflammatory response

Topical application of cantharidin leads to blister formation. As described in the introduction, the acantholysis is a result of enzymatic processes that are initiated by cantharidin, but that are not caused directly by the compound. Considering the wealth of literature that is coming from the potential use of cantharidin as an anti-cancer agent (Li, Wu et al. 2023), the diverse molecular mechanisms which it affects (Naz, Wu et al. 2020) and the demonstrated cell toxicity, it is likely that the compound may influence the inflammatory response that accompanies the formation of the blister which make it a suitable model for studying human inflammatory responses, but the direct effect of cantharidin on the stromal, resident and infiltrating immune cells has never been directly explored.

My experiments attempted to evaluate these potential biological effects of cantharidin in human tissues, first by studying its effect on whole blood leukocytes and derived PBMCs, where cantharidin appears to cause cell death once a concentration threshold is reached. Incubation for 24h with the compound affects cell viability at concentrations above 10 μM. The effect of such concentrations are also observed by other authors when assessing viability of cancer cell lines after treatment with cantharidin (Kern and Schroeder 2014).

Effect of cantharidin incubation at over 10 μM is observed in all leukocyte subsets, although baseline viability of monocytes appeared to be compromised even without cantharidin after 24h. Circulating monocytes activate and differentiate into macrophages when they leave circulation. In-vitro, this is commonly accomplished by the addition of M-CSF or GM-CSF and/or use of serum-derived preparations (Brugger, Kreutz and Andreesen 1991). In the absence of other co-factors, monocytes in culture progressively lose viability in the absence of serum, cytokines or other stimuli (Mangan, Welch and Wahl 1991, Bohnenkamp, Burchell et al. 2004), which explains the low viability of these cells in PBMC culture (unsupplemented) compared with whole blood.

Above the 10 μM threshold where viability is impacted, but not at lower concentrations of cantharidin, function is also compromised and there are changes to monocyte markers of activation and polarisation. A similar viability threshold was also observed for primary pooled fibroblasts and keratinocytes. In these experiments these cells were used as a proxy for skin cells present in the blister cavity and basal layer. With the highest concentrations of cantharidin, the number of events obtained after flow cytometry analysis was low, which may be due either to the loss of cells on harvesting, or most likely, by the elimination of FSC_{lo}/SSC_{lo} events below analysis threshold. The fact that treatment reduces cell size, as observed by microscopy, may will also change light scattering properties, making dead cells fall outside the gating strategy.

Interestingly, when analysing cell culture supernatants from primary skin cells exposed to concentrations of cantharidin under the viability threshold of 10 μM, we observed an increased release of pro-inflammatory cytokines. In these cultures, cantharidin exerts a direct effect, triggering the release of pro-inflammatory cytokines such as IL-2, IL-6, and IFN-γ from fibroblast cultures, and IL-1β from keratinocytes, in a dose-

dependent manner, with effects observed from concentrations as low as 0.1 μM. These same concentrations, when tested in either whole blood or PBMC cultures, did not produce the same effect. Cytokine levels were maintained, or even reduced by the treatment, showing a variable effect of cantharidin in different cell types. Similar numbers of fibroblasts and keratinocytes were seeded per well (20,000) for each experiment, making these two systems comparable for evaluating absolute cytokine concentration. In comparison, 0.25 – 1.0 million leukocytes in 50 μL of whole blood and 1 million PBMCs were used for the other conditions. Although with a much larger number of initial cells, blood derived cells produced lower basal levels of cytokines, except for TNF.

Cytokines can be involved in a multiplicity of pathways and can exert their effect in diverse cell types (Cohen and Cohen 1996). The release of cytokines into the blister milieu from cantharidin stimulated stromal cells may impact the proinflammatory status of the blister milieu, possibly via impacting resident immune cells place or recruiting immune cells from the microvasculature.

Enhanced IL-1β production by keratinocytes was observed in response to cantharidin. IL-1β directly is not able to recruit immune cells (Yoshimura, Matsushima et al. 1987), but it will drive neutrophil chemotaxis to inflamed tissue via the upregulation of chemokine production and release by other cells, such as endothelial cells (Sica, Matsushima et al. 1990, Sadik, Kim and Luster 2011). IL-1β can also recruit neutrophils via production of chemotactic lipids (Pyrillou, Burzynski and Clarke 2020).

The cytokine-enriched medium obtained after fibroblast cultures with cantharidin was observed to be a chemoattractant for PBMC in chemotaxis assays based on the Boyden chamber principle (Chen 2005) demonstrating another pro-inflammatory effect of fibroblast cantharidin treatment. Whilst only a selection of pro-inflammatory cytokines was enumerated in these experiments, other soluble mediators such as chemokines or lipid mediators could have provided interesting data to more clearly identify cantharidin as a factor in the production of chemoattractant molecules.

Fibroblasts may not be the most prevalent cell in the epidermis and in the blister cavity, but their ability to respond to cantharidin should be considered as a similar response may also occur as in other resident skin cell types. The ability of resident cells to

produce other cytokines such as IL-2 and IFN-γ may also help to explain the enhanced recruitment of immune cells, especially neutrophils in a first wave, to the site of action.

After leukocyte recruitment to blisters, cytokine secretion will create/maintain a pro-inflammatory milieu capable of recruiting and activating other immune cells, establishing a cascade. The proposed mechanism of action of cantharidin on both blister creation and in the initiation of the immune response is summarised in Figure 16.

We can thus postulate that in the cantharidin blister model, the cantharidin acts as more than an acantholytic reagent, indirectly affecting cell cohesion and probably initiating an inflammatory response via DAMPs, damage associated molecular patterns released from damaged cells. It directly acts on local cells, initiating inflammatory cascades that lead to the migration of leukocytes to site of inflammation and promotes pro-inflammatory mediator release from skin stromal cells (and possibly resident immune cells such as macrophages) that contribute to the observed inflammatory profile of these blisters.

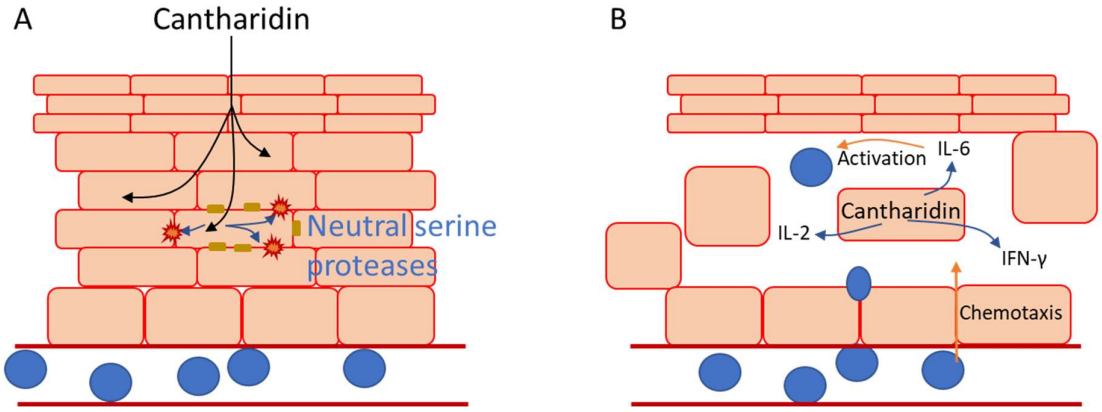


Figure 16: Proximal and distal mechanism of action proposed for cantharidin in the blister response. A: When cantharidin is applied topically, the compound is able to diffuse through the stratum corneum and act on cells in the stratum spinosum. Here, it promotes the release of neutral serine proteases, which specifically target the desmosomes, breaking up the anchor points between epithelial cells. B: Once the blister cavity is formed, local stromal or immune cells exposed to cantharidin will secrete cytokines to the extracellular matrix, where these will act on the basal layer, increasing the chemotactic gradient for leukocytes from the microvasculature, and once these cells enter in the blister cavity, the pro-inflammatory environment present will prime and activate these cells

3.4.2 Current limitations and future direction of research

The in-vitro data presented so far cannot fully replicate the in-vivo observations as there is no data available on the extent of cantharidin penetration to the tissue in human skin.

Cantharone is a 0.7 % cantharidin preparation, which corresponds to a 36 mM solution. In vivo, 25 µL of 5.1 mM preparations are commonly used in paper disc (Day, Harbord et al. 2001), whereas most data on this thesis uses 5 µL of a 10.2 mM preparation. Considering that most of the compound would permeate and could be present in the blister fluid, a maximum possible concentration of approximately 250 µM would be present in the fluid of an average blister. But it is likely that some or most of the compound would be retained in the top layers of the epidermis.

We have tried to explore this by looking at confocal Raman microscopy to detect the chemical in excised tissue (Jung, Namjoshi et al. 2022) and, although able to detect a compound signature, we were not able to follow the permeation of the compound after application to the epidermis. One other possibility would be to quantify cantharidin in blister supernatants or in skin punch biopsies. This was not pursued as not part of the original ethics application and not followed upon after sample collection but would be useful to understand the levels of the chemical in the blister milieu and in epithelial cells to understand whether the chemical is present at a sub-lethal dose and correlate with ex-vivo biological observations.

3.4.3 Direct cantharidin application to skin produces more consistent results compared with established methodology

The current established methodology for creating cantharidin blisters did not produce consistent and reliable data when used in GSK laboratories, and a novel approach was explored that theoretically allowed for a more homogeneous exposure at a clearly defined area. This direct application methodology used a lower volume (5 µL vs. range of 10–25 µL) with a higher concentration of cantharidin (0.2% vs. 0.1%) compared to the majority of previously published reports (Day, Harbord et al. 2001, Morris, Stables et al. 2009, Dinh, Corraza et al. 2011, Jenner and Gilroy 2012). This new methodology was tested in a dose escalation cohort, where increases in concentration above 0.2%

did not lead to any further increase in observed blister volume. Other effects of cantharidin on the inflammation were not evaluated in this cohort, but would be interesting to determine whether alternative parameters such as neutrophil infiltration, soluble mediators or cell surface activation markers were impacted by higher doses of the vesicant.

The optimised methodology produced more consistent blisters. A lower number of compromised blisters was observed (9.6% vs 2.6% of leaked or failed blisters) and a lower variability in the main outputs of volume and cellularity was obtained. Some of this difference may be explained by institutional and operator experience of blister creation and handling. This novel methodology was used for the work carried out throughout this thesis.

3.5 Chapter conclusions

- Cantharidin is a toxic compound which does not directly exert any direct activation effect in *in vitro* blood derived leukocyte cultures.
- Cantharidin does however have a direct stimulatory effect on stromal cells in culture, inducing pro-inflammatory cytokine release.
- Supernatants from stromal cell cultures with cantharidin induce activation of blood derived monocytes and promote leukocyte chemotaxis.
- Direct application of cantharidin to skin provides a more reliable model to the established paper disc protocol.

Chapter 4: The blister response over time – Immunological differences between suction and cantharidin challenges

4.1 Introduction

4.1.1 The inflammatory response

Inflammation is a defence mechanism of the body in which the immune system is activated in response to either harmful stimuli originating from the external environment (e.g. pathogens) or from tissue damage. The inflammatory response is an evolutionarily preserved host process that consists of a complex network of molecular and cellular events, both local and systemic, that aims to protect and repair tissues prior to returning them to homeostasis.

The course of an inflammatory response will depend on the initial trigger and the tissues involved, but is clinically characterised by the cardinal signs of *calor, rubor, tumor* and *dolor* (heat, redness, swelling and pain) and it is biologically underpinned by signalling pathways that lead to release of inflammatory markers and cause increased blood flow (vasodilation) to allow trafficking of leukocyte sub-populations to the affected tissue to contain or remove the triggering agent. This inflammatory response is normally followed by an active process of resolution, characterised by the clearance of the triggering agents, establishment of memory response and restoration of tissue function (Fullerton and Gilroy 2016).

Inflammation is classically associated with many acute disease states, such as infection or sepsis, but when unchecked, or unbalanced, is now recognised to play a key role in chronic pathologies including rheumatoid arthritis, cardiovascular disease and Alzheimer's (Duan, Rao and Sigdel 2019). These are associated with substantial mortality and morbidity; however, neither the mechanisms that trigger and regulate the inflammatory response, nor the biological basis for inter-individual variability, are fully understood (Andreasen, Krabbe et al. 2008). Increased understanding of the molecular and cellular pathways controlling inflammation in humans would permit a deeper understanding of, and ability to address pathophysiological responses.

4.1.2 The initiation of the immune response

The innate immune system protects organisms through nonspecific defence and surveillance by cells such as monocytes, neutrophils, dendritic cells, natural killer (NK) cells, mast cells, eosinophils and basophils. These cells recognise and bind common

molecules found on the surface of pathogens or apoptotic and damaged cells via pattern recognition receptors (PRRs) (Li and Wu 2021).

The first of these, the Drosophila Toll protein, was characterised in 1988 (Hashimoto, Hudson and Anderson 1988) and found to play a role in the resistance of these organisms to fungal infection (Lemaitre, Nicolas et al. 1996). CTL4, a human homologue to the Toll protein was described in 1997 (Medzhitov, Preston-Hurlburt and Janeway 1997) and since then, many other PRRs have been described.

PRRs can be classified into different families due to structural similarities. Toll-like receptors (TLRs) exist as transmembrane glycoproteins, composed of an extracellular region that recognises the pathogens, a transmembrane region, and an intracellular region containing a domain similar to IL-1R that initiates the signalling cascades. In the cytosol, other types of receptors can be found, such as NOD-like receptors that detect bacterial and viral particles and RIG-I-like receptors that are specialised in detected viral double stranded RNA before initiating the inflammatory signalling cascade (Chen, Deng et al. 2018).

The molecular entities that these receptors recognise are the pathogen-associated molecular patterns (PAMPs), highly conserved carbohydrates, lipoproteins or nucleic acids that are present in pathogenic organisms, that are essential for pathogen survival and are not found in the host, such as LPS, lipoteichoic acid (LTA), and bacterial DNA, or damage-associated molecular patterns (DAMPs) that are created in the host after tissue damage or cellular necrosis.

4.1.3 Inflammatory signalling pathways

Different PRRs are triggered by their respective ligands in separate cellular locations by different mechanisms, but as the signalling cascade always involve adaptor proteins, protein kinases and transcription factors, and these have similar structures and functions, there is substantial cross-talking between these cascades which converge into common signalling pathways (Oeckinghaus, Hayden and Ghosh 2011).

Each PRR has a pattern recognition domain and other domains that will couple with receptor adaptor molecules. Binding of PAMPs or DAMPs to the binding regions of PRR will cause the triggering of different signalling pathways, the specificity of which

can be determined, in part, by the type of adaptor molecules bound. Recruitment of one or several adaptor molecules to a given PRR is followed by activation of downstream signal transduction pathways via phosphorylation, ubiquitination, or protein-protein interactions, that will ultimately lead to activation and nuclear translocation of transcription factors, such as activator protein-1 (AP-1), NF-κB or interferon regulatory factor 3 (IRF3) that will regulate expression of genes involved in inflammation and host defence.

Although with slightly different outcomes in different types of cells, the activation of the canonical NF-κB pathway through these PRRs induces pro-inflammatory cytokines, chemokines, and other inflammatory mediators (Mogensen 2009). p38 MAPK also regulates cytokine expression and plays a similar role in the activation of the host immune response (de Souza, Vale et al. 2014) whereas IRF-3 is an initiator of the type I interferon response which plays a crucial role in antiviral immune response (McNab, Mayer-Barber et al. 2015).

4.1.4 Cytokine secretion and cell migration in inflammation

The main effectors of the inflammatory signalling cascades are cytokines, small proteins (<40 kDa) secreted by cells that are the main means of cellular communication and regulation of the immune response.

Historically, different nomenclatures have been given to cytokines for their different roles in cell migration (chemokines), communication between leukocytes (interleukines) or growth factors. Cytokines are part of a highly complex signalling network, where different cell types can secrete the same cytokine and single cytokine can act on different cell types (pleiotropy). Cytokine effects are influenced by simultaneous presence of other cytokines, hormones, antagonists or isolated receptors and are usually redundant in their activity with similar functions being provoked by different cytokines. They are usually part of complex cascades where one cytokine enhances the production of more cytokines in the target cell, potentially initiating a major response from a very small initial signal.

Inflammatory triggers will initiate a pro-inflammatory response, with the production of classical pro-inflammatory cytokines such as IL-1β, IL-6, and TNF, predominantly by

activated macrophages, but also by non-immune cells such as fibroblasts and endothelial cells. As part of the complex signalling, even in the early stages of the inflammatory response, anti-inflammatory cytokines such as IL-10 are also produced (Geginat, Larghi et al. 2016).

Along with pro-inflammatory cytokines, chemokines are also produced in response to these inflammatory triggers. These are crucial in the recruitment of immune cells to the site of inflammation and are involved in both extravasation and chemotaxis. First, they induce changes in receptors in both the endothelium and the circulating leukocytes resulting in a transient leukocyte adhesion to the endothelial wall, followed by adhesion, crawling and migration (Laudanna, Kim et al. 2002), a series of events mediated mainly by selectins and integrins (Ley, Laudanna et al. 2007). They then target these extravasated leukocytes to the site of injury through a chemokine gradient (Foxman, Campbell and Butcher 1997).

In a typical acute inflammatory response, there is a hierarchy of cellular accumulation required for an effective response. Specialised leukocyte subsets are needed for an initial phase of pathogen elimination, followed by clearance of apoptotic cells, restoration of tissue and activation of the adaptive immune system. Neutrophils are the first responders, migrating to the site of injury where they cluster to eliminate the trigger by phagocytosis, neutralisation of foreign materials and formation of Neutrophil Extracellular Traps (NETs) (Leick, Azcutia et al. 2014). Activated neutrophils can promote the arrival of a second wave of monocytes, by releasing soluble factors at the site of inflammation (Swirski and Robbins 2013); these cells maintain inflammation levels by producing pro-inflammatory cytokines and mature into inflammatory macrophages that remove PAMPs, cell debris and apoptotic neutrophils.

Efficient leukocyte accumulation in injured or infected sites is crucial for pathogen elimination and tissue healing, but uncontrolled accumulation is a feature of chronic diseases, such as atherosclerosis (Viola and Soehnlein 2015).

4.1.5 Resolution of the immune response

After peak inflammation, the dampening of inflammation, clearance of inflammatory cells and return to tissue homeostasis is an active process that is tightly controlled.

After the removal of the inflammatory triggers, the synthesis of pro-inflammatory cytokines is suspended and any soluble mediators present are catabolised, stopping the positive feedback loop that drives the initial inflammatory response and halts leukocyte recruitment and oedema formation. When this stage is not under control, sepsis may occur (Stoecklin and Anderson 2006).

Immune cells are then cleared from tissue either by re-entry in circulation, lymphatic drainage (enabling the initiation of an adaptive immune response), or most commonly, cells will undergo apoptosis (or other types of cell death such as NETosis or necrosis) and will then be targeted for efferocytosis by macrophages (Fullerton and Gilroy 2016).

Similarly to the inflammatory cascades, resolution has its own large panel of mediators, which do not just overlap with already mentioned anti-inflammatory cytokines and have different effects. Anti-inflammatory mediators will inhibit cell migration or block signalling, whereas pro-resolving mediators will promote processes such as apoptosis or efferocytosis (Sugimoto, Sousa et al. 2016).

When resolution is achieved and homeostasis is reestablished, a further phase is initiating where the adaptive immune system is activated, memory and regulatory lymphocytes are expanded in circulation and resident macrophages return to the healed site (Newson, Stables et al. 2014).

4.1.6 Chapter aims:

Investigate, for the first time, the early cellular and humoral changes that follow topical cantharidin application and identify the processes underling this immune challenge.

Compare the kinetics of the blister response in response to suction and cantharidin, establishing whether the immune responses differ qualitatively and quantitatively.

4.2 Additional methods:

4.2.1 Clinical study designed for studying early blister events

Four subjects had suction and cantharidin blisters raised on their forearms. Suction blisters were collected at 4, 8 and 24h post start of blistering procedure. For cantharidin

challenges, at both 4 and 8h, there were no fully formed blisters at the exposure sites. To obtain these early blisters over cantharidin sites, suction was applied over the exposure sites following the same procedure and pressure gradient as for those formed over untreated skin (described in 2.1.3) until the formation of a bleb, with pressure kept at that value until the formation of a suitable blister for harvesting (application of pressure was stopped when blister occupied approximately 50% of the chamber). For 16h and 24h, blisters were harvested as previously described.

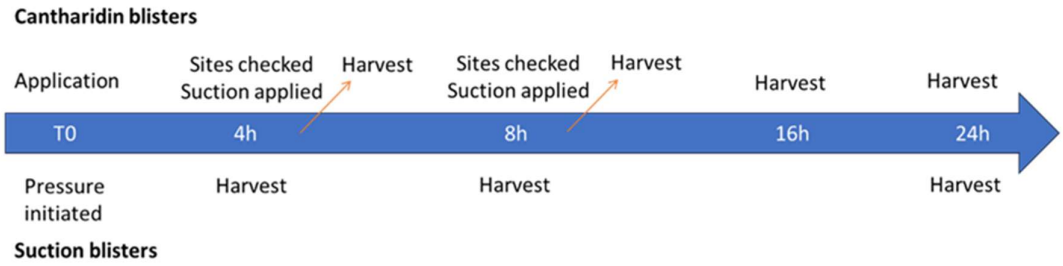


Figure 17: Study design. Timeline of clinical events that followed application of cantharidin or initiation of suction procedure, obtaining blister samples over the first 24h

4.2.2 Studies used for data comparing 24h and 48h blisters

As part of the same ethics application as 4.2.1, a second part of this study was carried out to determine pharmacological modulation of blister contents. In this second part, a cohort of 6 volunteers had one placebo blister taken at both 24h and 48h.

Also used was data from 'validation cohort 2' defined in 3.2.9. As described before, 12 volunteers were enrolled (mean age 32.2, range 20-44) who had replicate placebo blisters collected at both 24h and 48h.

4.3 Results:

4.3.1 Suction application to cantharidin challenge sites allows exploration of early stages of cantharidin blisters

A study was designed to deliberately investigate the time courses of both suction and cantharidin methods. For the suction model, blisters were harvested 4h and 8h following the initiation of each blister (e.g. after negative pressure was started). For the cantharidin method, as no primary blister had formed at 4h and 8h post cantharidin

dosing (Figure 18 A), negative pressure was applied to raise a blister on the site of application. Blisters were then harvested immediately upon formation of a blister suitable for collection.

When observing the cantharidin challenge sites after 4h of application the skin appeared to show macroscopic signs of damage. Small areas of skin appeared loosened when compared with surrounding tissue. At 8 hours, 2 subjects already had partial blistering, whereas the other 2 subjects had similar changes to those observed at 4 hours. Interestingly, there appeared to be a consistent annular formation on the 8-hour blisters, indicating that acantholysis commences from the edge of the application site.

At the 4h time point, the time and pressure required to form an initial bleb was lower on sites pre-treated with cantharidin when compared with naïve skin (Figure 18 B). In two of the subjects, the initial bleb occurred at the centre of application, but for the other two, the bleb formed in the edge of the aperture. The time required for blister growth was also shorter, although for these blisters, the study was designed for stopping the procedure once a blister is sufficiently large as to collect approximately 100 µL (so that the contents could be captured as close to the timepoint as possible), instead of the normal process for raising a suction blister, where suction would be maintained until the blister completely fills the aperture.

At the 8h time point, blebs were either already present or formed rapidly following the application of minimal pressure. However, blister formation time remained substantial (>30 minutes), likely because portions of the epidermis were still strongly anchored to the lower layers.

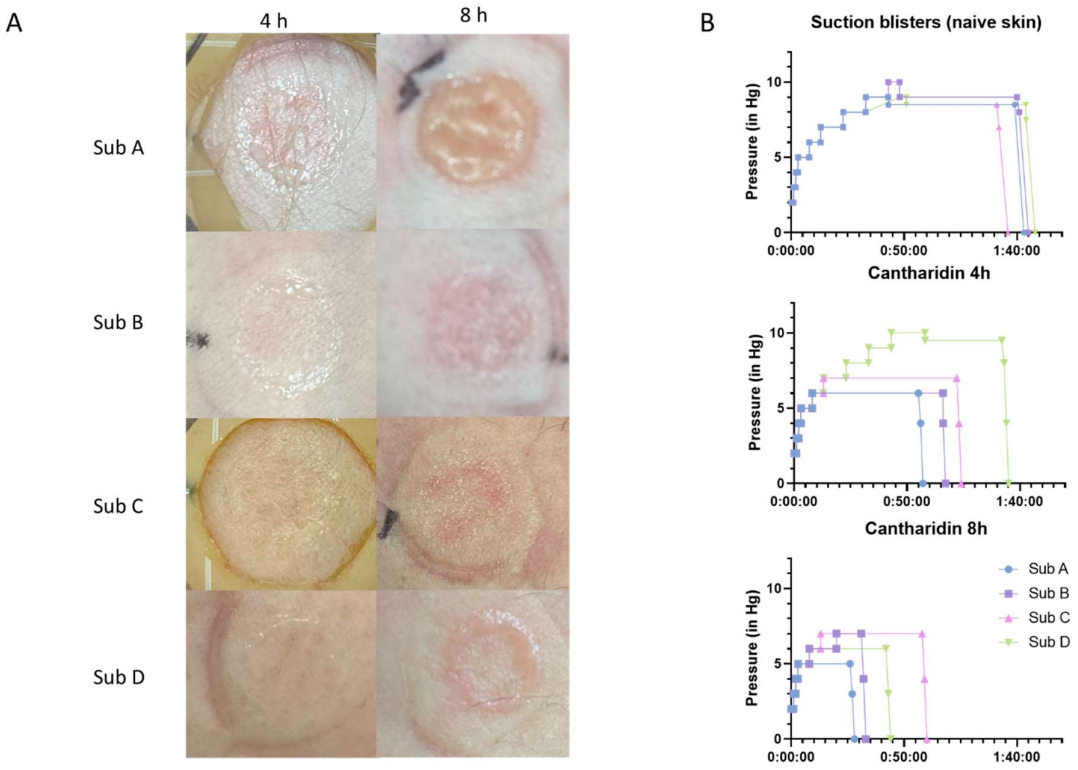


Figure 18: Suction application allows for the formation of harvestable blisters in cantharidin challenge areas. A: photographs of cantharidin application sites after 4 hours and 8 hours of cantharidin exposure and prior to suction being applied; B: pressure profiles for individual blisters formed by each of the 4 participants in naïve skin or after 4 hours and 8 hours of cantharidin application

4.3.2 Use of suction on cantharidin sites appears safe and does not impact healing

There were no reports of pain or discomfort from any of the 4 participants undergoing the study. All subjects had each of their 4h and 8h cantharidin blisters raised using negative pressure on the application site, but no adverse events were reported in any case. Also, the use of mechanical force on an already challenged site appeared to not impact skin healing. No observable changes were noted between sites with and without suction use, both in the early stage of healing or in the longer term.

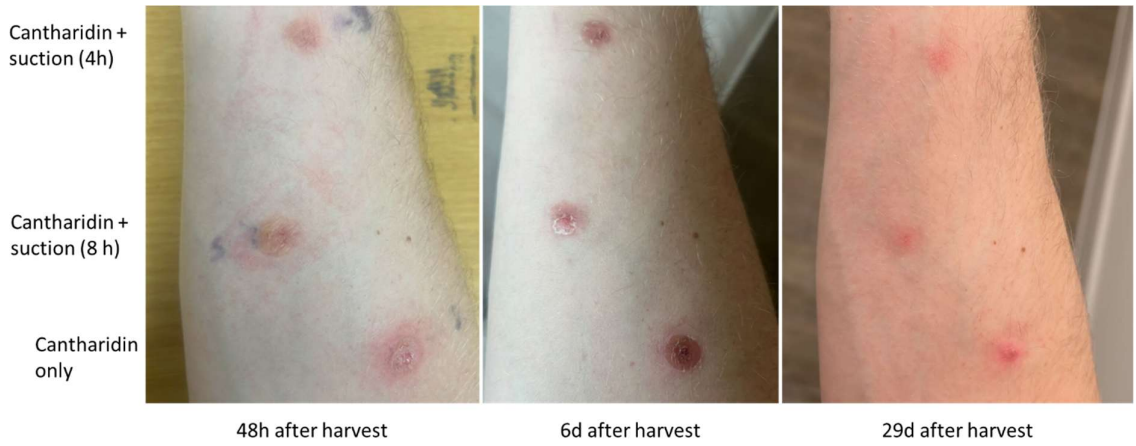


Figure 19: Healing profile of cantharidin blisters with and without use of suction

4.3.3 Detailed analysis on progression of cellular content in blisters over 24h

Data were available for the full time course of 4 subjects with the cantharidin method (4h, 8h, 16h and 24h) but one sample was not collected for the 24h suction blister (Subject B). Suction blister samples were also not collected for the 16h time point due to the practical challenges this would pose regarding formation time.

At 4 hours after challenge, the suction blisters contain some cells, mainly neutrophils and lymphocytes, but in very low numbers (range 223 - 9650 cells per blister). Cantharidin application sites, having a blister formed by negative pressure, contained a similar, low number of total cells (range 162 – 1679 cells per blister). Cell phenotypes present appear to be mostly lymphocytes and non-classical (CD3-, CD19-, HLA-DR-, CD16-) CD45+ leukocytes.

At 8 hours, the recruitment of cells to suction blisters increased slightly (6092 – 28413 cells per blister), and patterns of leukocyte influx appear to differ per subject: neutrophils becoming the most dominant cell subset in two subjects, lymphocytes in the other two. In cantharidin-site blisters, the total number of cells remains low in most subjects (range 1293 – 19006 cells per blister) with lymphocytes and non-classical CD45+ cells continuing to represent the majority of cells, except in the one subject where a primary blister was already apparent at this timepoint (Sub A - Figure 18). In this individual a larger number of cells were present, the majority neutrophils.

In cantharidin blisters, the number of cells increased dramatically from 8 to 16 hours (median (range) 10,063 (1,293-19,006) vs 234,702 (14,217-417,806) cells/blister), when the blisters appeared fully formed. The kinetics of exudate ingress were however discrepant to cellular migration as the volume of blisters increased by approximately 50% between 16h and 24h in 3 participants (Figure 20 C). The increase in volume was also accompanied by a limited increase in cell number (312,761 (114,898-1,580,530) cells/blister at 24h), with the highest increase coming from monocytes (21,048 (6,113-39,691) cells/blister at 16h vs 100,941 (22,405-192,263) cells per blister at 24h) and lymphocytes (10,910 (2,653-26,315) cells/blister at 16h vs 23,001 (12,524-57,200) cells per blister at 24h), which appear to start to migrate in larger numbers only at this later timepoint. Overall inter-subject variability was very high for both blister volume and cell number, with subset number and phenotype of migrated cells also appearing to differ between individuals.

In suction blisters, where the 16h time point was not possible to analyse, there is a big difference between 8h and 24h. Total cell number increased at this timepoint, (median 10-fold increase for subjects A and C where we have data), with all major subsets increasing except for lymphocytes. For this type of blister, the monocyte subset also appears to be a major contributor for increase in cell number at this later timepoint, as they were almost absent for most subjects at 8h (median 1,178 (172-4,900) cells/blister at 8h vs 9346 (8,328-70077) cells/blister at 24h).

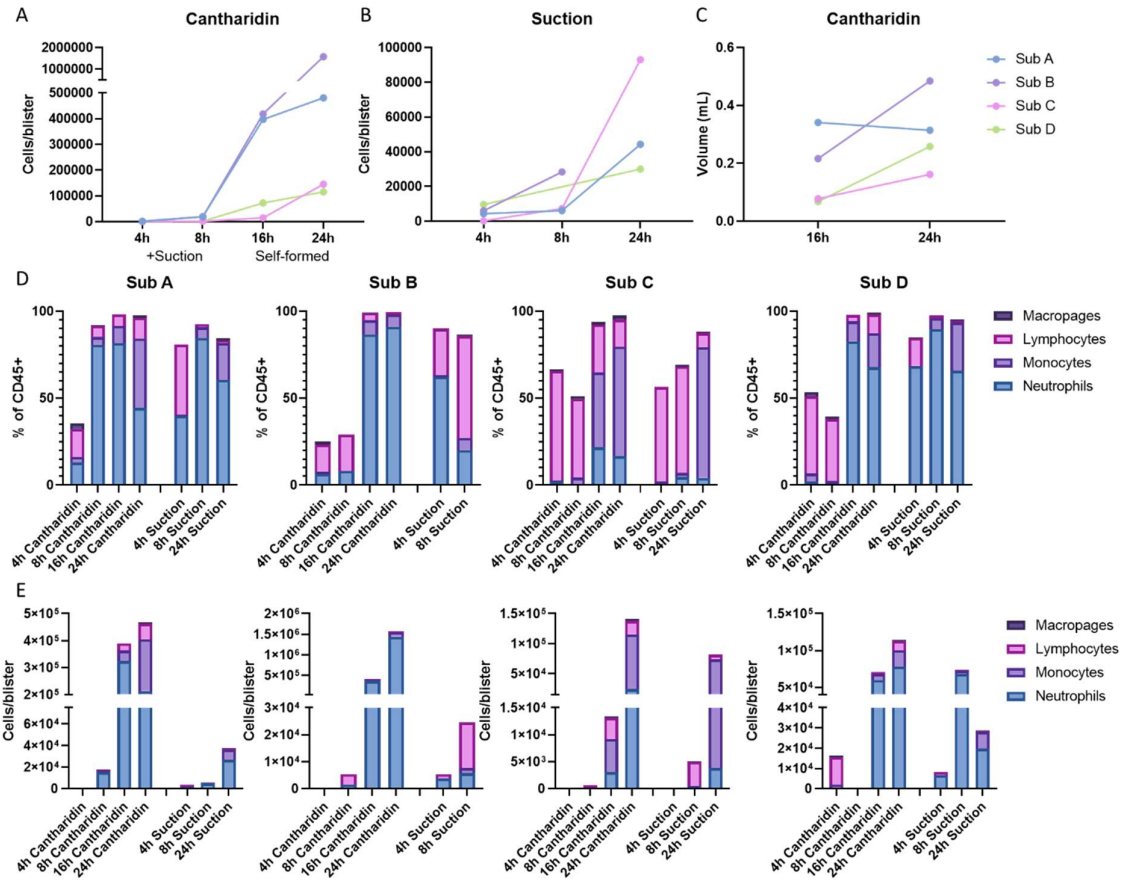


Figure 20: Temporal kinetics of cell number and phenotypes for both suction and cantharidin models. Total number of cells observed in blisters raised via A: Cantharidin and B: Suction at each time point. For cantharidin methodology at 4 and 8 hours, blisters had to be raised by suction, delaying harvesting. C: Volume of blister supernatant on self-formed cantharidin blisters. Cellular make up of each evaluable blister expressed as percentage of leukocytes (D) and as total number of cells (E).

4.3.4 Monocyte phenotype evolves with time after influx to blister

Blister cells were processed for flow cytometry in a panel that included activation/polarisation markers. When analysing the monocyte population (gated on FSC^{mid}/SSC^{mid} and $HLA\text{-}DR^{+}$), we can observe that in the cantharidin model, the first monocytes only appear to enter the blister at the 8h timepoint. At that timepoint, these tissue monocytes appear to be almost exclusively from a classical (CD14+/CD16-) phenotype (median intermediate phenotype 0%). By 16h (only observed for cantharidin blisters), a significant proportion of these cells are staining for CD16 (median intermediate phenotype 19.0%), and at 24h the intermediate phenotype (CD14+/CD16+) appears to be the most common (median intermediate phenotype

37.9%; Figure 21 A). For the suction model, a similar percentage of intermediate monocytes was observed for 4 and 8 hours, with 24h blisters containing more than 50% of CD14+/CD16+ intermediate monocytes (Figure 21 B). In contrast, no discernible patterns of change in activation/polarisation markers were observed including monocyte CD163, CD206 and CD64 (Figure 21 C,D).

Regarding other cell subsets, lymphocytes were mainly CD3+/CD4+ helper T cells and CD3+/CD4- cytotoxic T cells. The relative proportion of the latter decreased in the at the 16h and 24h timepoints when compared to the earlier time points. No CD19+ B-cells and very few NK cells (defined with CD16+ as a surrogate for CD56 as most NK cells express this marker (Cooper, Fehniger and Caligiuri 2001)) were present at any stage (Figure 21 E). Neutrophil activation was assessed by measuring CD64 expression and no patterns emerged for any subject, although subjects A and B started with a higher background CD64 MFI (Figure 21 F).

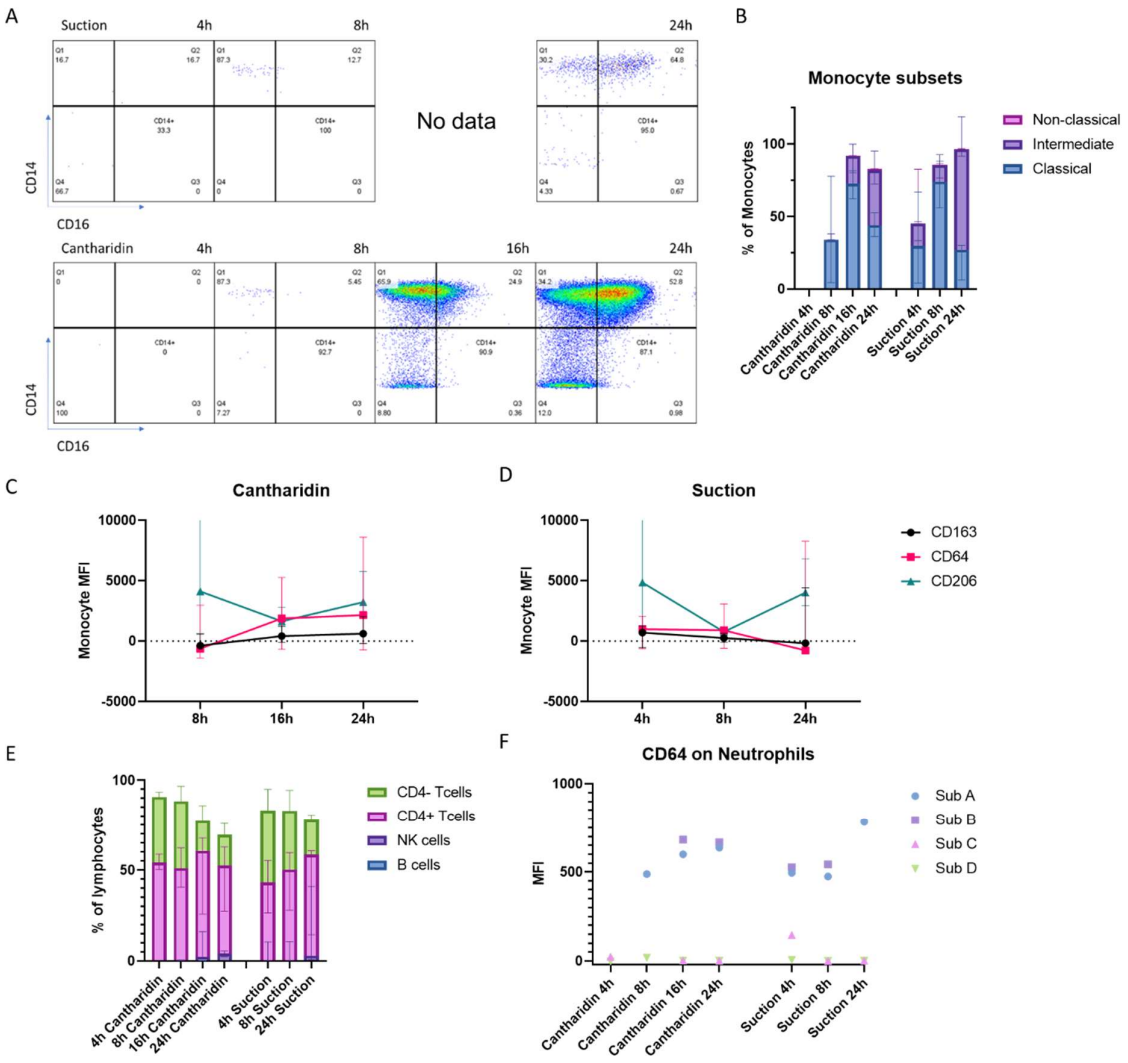


Figure 21: Monocyte maturation after influx to blister milieu. Monocytes enter the blister as CD14+/CD16- classical monocytes from 8h and appear to mature to CD14+/CD16+ intermediate phenotype with the presence in the tissue. A: Representative dot plots for CD14 vs CD16 showing the different stages of maturation over time in both blister models; B: median monocyte population phenotype (n=4 blisters per condition, median with interquartile range); Monocyte activation markers do not show any obvious trends over time in either C: cantharidin and D: suction models.

4.3.5 Clear and consistent patterns of soluble mediator release are observed in the cantharidin model

Cytokine and chemokine data were obtained using multiplex kits from Meso Scale Discovery ran at optimised dilutions (1/4 for cytokine, 1/25 for chemokine) to allow most data to fall within quantification range. Limited data fell below limit of quantification, especially for suction blisters or early cantharidin blister supernatants,

and these were imputed with a value of half the assay's lower limit of detection to allow plotting and statistical calculations.

Data for suction blisters was highly consistent and shows very low levels of pro-inflammatory cytokines throughout the time course for all subjects. Except for a few samples (Sub A 24h for IL-4, IL-6, IL-12-p70 and IL-1β and Sub B 8h for IL-13 and IL-2), all other samples do not show any time profile and are mostly in the lower limits of quantification.

In contrast, for cantharidin blisters, there are consistent temporal profiles between individuals. Sub A consistently presents a more rapid cytokine response in comparison to the other 3. Notably, his cantharidin blister at 8h was the most advanced just by visual observation of the site (Figure 18).

Two cytokines appear to have a much higher magnitude of activation than the others – TNF and IL-6, which increase concentration up to the ng/mL range from very low pg/mL baselines. TNF is also peculiar in that the peak response occurs very early – 8h for 2 subjects and 16h for the other 2. IL-2, IL-4, IL-6, IFN-γ, IL-12p70 and IL-1β increase from baseline at 16h and appear to peak at 24h. IL-10 and IL-13 do not appear to show clear trends.

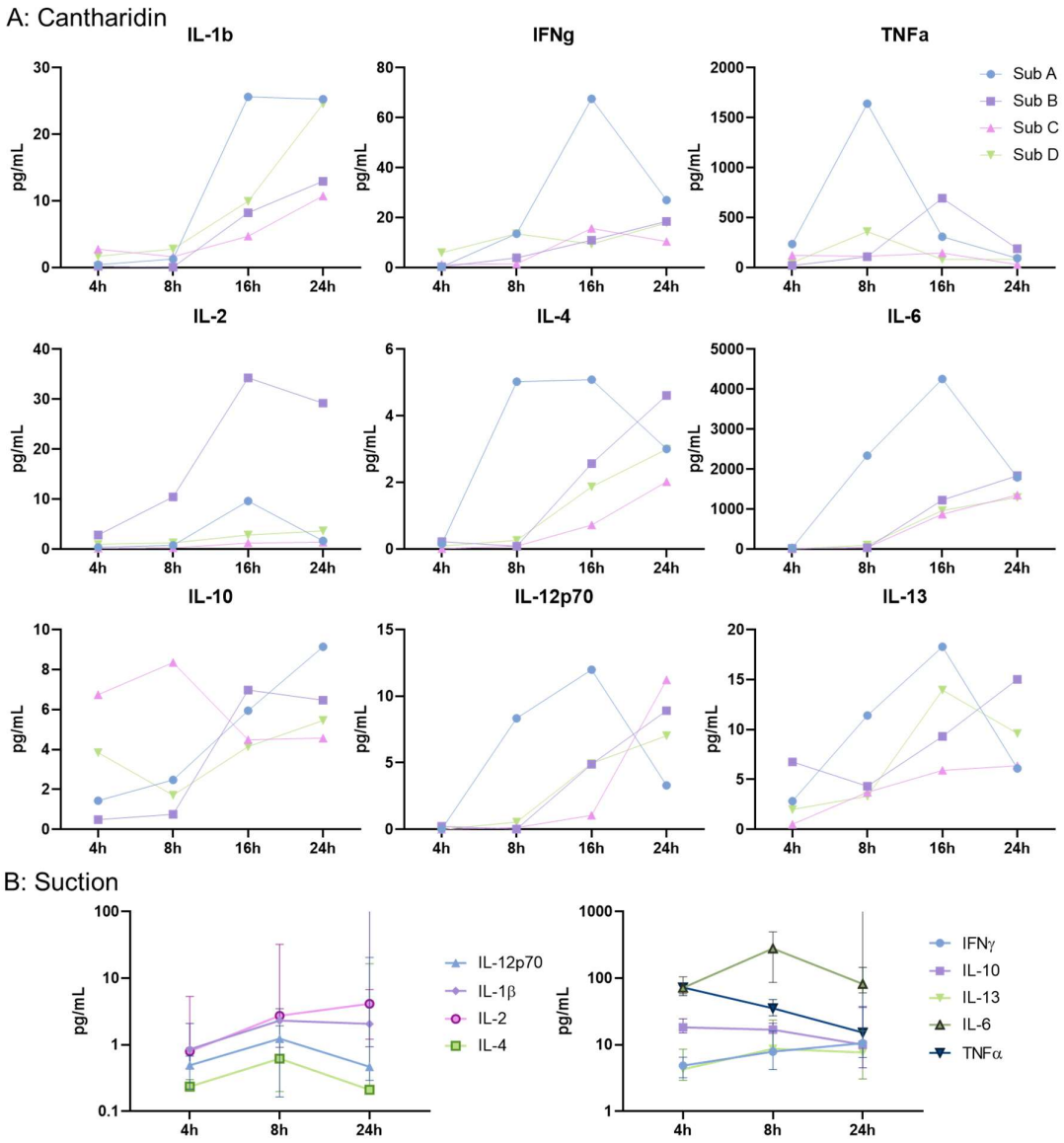


Figure 22: Cytokine profile in blisters over time. A: Figures display the time course for each participant in the cantharidin model (n=1 blister per timepoint) for each analysed cytokine. B: median and interquartile range of data obtained for the suction model (n=4)

A panel of chemokines was also analysed for all blister samples. In suction blisters, a clear response at 24h is observed for TARC and the only other chemokine where a profile is observed is IP-10 where 2 subjects peak at 24h and 2 others appear to increase much earlier at 8h. For subject A, the response returns to baseline at 24h, whereas there is no 24h data for subject B.

In cantharidin blisters, there are again consistent individual trends observed for most analytes, excluding Eotaxin-3, where data appears to remain at baseline for all timepoints. IL-8 appears to have a very clear response, with peak chemokine values at 16h post challenge and then a reduction at 24h for all subjects. The other chemokines studied (IP-10, MIP-1α, MIP-1β and TARC) appear to show levels raised at both 16 and 24h.

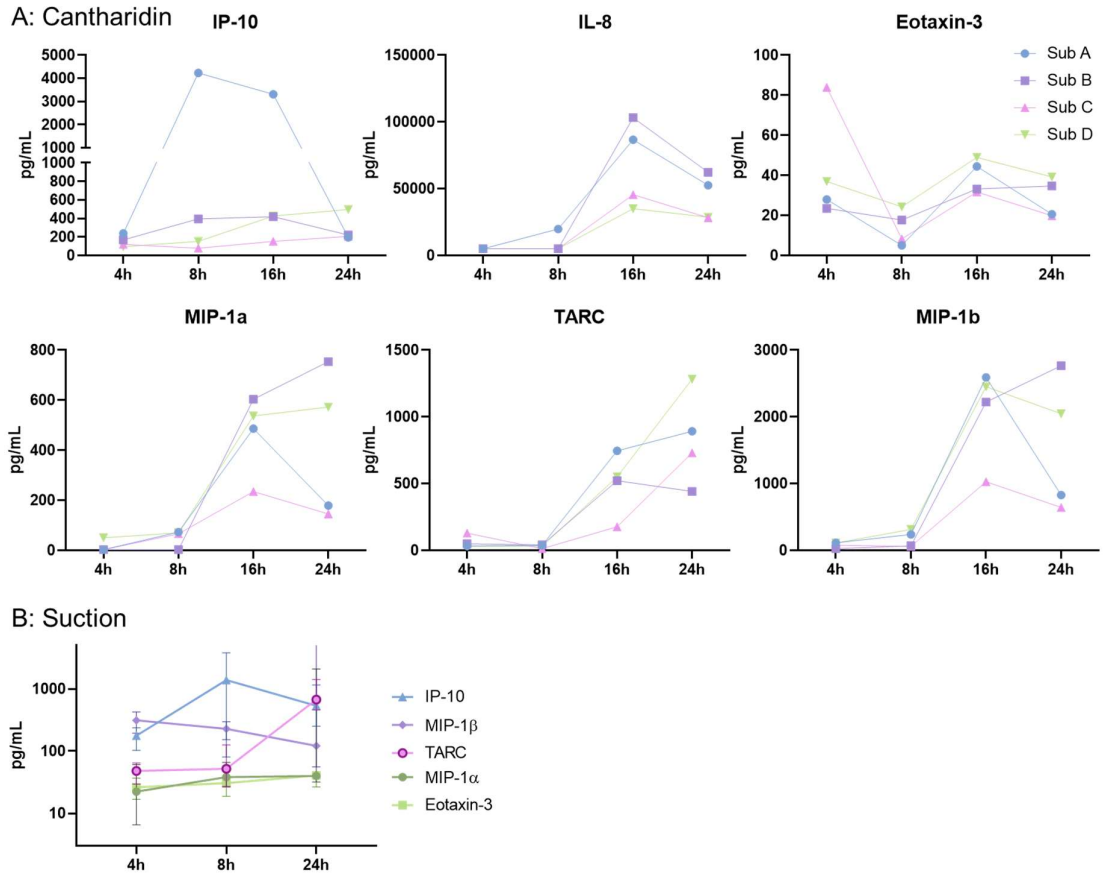


Figure 23: Chemokine time course. A: Figures display the time course for each participant in the cantharidin model (n=1 blister per timepoint) for each analysed cytokine. B: median and interquartile range of data obtained for the suction model (n=4)

4.3.6 The resolution of the cantharidin blister response

Traditionally, the cantharidin blister model is used to describe both acute (24h) and resolving (48h/72h) phases of inflammation. Although the time course data generated for this sub-study was only designed to describe the early inflammatory process, as

part of this project, data was also generated regarding progression to the resolving stage (24h vs 48h) and can be interpreted in this context to provide a complete narrative of the inflammatory process observed in the cantharidin model.

The data presented here originates from 2 separate studies run in UCL and GSK, with many common outputs, providing independent conclusions that align. In the figures below, these cohorts will be designated by Coh1 and Coh2, respectively. The correlation data below was calculated with a non-parametrical paired test (Wilcoxon) and the median difference between pairs along with 95% CI of the differences are displayed.

In terms of volume and cellularity of the blister, the 24 and 48h timepoints have equivalent contents, i.e. there are no significant differences between 24 and 48 hours for any of the parameters tested ($p>0.05$). In terms of volume, it is interesting to note that there is a very close correlation between volumes of blisters for 24 and 48h for each subject ($r=0.8473$; $p<0.0001$), but that same correlation is not observed for the cellularity measures.

Although total cell number appears unchanged, there are alterations when looking at the individual leukocyte phenotypes in the blister supernatants. The number of neutrophils and lymphocytes appear to remain constant between the two timepoints, but there appears to be an increased number of monocytes and macrophages at 48h. The maturation levels of these monocytes also appear to evolve with time with a significant increase in the absolute number of intermediate monocytes at 48h ($p<0.05$), although the number of classical monocytes appears not to change. Interestingly, the monocyte phenotype does not appear to evolve to the non-classical CD14-/CD16+ phenotype with no sign of loss of CD14 from monocytes harvested at 48h.

The change of monocyte phenotype is not only apparent in the CD14/CD16 activation process, but also in some other markers of activation/polarisation. Some polarisation markers were common for both studies, and there appears to be an increase in CD163 expression (median increase: 2057, (95% CI of difference between pairs: 282.7 – 3212), $p=0.0312$ Coh1; 640.4, (359.6 – 921.2), $p=0.0005$ Coh2) but this was not consistently observed for CD206 (4523, (-2215 – 19023), $p=0.0312$ Coh1, -38.0, (-752.6 – 3179), $p=0.492$ Coh2).

In the study carried out at GSK (Coh2), additional myeloid markers were evaluated that were not replicated in the study carried out in UCL. In this cohort, convincing evidence was shown for increase of CD209 (median difference between pairs: 821.7,(with 95% CI of the difference: 504.9 – 1237); p=0.0005), CD40 (68.0, (20.19 – 162.3); p=0.0269), CD80 (315.3, (267.1 – 470.9); p=0.0005) and a decrease in CD83 (-803.2, (-1049 – -532.5); p=0.001) and CD86 (-336, (-520.9 – -98.4); p=0.0122).

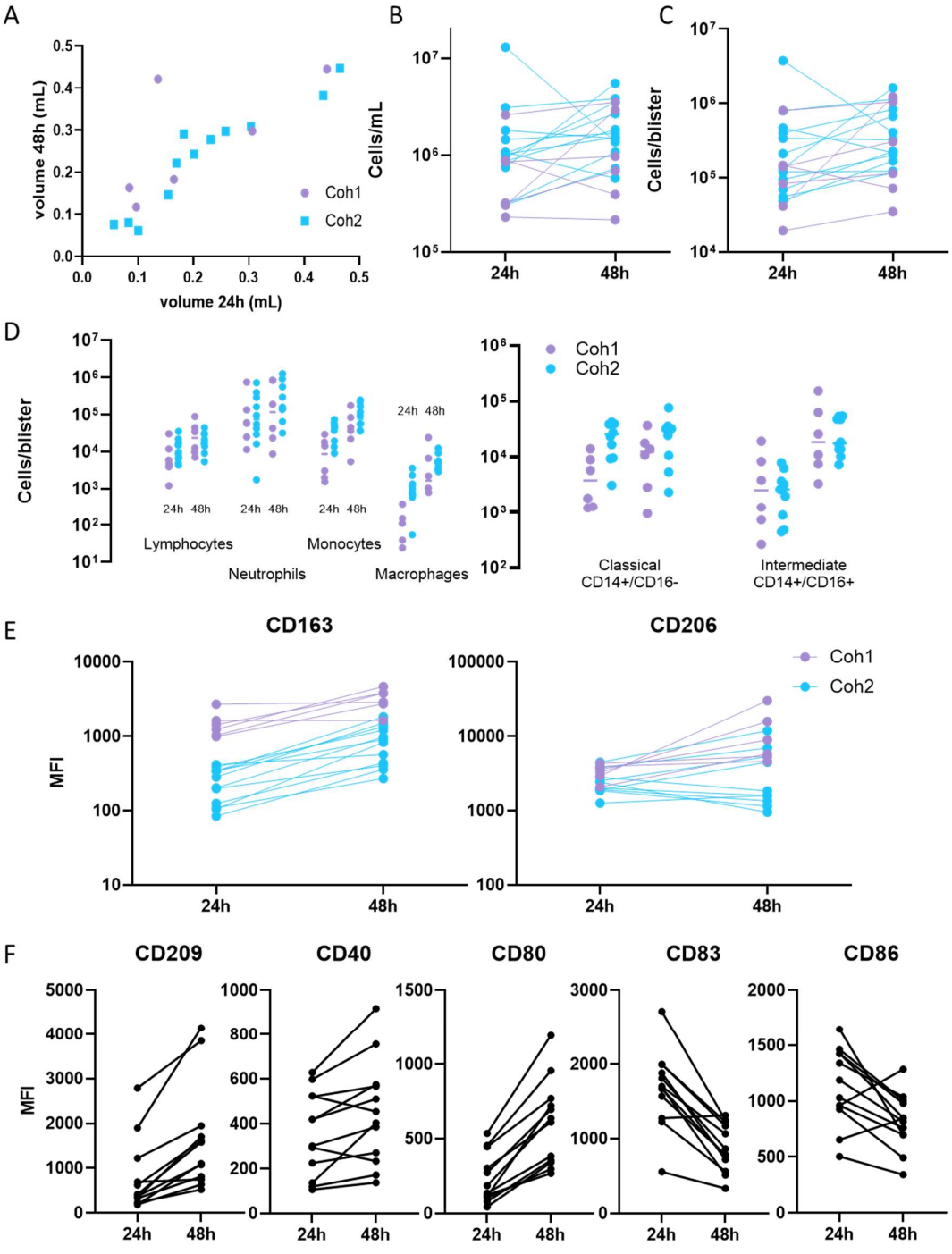


Figure 24: Differences in cellular outputs between 24 and 48h cantharidin blisters. [A]: Correlation of blister volume between 24 and 48 hours in same subject (Coh 1: n=6, purple; Coh 2: n=12, blue). Differences in cellularity between 24h and 48h on same subject as measured as cells/mL [B] and cells/blister [C].[D]: Number of leukocyte subsets at 24 and 48h expressed as cells/blister on the left, with a focus on the monocyte subsets on the right; Monocyte polarisation/activation markers with data for both cohorts [E] and where data was only available for cohort 2 [F]

The cytokine and chemokine profiles at these 2 timepoints was also assessed in both the UCL and GSK studies (Figure 25). For the cytokines most highly expressed in the blister response, there was a consistent reduction in both studies of TNF (-131.9, (-249.9 – -21.9) p=0.0312 for Coh1; -59.8, (-91.31 – -12.9), p=0.0195 Coh2) and IL-8 (-86877, (-162244 – -34633), p=0.0312 for Coh1; -46252, (-239319 – 69381), p=0.125 Coh2) but reduction of IL-6 in only cohort 1 (-621.2, (-1067 – -112.1), p=0.0312 Coh1; -668.3, (-4322 – 4270), p=0.875 Coh2). Other cytokines did not exhibit consistent temporal trends.

In relation to chemokines, TARC was consistently increased in both datasets (3725, (2592 – 5768), p=0.0312 Coh1; 2135, (1279 – 2833), p=0.0005 Coh2), with MDC also being observed to significantly increase in the GSK study. In contrast, MIP-1β (-873.2, (-2746 – -85.2), p=0.0312 Coh1; -1215, (-3359 – -432.8), p=0.0039 Coh2) and Eotaxin-3 (-7.8, (-21.8 – 2.82), p=0.1563 Coh1, -35.7, (-77.9 – -16.5), p=0.0039 Coh2) appear to show a decrease at 48h. MCP-1 was only assessed on Cohort 2 and is also significantly reduced at 48h (-13303, (-23822 – -8315), p=0.0005).

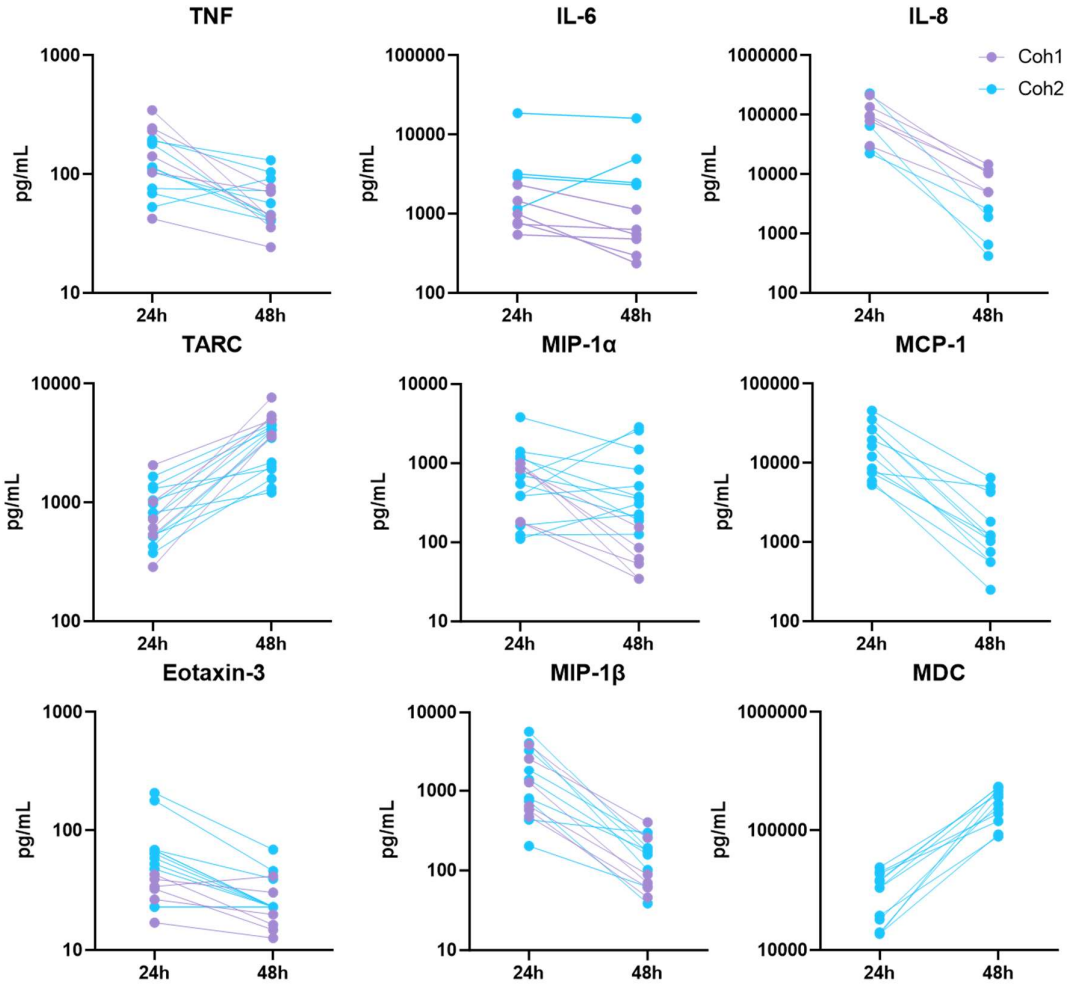


Figure 25: Progression in cantharidin blister cytokine and chemokine profile between 24 and 48h. Pairs of 24/48h blisters of same participant show progression of cytokine and chemokine levels (n=6 Coh1, n=12 Coh2). Wilcoxon test carried out for paired analysis * p<0.05; ** p<0.01; *** p<0.001, **** p<0.0001

4.4 Discussion

4.4.1 Suction blistering allows for safe exploration of early cantharidin exposure

This study is the first report on the early events that drive cantharidin blister formation and adds to the limited information available on the time course of the suction blister reaction (Davidsson, Bjorkman et al. 2013).

Data on the early biological events following cantharidin exposure to date are limited to microscopy observations on skin biopsies obtained after in-vivo challenge (Piérard-

Franchimont and Piérard 1988) and more detailed observations from excised human skin (Bertaux, Prost et al. 1988). These reports are still the best description of the structural events that lead to acantholysis but fail to report on the immunological sequelae that occur in parallel with the formation of the blister chamber.

To do so, suction was employed to draw the cellular and humoral mediators that are present in the challenge site prior to acantholysis. This is the first report of the use of negative pressure following cantharidin exposure, but this method is frequently employed to obtain material from other local immune challenges such PPD (Akbar, Reed et al. 2013), LPS (Buters, Hameeteman et al. 2022) or varicella-zoster virus (VZV) (Patel, Vukmanovic-Stejic et al. 2018).

The approach was well tolerated by the volunteers, there were no reported adverse effects, and the progression to blister healing was not different to either of the blister methods alone.

When comparing the methods for blister formation, suction has the advantage of creating a harvestable blister in 2-3 hours, which opens the door to observation of early events after a challenge. The fact that it still takes hours of continuous suction, paired with the fact that this needs complete attention from an experienced operator to control the pressure during formation makes this method less practical for general use. Using cantharidin prior to application of suction could in theory allow a quicker and more standardised approach for creating a blister, but we have observed that 4 hours after application there is still significant cohesion within the epidermis and that significant pressure and time are still required for the formation of a blister. Also, inter-subject variability in the level of skin integrity still requires an experienced operator to apply variable amounts of pressure to create a blister, so it is not possible to completely standardise the procedure on a ‘one-size-fits-all’ basis.

At the 4 and 8 hour time points, it was observed that acantholysis commences from the edge of the application site. The direct application methodology was devised to allow a more homogeneous distribution of cantharidin over the application site when compared to the paper disc method, but this observation appears to suggest that capillary action may force the acetone to aggregate on the barrier and creates a concentration gradient from the outside to the inner ring.

4.4.2 Cantharidin blister response appears to be driven by TNF release from stromal cells

The cantharidin blister model is used both as a model for acute and resolving phases of inflammation, usually by harvesting blisters formed 16-24h (acute) or 48-72h (resolving) after the cantharidin application. The 24/48/72h collections are also the most useful practically, but little is known about the full time course of events following cantharidin exposure.

One limitation to this method is the time to acantholysis, which is known to happen overnight after cantharidin application, but this is poorly understood and has led to the earliest time of collection be around 24h, as all those exposed will have generated a blister by this point. In the described time course experimental study carried out in 4 volunteers, the first visible signs of blistering are observed at 4 hours as small patches of loose skin in some volunteers, and at 8h these patches tend to form larger areas, usually at the edges of the cantharidin application area. By 16 hours the blisters had a normal appearance in all 4 participants, with full acantholysis having occurred. Volume probably only peaks at 24h and it is maintained up to 48h.

The application of cantharidin appears to initiate an inflammatory reaction driven by TNF, that appears to peak at around the time of initial acantholysis. This peak was only possible to evaluate due to the use of suction to be able to capture the local interstitial fluid, and as the procedure will pull fluid from surrounding tissue in a short period of time, diluting the local mediators, the values are probably an underestimation. This early TNF peak precedes the influx of immune cells and so must be produced by local cells.

Stromal cells are known to produce TNF in response to bacterial infection or damage (Kock, Schwarz et al. 1990, Fahey, Turbeville and McIntyre 1995, Banno, Gazel and Blumenberg 2004) and may be the initial source of this cytokine. Our data supports this observation as we have shown pro-inflammatory cytokine release in response to cantharidin in in-vitro experiments with fibroblast and keratinocyte cell lines.

In animal wound models, TNF is one of the first mediators to be released by immune and non-immune cells such as vascular endothelial cells, keratinocytes and fibroblasts in the injured area, that leads to the initiation of the inflammatory phase, promoting leukocyte recruitment to the tissue (Ritsu, Kawakami et al. 2017). This response is

driven by the tissue damage, specifically by the Damage-Associated Molecular Patterns (DAMPs), which are able to trigger a TNF response and initiate innate immune response (Rani, Nicholson et al. 2017).

This production may follow tissue damage initiated by cantharidin, or could, in theory, be directly caused by the enzymatic mechanisms presented by cantharidin. Okadaic acid, another phosphatase 1 and 2A inhibitor (Honkanen 1993), has been shown to control cellular functions by increasing the phosphorylation status of phosphoproteins. It can, by itself stimulate TNF mRNA accumulation and TNF synthesis in monocytes. Calyculin A, another potent inhibitor of phosphatase 1 has similar effects (Sung, Walters and Fu 1992).

In an attempt to validate the cantharidin blister methodology, Dinh et al. used Adalimumab, Humira®, an anti-TNF drug, and observed a suppression of neutrophil ingress to blister sites after treatment, which is probably the most immediate cellular change induced by TNF. Unfortunately, this paper only focusses on immune cell changes and does not report mediator analysis (Dinh, Corraza et al. 2011).

4.4.3 TNF initiates an inflammatory response characterised by well-defined soluble mediator and cellular patterns

Shortly after the TNF peak, a clear IL-8 profile that peaks at 16h and already appears reduced at 24h in all 4 subjects is observed. IL-8 is a chemokine produced by a variety of immune and tissue cells that, unlike other cytokines, has a very high specificity for targeting neutrophils. IL-8 exists as a preformed pool in endothelial cells which results in a rapid drive for neutrophil chemotaxis to site of action (David, Dominguez et al. 2016) and, in the site of action, will promote resolution by inducing phagocytosis and production of neutrophil extracellular traps (NETs) (David, Dominguez et al. 2016). The IL-8 peak observed at 16 hours indeed coincides with the initial drive of neutrophil recruitment to the blisters, which are the first effector cells in the innate immune response. These cells appear to peak around 24h, when a transition to monocyte recruitment is observed as is classically recognised in acute inflammation (Kaplanski, Marin et al. 2003).

In the blister milieu, this stage (16 to 24h) appears to be characterised by the peak in pro-inflammatory cytokines such as IL-6, IL-1β and IFN-γ. Their levels slightly reduce until 48h. These play important roles in the acute phase inflammatory response, and levels are maintained up to initiation of resolution.

Chemokines such as MIP-1α and MIP-1β (also known as CCL3 and CCL4), mainly produced by monocytes/macrophages and responsible for the recruitment of multiple leukocytes, peaked at 24 hours and declined toward baseline by 48 hours, potentially indicating a reduction in chemotactic signals attracting leukocytes to the blister milieu. Another major chemotactic mediator involved in the recruitment of monocytes into tissue is MCP-1 (also known as CCL2) which was not measured in the early time course experiment but is dramatically reduced at 48h compared with 24h as measured in GSK data. Their levels appear to track the flux of classical monocytes, which appears to peak at 24h.

4.4.4 Blister milieu promotes M2-like monocyte polarisation

In circulation, there are 3 well characterised populations of monocytes that evolve in sequential stages: classical (CD14+/CD16-), intermediate (CD14+/CD16+ and non-classical (CD14-/CD16+) subsets (Ziegler-Heitbrock, Ancuta et al. 2010). Classical monocytes derive from precursors in the bone marrow, circulate for approximately 1 day and either move out of circulation or differentiate into intermediate monocytes. These have a longer lifespan (4 days) and again either disappear from circulation or transition to fully differentiated non-classical monocytes that remain in circulation for 7 days, migrate to tissue or die (Patel, Zhang et al. 2017). Further transcriptional analysis identified smaller subsets within each group (Gren, Rasmussen et al. 2015).

Classical monocytes appear to express higher level of chemokine receptors, indicating a higher potential to migrate towards injury and inflammation stimuli, and appear to produce higher levels of pro-inflammatory cytokines when stimulated. Intermediate monocytes also produce cytokines, but may be a source of IL-10, an anti-inflammatory cytokine, and have a higher level of antigen presenting molecules. Non-classical monocytes can also present antigens and are associated with wound healing (Kapellos, Bonaguro et al. 2019).

In the blister model, monocytes appear to arrive from the periphery as CD14+/CD16- classical monocytes, as the first monocytes observed at 8h had this phenotype. At 16h, a large proportion of the blister monocytes had already gained CD16 expression, evolving into CD14+/CD16+ intermediate monocytes. The proportion of intermediate monocytes continue to increase for 24h and 48h, never observing any monocytes losing CD16 and moving to the last stage of differentiation, even at the 48h timepoint.

Although no significant trends are observed in the early time course data, between 24h and 48h, changes in surface expression of activation/polarisation markers were observed. CD163 MFI is increased in both UCL observations and data from GSK which was much more focussed on myeloid cells. In the latter dataset, it is also possible to observe an increase of CD209, CD40 and CD80 and a decrease in CD83 and CD86 at 48h.

CD83 is mainly expressed on the surface of dendritic cells and is a marker of mature DCs (Li, Ju et al. 2019), although it is present intracellularly in many other immune cells (Cao, Lee and Lu 2005). Thus, a reduction in this marker may indicate a stronger pull towards macrophage differentiation rather than DC maturation for the early infiltrating monocytes.

When leaving circulation and after activation via molecules such as PAMPs or DAMPs, monocytes will undergo morphological and functional changes to mature into phagocytic macrophages. These cells play a central role in tissue homeostasis and repair, and can differentiate specifically in response to environmental cues into what have been classically described as M1 macrophages (pro-inflammatory cells induced by IFNγ or microbial stimuli, which produce TNF and IL-6, and are critical for host defence), or alternatively activated M2 macrophages (cells associated with tissue repair, induced by IL-10 and IL-4, which in turn produce IL-10 and TGF-β) (Italiani and Boraschi 2014). Looking at classical markers of these 2 main subsets, M1 express high levels of markers associated with antigen presentation (such as CD86) and M2 will express high levels of surface markers such as CD163 and CD206, which are associated with tissue repair and immunosuppression (Yao, Xu and Jin 2019). Cells observed in the 48h cantharidin blisters appear to be polarising into an M2-like, anti-inflammatory phenotype, although CD80, a co-stimulatory molecule, similar to CD86 and also associated with M1, is increased at 48h.

Interestingly, CD163 is reported to be present in classical monocytes, and induced by LPS stimulation in this population, but reduced or absent in intermediate (CD16+) monocytes (Tippett, Cheng et al. 2011). In this study, CD163 expression is increased at the same time as monocytes are gaining CD16 and transitioning to an intermediate phenotype.

4.4.5 Cantharidin blister chemokine profile promotes ingress of anti-inflammatory lymphocyte at 48h

TARC showed a very consistent pattern of expression on the cantharidin model. From background levels at 8h, starts to rise at 16h and a gradual increase is observed at 24h, which continues to 48h. A similar significant increase is observed for MDC from 24 to 48 hours. These two chemokines, although produced by different cell types – TARC is constitutively expressed in the thymus but also produced by dendritic cells, fibroblasts, keratinocytes and endothelial cells (Saeki and Tamaki 2006) whereas MDC is mainly produced by macrophages and dendritic cells (Korobova, Arsentieva and Totolian 2023), are the 2 ligands of CCR4, a chemokine receptor mainly found in Th2 cells, skin-homing T cells and Treg cells. This chemokine is the main driver for these lymphocytes and has been targeted for treatment of conditions such as atopic dermatitis and asthma (Yoshie and Matsushima 2015). In the cantharidin model, a steady increase of lymphocytes from 16 hours to 24 hours and a trend to a higher percentage of CD4+ cells (and reduction of CD3+ CD4-) is observed at the later timepoints. Unpublished data from earlier studies at GSK showed a much higher percentage of Treg cells present in 24h suction blisters than in periphery, possibly due to selection via CCR4 engagement.

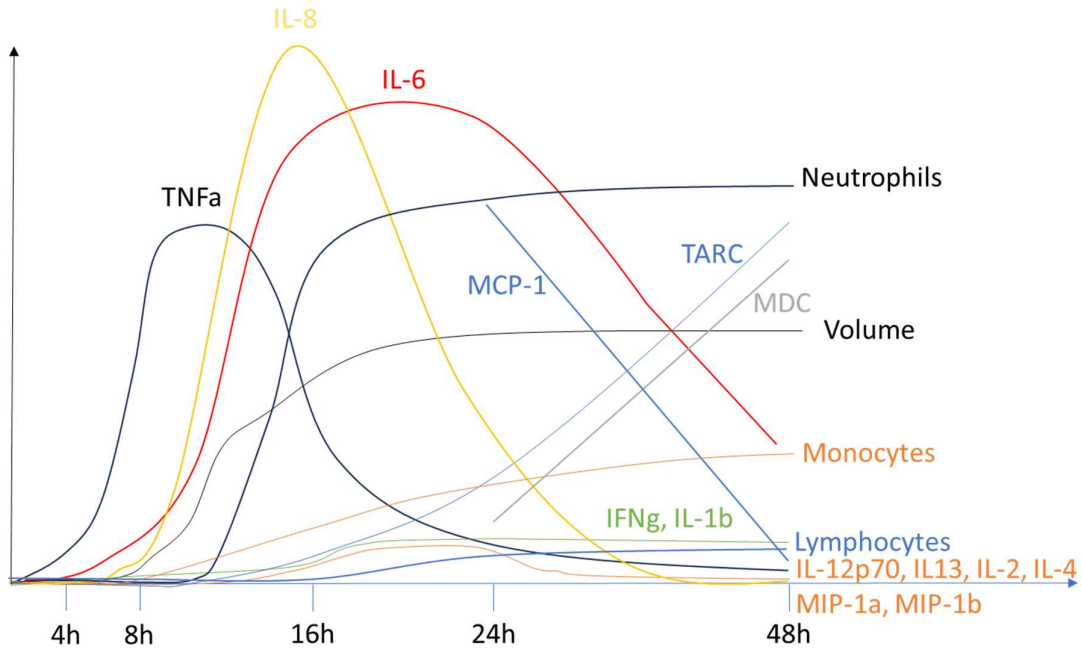


Figure 26: Immune response to cantharidin challenge. Cellular and soluble mediator time course after cantharidin application

4.4.6 Cellular damage caused by suction creates an earlier, but weaker immune response

In contrast with the observed immune response to a cantharidin challenge, the responses observed to the creation of a suction blister were less consistent.

Although the suction time course lacks some of the detail, as the 16h time point was not feasible, it is clear that most of the observations from the cantharidin profile are not recapitulated in this model.

The trigger in this case is a mechanical force, rupturing the layers of the epidermis, inducing cellular damage and thus initiating a response triggered by DAMPs, endogenous danger molecules, that initiate the inflammatory response via TLR engagement. TLRs induce pro-inflammatory cytokine secretion with TNF being one of the primary effectors in the response (Relja and Land 2020). In this model, TNF is already elevated at 4h, clearly dropping at 8h, whereas in cantharidin, this first effector only peaks at 8h. IL-6 also appears to peak earlier, with 24h levels being reduced when compared to 8h. This suggests that the initiation of the immune response in the

suction blister happens sooner than in the cantharidin blister and that can be seen in the maturation of monocytes, which start to gain CD16 earlier in suction.

The magnitude of the response is clearly lower, as levels of pro-inflammatory cytokines are lower compared to cantharidin and the immune trafficking to the blister site is much reduced, recruiting a much lower percentage of neutrophils, the first responders to an inflammatory challenge.

Comparing the two models, a milder inflammatory challenge is observed in suction, that happens very close to the formation of the blister, compared to a delayed, but much more pronounced response to damage induced by cantharidin. Both immune responses appear to be driven by an early peak of TNF that initiates the recruitment of immune cells to the site, but their magnitudes are very different, with cantharidin eliciting much higher levels of pro-inflammatory cytokines that culminate in a much more cellular blister.

4.5 Chapter conclusions

- Suction blistering over cantharidin exposed sites allows the study of early biological events in the skin, allowing the mapping of the cantharidin response.
- TNF secretion from stromal cells appears to be the initial inflammatory trigger in both models.
- A full inflammatory response was observed in the cantharidin model through a well characterised sequence of humoral and cellular events.
- Suction creates immediate damage, but initiates a weaker immune response.

Chapter 5: Characterisation and comparison of blister models

5.1 Introduction

5.1.1 The utility of the cantharidin model of inflammation

The use of cantharidin to study inflammatory neutrophils was first described in 1964 (Boggs, Athens et al. 1964). Since then, this methodology has been used as a tool to induce and study skin inflammation in academic research and to observe pharmacology in skin in drug development.

Most of the current literature is based on the method described by Day et al. (Day, Harbord et al. 2001), which has been further defined (Jenner and Gilroy 2012) and validated by using common anti-inflammatory compounds to modulate the blister inflammatory reaction (Dinh, Corraza et al. 2011).

The temporal changes in cellular and soluble components of the cantharidin blister have been covered in Chapter 4. Early onset blisters, at 24h post-application, are characterised by a less cellular fluid, dominated by neutrophils and the blister milieu evolves to a resolving phase at 72h, where differentiation/activation is observed in monocytes/macrophages through upregulation of CD16 and CD163 and an influx of eosinophils and dendritic cells is observed (Jenner, Motwani et al. 2014). This resolving stage is actively controlled by the inhibition of neutrophil trafficking, dampening of pro-inflammatory signalling and cytokine catabolism, followed by granulocyte apoptosis and phagocytosis (De Maeyer, van de Merwe et al. 2020). Rapid changes in cytokine profiles have also been described with reduction of pro-inflammatory cytokines such as IL-6 and TNF being accompanied by a rapid increase in immunoregulatory factors such as TGFβ and MDC (Evans, Haskard et al. 2013).

Resolution of inflammation is one of the main areas of study where this methodology is employed, and different immunophenotypes have been observed in blister samples with subjects responding either with immediate leukocyte accumulation and cytokine synthesis or with a gradual increase in inflammation and a delayed resolution depending on presence of preresolution mediators in blister fluid (Morris, Stables et al. 2010). Substantial changes in resolution pathways were also detected in blisters of subjects with type 2 diabetes. Although cell numbers are similar in early blisters when compared to healthy subjects, the TNF response is dysregulated with very low levels of this cytokine present at early onset and a much higher concentration in later blisters, promoting longer-term, non-resolving inflammation (Landis, Evans et al. 2010).

This model has been used to study immunological differences between populations. By comparing early and late blisters in young and elderly participants, differences in resolution profiles were found between the groups whereas the mechanisms driving onset of inflammation was found to be maintained. Resolution was impacted mainly due to a reduction of TIM-4 in macrophages which enables efferocytosis (De Maeyer, van de Merwe et al. 2020). Striking sex differences, have also been found in the resolving blisters, with females showing lower expression of cell surface inflammatory markers and an enhanced resolution profile on lipid mediator analysis (Rathod, Kapil et al. 2017).

Another area where blister methodologies can be useful is the study of chemotaxis. Adhesion molecules have central roles in this process, with LFA-1 been seen to be shed into the blister fluid after transmigration of monocytes and neutrophils from blood to tissue (Evans, McDowall et al. 2006) and CXCL6 shown to be central to gender differences in neutrophil numbers in blisters (Madalli, Beyrau et al. 2015).

Major surgical procedures trigger a systemic pathological inflammatory response, which may be caused by leukocyte extravasation into tissue. Surgical intervention with cardiopulmonary bypass was shown to increase the extravasation of leukocytes into perioperative skin blisters (by 381%) when compared to blisters done preoperatively, proving the hypothesis. This trafficking was attenuated when using Aprotinin, demonstrating the efficacy of the anti-inflammatory intervention (Evans, Haskard et al. 2008). In another surgical study, the impact of conventional and miniaturised coronary artery bypass in inducing systemic inflammation were compared by analysing blister contents. Authors observed the same accumulation of leukocytes in response to surgery but were not able to find differences between the 2 surgery methods by looking at blister-infiltrating leukocyte numbers (Nguyen, Fiorentino et al. 2016).

Cantharidin application has mostly been used as a stand-alone methodology to look at inflammatory events but has also been used a means of obtaining cells to study the effect of parallel stimuli. The first publication of this method evaluates the transformation of early formed blisters, described as not very cellular, into a cellular exudate by the direct injection of killed staphylococci to the blister cavity (Boggs, Athens et al. 1964).

5.1.2 The suction blister model

Skin blisters can also be formed by application of controlled negative pressure to a delimited area of exposed skin, forcing separation of the epidermis and dermis. Like chemical blistering, this is a minimally invasive, painless, nonscarring, tissue sampling technique, allowing access to inflammatory cells and exudate.

The first reproducible method to form these epidermal blisters was described in 1968 (Kiistala 1968), and the same basic principles are maintained today. Whilst some attempts have been made to standardise the procedure (Davidsson, Bjorkman et al. 2013, Holm, Vukmanovic-Stejic et al. 2018) there is still a large variation in the technique used to produce such blisters. Differences in aperture sizes, pressure gradients and length of procedure are found between different groups, that may explain some of the variability in outcomes observed.

The separation between dermis and epidermis is accompanied by the filling of the blister cavity with an almost acellular interstitial fluid. Active migration to the site of action starts with the arrival of neutrophils, with monocytes appearing slightly later and increasing in frequency over time, with a lymphocyte population present only after 12-24h. During this period, the volume of fluid remains constant, but a build-up of cells and inflammatory mediators is observed (Davidsson, Bjorkman et al. 2013, Smith, Wilson et al. 2015). The simplicity of the method, allied to the possibilities of adaptations, made it useful for several areas of study.

The analysis of resident skin cells is possibly, one of the most obvious applications of this method. The biology of skin resident memory CD8+ T cells (Seidel, Vukmanovic-Stejic et al. 2018) or the activation and migration of epidermal Langerhans cells (Dearman, Bhushan et al. 2004) have been studied using cells obtained with this methodology.

In dermatology, the suction blister method has been used as a model for the creation of a standardised wound and to assess epidermal regeneration (Kottner, Hillmann et al. 2013, Rakita, Nikolic et al. 2020). This wound healing model has also been applied in the study of conditions where healing is impacted, such as diabetes and jaundice (Koivukangas, Annala et al. 1999, Koivukangas, Oikarinen et al. 2005).

The suction blister method was also applied in psychology/psychiatry to evaluate the influence of stress and anger in the inflammation and healing process, to understand whether stressful events have an impact in both the cytokine profile in the blister milieu (Glaser, Kiecolt-Glaser et al. 1999, Kiecolt-Glaser, Loving et al. 2005) and the healing process of the wound, as observed by differences in trans-epidermal water loss (Gouin, Kiecolt-Glaser et al. 2008). It is also used clinically to create patches of epidermis with functional and viable melanocytes to be grafted on top of vitiligo lesions to improve pigmentation (Khunger, Kathuria and Ramesh 2009, Rodrigues, Ezzedine et al. 2017).

5.1.3 Suction blisters as a means to obtain cells from a primary inflammatory challenge

As the negative pressure blistering process is thought to be relatively benign and elicit a standardised response within and between individuals, researchers have additionally used this technique to sample tissue previously exposed to independent inflammatory triggers.

Delayed-type hypersensitivity (DTH) reactions are an example of cell mediated immune response, used in clinic to measure the immune function of individuals, both as verification of efficacy of prior vaccination or to elucidate the effect of drugs or disease conditions in the normal immune response. A DTH response is characterised by the appearance of a local response, redness surrounding the site of antigen challenge, with a vigorous reaction reflecting an effective response. These reactions have been used in conjunction with the suction blister model to allow cells responding to the local antigen to be collected and studied. This DTH blister model, using tuberculin purified protein derivative (PPD) as the antigen, has been described in 1987, with description of cellular kinetics on both the non-specific naïve blister inflammation and the DTH response in blisters and how the 2 interact, which is an important point, sometimes missed when considering only one of the variables (Kenney, Rangdaeng and Scollard 1987).

The impact of age on the immune response has also been studied using the DTH-suction blister paradigm. Clinical response to challenge was lower in older individuals, with blister macrophages in older subjects still capable of producing TNF, but a

decreased function may be due to higher number of skin resident T regulatory cells (Treg) (Agius, Lacy et al. 2009). The capacity to mount a specific response has also been studied, with older subjects having the same rate of circulating antigen-specific T cells, but a much reduced frequency of these cells in blister cells extracted from a DTH site (Akbar, Reed et al. 2013). An interesting observation when studying the DTH response in blister samples was the fact that the challenge with PBS as a control, non-inflammatory stimuli, resulted in a major influx of leukocytes (non-lymphocytes), when compared with unchallenged skin, probably resulting from local trauma and which can be further studied as a possible future model (Belson, Schmidt et al. 2016).

By accessing the DTH response with suction blisters at different timepoints, different cell populations can be explored, including the accumulation and expansion of Treg cells in the response (Vukmanovic-Stejic, Agius et al. 2008) and antigen-specific T cell accumulation in blister fluid after varicella zoster challenge. These cells have a central memory phenotype and their accumulation is in parallel to the expansion and accumulation of cells with a regulatory phenotype to control inflammation during antigen specific response in tissue (Vukmanovic-Stejic, Sandhu et al. 2013).

Another model of acute inflammation used in parallel with suction blisters is the intradermal injection of UV-killed Escherichia coli, characterised characterized by an increased blood flow, accumulation of neutrophils and peak levels of pro-inflammatory cytokines at onset and a resolution phase accompanied by a reduction of blood flow, increase of monocyte/macrophage frequency, and later arrival of lymphocytes and reduction of pro-inflammatory cytokines (Motwani, Flint et al. 2016). This model allowed the study of the resolution phase of inflammation, gaining insight on the local immune alterations following resolution phase (Motwani, Newson et al. 2017) and the identification of pro-resolution mediators (Motwani, Colas et al. 2018).

UV radiation has also been used to trigger a cutaneous response to be assessed through blister contents. The radiation was observed to be sufficient to increase IL-6 secretion and to promote the release of ROS in blister fluid (Kuhn, Wolber et al. 2006). This dual UV/Blister method has also been proposed as a burn wound model (Svedman, Hammarlund et al. 1991) and has been used to test the effectiveness of sunscreen protection (Josse, Douki et al. 2018).

The de-roofing of the blister, by careful cutting of the epidermis once a blister is formed, has been used many times for creating a non-traumatic skin window that allows a more direct access to the lower layers of the skin to study cell trafficking and mediator secretion (Follin 1999). De-roofing of the blister has also been used to model open wounds, which because of the minimal invasiveness of the procedure, is well adapted for the study of wound healing (Kottner, Hillmann et al. 2013).

5.1.4 Blister models used in pharmaceutical development

Skin blisters have been employed in several stages of pharmaceutical development, starting from early pre-clinical development where they can be used in in-vivo animal models. Drug efficacy biomarkers can be tested in mouse ear inflammation models that use cantharidin to induce a similar response to the one observed in men (Gábor 2003, Dawson and Vogelsanger 2021), and can also be modulated by oral anti-inflammatories, as observed in the human model (Ivetic Tkalcevic, Hrvacic et al. 2012). A suction model in hairless mice has also been proposed for the study of anti-inflammatory and immunomodulatory drugs (Rommain, Brossard et al. 1991).

Probably the most used application of these models is for tissue pharmacokinetics. PK studies are usually limited to evaluation of absorption and elimination of a drug, but sufficient drug concentration at site of action, which is frequently outside the plasma compartment, is necessary for a desired drug effect, and does not always track the plasma levels (Gonzalez, Schmidt and Derendorf 2013).

Blister fluid is one of the different tissues where PK can be determined, alongside other fluids such as microdialysates and saliva (Brunner, Schmiedberger et al. 1998); this allows the demonstration of theoretical effect of antibiotics by comparing the concentration obtained to the minimum inhibitory concentration (MIC) for treatments for skin conditions (Klimowicz, Nowak and Bielecka-Grzela 1992, Fetterly, Ong et al. 2005, Sun, Ong et al. 2005, Nicolau, Sun et al. 2007). Studies have showed differences in fluid penetration, usually with a delayed peak concentration and a delayed half-life in blister when compared with plasma (Zimmerli, Sansano and Wittke 1996, Maglio, Teng et al. 2003).

Most PK studies to date have been performed in cantharidin blisters, possibly due to the larger volumes usually obtained in these blisters, or the ease of application, but suction blisters have also been used with similar time/concentration profiles for antibiotics (Bernard, Bensoussan et al. 1994), in first-in-human monoclonal antibody studies (Bouma, Zamuner et al. 2017, Reid, Zamuner et al. 2018), and a UV-burn/suction blister model was used to measure both tissue PK and PD in a NSAID drug (Oelkers, Neupert et al. 1997). A lower penetration and larger than desired variability of data was observed in this study and that may be due to the blisters in this study being raised immediately post drug administration, a different method than for the other studies discussed here. Tissue PK has also been determined from blisters from burns patients (Walstad, Aanderud and Thurmann-Nielsen 1988, Hua, Xu et al. 2015).

But perhaps, the major contribution of these methods may be for detecting pharmacodynamics, the effects the drugs do in relevant tissues. Non-targeted analysis of lipidomics (Nilsson, Sjobom et al. 2019), proteomics (Kool, Reubsaet et al. 2007) and metabolomics (Niedzwiecki, Samant et al. 2018) has been performed in suction blister fluid and differences and similarities between biomarkers in tissue and in plasma have been described. Knowledge of baseline values for many biomarkers may lead to their use in future studies.

The suction blister wound model (Kottner, Hillmann et al. 2013) was used in clinical trials to explore the modulation of epidermal regeneration, vascularisation and infection levels in wounds by zinc sulphate (Larsen, Ahlstrom et al. 2017), and wound healing by omega-3 supplementation (McDaniel, Belury et al. 2008).

Whilst the DTH model has been studied and developed within academia, it has been validated by the pharmaceutical industry as a model to test T-cell targeted drug interventions (Belson, Schmidt et al. 2016). The authors have used both the recall antigen PPD and the neo-antigen KLH, showing very similar clinical and cellular responses to both, and for the first time using blister and biopsies in parallel in these 2 challenges. A robust T-cell response was obtained at 48h post challenge, with activated T-cells being the major component in the blister response, markedly different to that observed in unchallenged, or PBS challenged skin, or described in the literature for normal blister response.

As discussed, the search for efficacy biomarkers is being pushed to the earliest stages of the clinical research. The suction blister model was used to demonstrate the modulation of chemotaxis due to neutralisation of a chemokine ligand (CCL20) by monoclonal antibody therapy in a first-in-human study (Bouma, Zamuner et al. 2017). This study was able to show a relationship between tissue PK, target engagement and the desired biological response – the inhibition of recruitment of CCR6+ pro-inflammatory cells to the site of action. On the other hand, the inhibition of the migration of these cells was limited and achieved a plateau at the higher doses of the drug, showing the redundancy in chemokine pathways. This study was successful in demonstrating the mechanism of the drug but critically showed that the limited effect observed in tissue is not sufficient to stop activated cells migrating to site of action and thus provided invaluable data on the possible clinical utility of the compound.

5.1.5 Current understanding and existing gaps on blistering models

The blister inflammatory response is characterised by both humoral and cellular elements. Both outputs exhibit significant inter-subject variability. In the original paper by Day *et al.* (Day, Harbord et al. 2001), the blister volume collected from 12 volunteers varied from 40 - 800 µL, leukocyte numbers per blister ranged between 20,000 and 3,000,000 and pro-inflammatory cytokines also had several fold difference between volunteers. Negative pressure blisters also demonstrate similar variation on cellular and mediator data (Davidsson, Bjorkman et al. 2013). To assess efficacy of an intervention or treatment, the signal/noise ratio needs to allow the effect of the procedure to be higher than the variation observed in the method. High inter-subject variability will impact on ability in pharmaceutical development to observe significant changes due to treatment, necessitating a larger sample size to achieve adequate power. Intra-subject variability is crucial for within-subject designs and estimates of short- or medium-term repeatability of challenge models are not currently available in the literature to permit estimation of subject specific change from baseline over time.

The outcome of both interventions is a macroscopically similar inflammatory site which has been successfully used to elucidate biological and pharmacological questions. Although as discussed in Chapter 4, the soluble mediators present in each method and the cellular kinetics are considerably different. Elucidating the commonalities and

differences between the inflammatory pathways and key immune cells triggered by each of the methodologies may allow for a more rational selection of the model to study unique biological pathways or specific activated immune cells.

In-depth understanding of both the intrinsic variability, but also the specific biological effects of each methodology, will thus allow their use for appropriate pre-clinical applications and help establish the early proof-of-mechanism and pharmacology required to improve translational science and drug discovery.

5.1.6 Hypothesis and aims for this chapter

Fundamental biological differences exist between the cantharidin and negative pressure blistering techniques that are associated with distinct inflammatory pathways. Understanding these differences will enable targeted application to specific scientific questions and drug development programmes

To address this hypothesis, I will aim to:

1. Identify optimisation opportunities in the procedures, or in the selection of blistering sites, to reduce variability and improve utility.
2. Analyse biological outputs of both methodologies and identify differences that could impact the selection of the model.
3. Assess intra- and inter- subject variability of the response elicited by the different challenges.
4. Obtain a deeper understanding of the biological processes ongoing during the blister development process.
5. Use the models to assess PK and PD effects of systemic and local anti-inflammatory therapies.

5.2 Additional methods:

5.2.1 Study design

To directly compare the two methods of blister induction, suction and chemical, both methods were applied concurrently to volunteers in a study specifically designed to compare biological readouts from, and variability in, responses.

The intra-individual variability of replicate blisters formed both concurrently and over time was investigated over a three-week period: blisters being formed at 1- and 2-week intervals following first induction.

Initially, eight blister locations were selected on the ventral aspect of each subjects' forearms. The sites were mapped on pieces of parafilm® to act as a template for location of sites in subsequent visits. Two sites were selected per arm for blistering during visit 1, with replicate blisters being generated in different arms via each method. One blister from each method was raised in the same arm in visits 2 and 3. A minimum distance of 5 cm was maintained between each inflammatory challenge.

At all time-points, the cellular profile of the blisters was analysed concurrently with peripheral blood samples taken at same time points using a comprehensive pan-leukocyte flow cytometry panel. Soluble mediators were analysed from the blister supernatants and plasma collected. A total of 40 cantharidin and 40 suction blisters were collected, along with 60 blood samples.

After the last visit, volunteers were asked to complete a questionnaire on their experience that addressed recruitment (anxiety towards blister formation) through to the procedures (pain, discomfort, convenience) and willingness to participate in further studies.

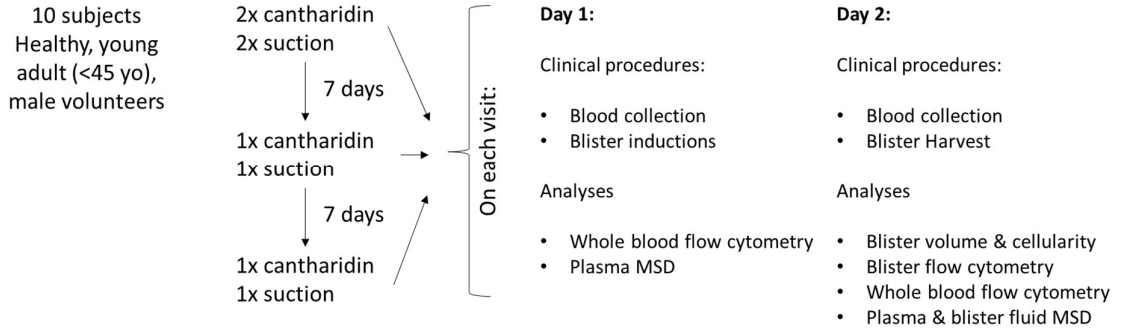


Figure 27: Study schematics. After obtaining informed consent, subjects visited the unit to have a blood sample taken before blister inductions. 24 hours later, blood samples were obtained prior to harvesting the blisters. Three visits were carried out with replicate blisters raised on the first visit and single blisters on days 7 and 14.

5.2.2 Questionnaire for volunteer experience

A questionnaire to obtain volunteer perception of the blistering methods was designed to obtain feedback on pre-participation expectations, procedural pain, discomfort and inconvenience and potential participation in future blistering studies.

Feedback was requested from the volunteer after their last visit. Study data were collected and managed using REDCap electronic data capture tools. REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies. A copy of the questionnaire can be found in the clinical protocol documentation (Appendix 1).

5.3 Results

5.3.1 Larger and more cellular blisters are formed using cantharidin

The main blister outputs of volume and cellularity are displayed in Figure 28. Median volume observed for cantharidin blisters was 159.2 µL (95% CI: 127.3–195.2 µL) and 98.44 µL (66.05–116.2 µL) for suction blisters.

The number of cells migrating into the suction blisters was observed to be significantly smaller compared with the cantharidin blisters both expressed as cells per mL of blister fluid and as total cells per blister (median (95% CI): $2.11x10^5$ ($1.55x10^5$–$2.40x10^5$) cells /mL vs $1.50x10^6$ ($9.15x10^5$–$1.85x10^6$, $p<0.0001$) cells/mL; $1.56x10^4$ ($1.13x10^4$–$2.75x10^4$) cells/blister vs $1.79x10^5$ ($1.41x10^5$–$2.98x10^5$, $p<0.0001$) cells/blister).

There was a large inter-individual spread in volume and cellularity data (Figure 28, panels A and C), but data appears to cluster per subject, both for volume and cellularity (Figure 28, panels B and D).

A clear correlation between volume and cellularity can be seen for suction blisters ($p=0.0001$, $r=0.5654$, Panel E), where larger blisters appear to be associated with a bigger number of extravasated cells, but the same is not observed for the cantharidin method.

Blister data obtained using the cantharidin method closely mirrored results from the GSK validation cohorts, both in absolute values and variability, further emphasizing the robustness and reproducibility of this application method (see Figure 15).

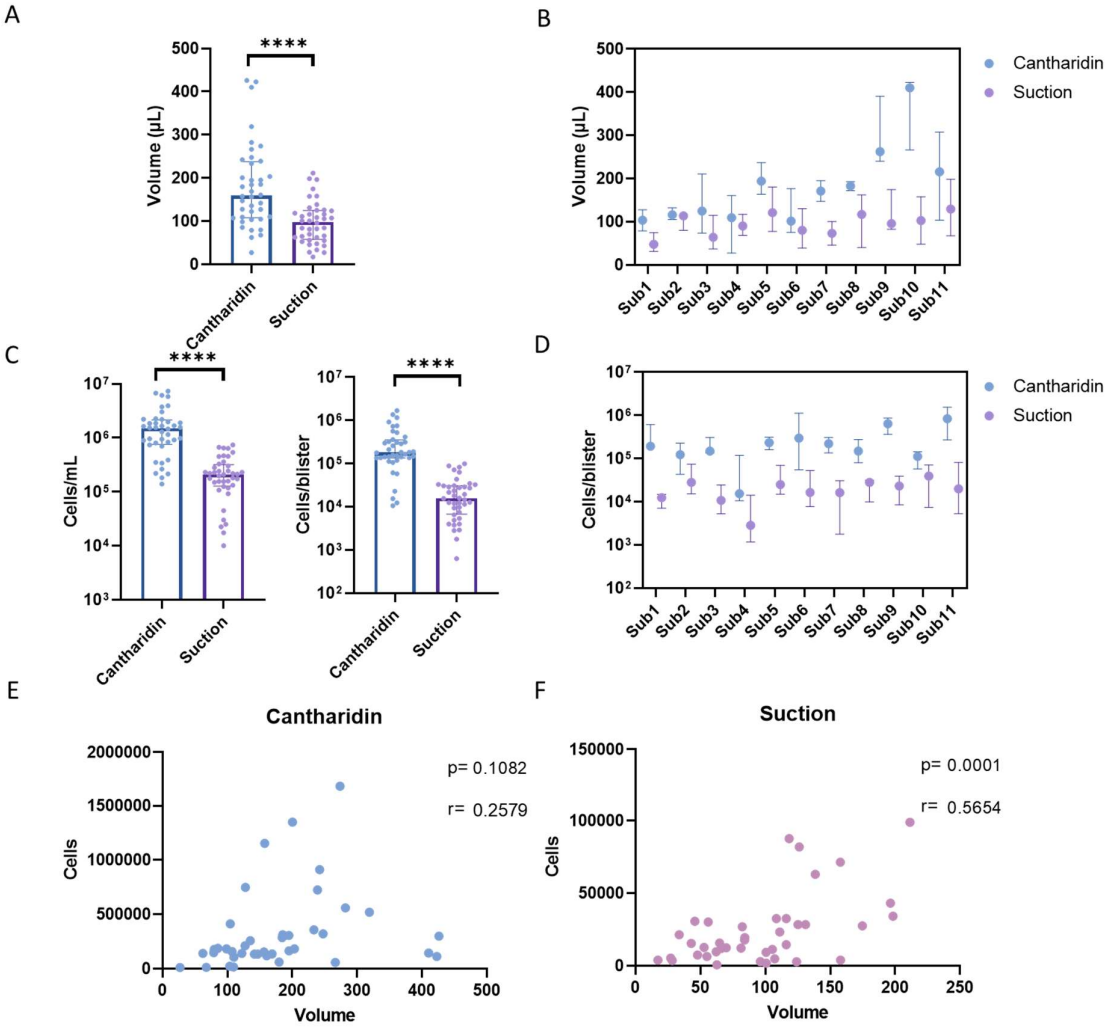


Figure 28: Differences in volume and cellularity outputs between methods. Median blister volume per method (A; n=40 blisters per method, derived from 11 subjects, median with interquartile range) and separated per individual (B; n=4 blisters of each type per participant); Overall cellularity expressed as cells per mL and cells per blister for both methods (C; n=40 blisters per method) and separated per individual (D; as cells/blister, n=4 blisters of each type per participant,); Correlation between blister volume and total number of infiltrating cells for cantharidin (E) and suction (F) blisters

5.3.2 Blistering methodology impacts the cellular make-up

Three sample types (peripheral blood, cantharidin and negative pressure induced blister exudates) were collected from each subject, at each timepoint, allowing direct comparison of circulating leukocyte populations to those in blister exudates. In total, 60 blood samples and 80 blisters (40 cantharidin and 40 suction) were obtained from the 11 enrolled participants.

Figure 29 summarises observed leukocyte subset distributions. Suction blisters displayed a lower percentage of neutrophils (median: 23.6%, 95% CI (21.2-33.1%)) compared with either peripheral blood (59.4% (56.2-62.0%)) or cantharidin blister (65.8% (48.8-66.6%)) samples ($p<0.0001$ for both) but contained a higher proportion of monocytes (29.1% (25.0-35.9%)) compared with cantharidin blisters (17.5% (15.6-25.3%), $p<0.01$). Both blister models presented a significantly higher percentage of monocytes compared with peripheral blood samples (5.8% (5.6-6.3%), $p<0.0001$ for both). Dendritic cells were also elevated in both types of blister samples (cantharidin 1.55% (1.35-2.07%), suction 1.92% (1.57-2.70%)) when compared with the levels in peripheral blood samples (0.39% (0.34-0.42%), $p<0.0001$).

The overall proportion of lymphocytes was greatly reduced in the blister exudates (cantharidin 8.54% (9.02-17.3%), suction: 13.6% (12.2-20.7%)) compared with the peripheral blood samples (30.15% (29.1-34.8%), $p<0.0001$ for both). The lymphocyte population was further sub-divided into 3 major groups: T cells (CD3+), B cells (CD19+) and NK cells (CD16+, used in this panel as a surrogate for CD56 as most NK cells express this marker (Cooper, Fehniger and Caligiuri 2001)). The vast majority of lymphocytes seen in both blister samples were T cells (75.4% (63.2-74.8%) cantharidin, 80.2% (69.9-81.2%) suction), with B cells absent in most blisters (median of 0% on both blisters) and the percentage of NK cells (cantharidin: 1.85% (2.12-4.84%), suction: 2.19% (2.21-4.72%)) significantly reduced compared to blood (11.9% (11.02-14.16%), $p<0.0001$ for both).

For dendritic cells, selected by the presence of HLA-DR and absence of most other lineage markers (CD14, CD3, CD16 and CD19), observed subpopulations differed between the two types of blister induction. Suction blister samples exhibited a similar percentage of the plasmacytoid (pDC, CD123+/CD11c-) and myeloid (mDC, CD11c+/CD123-) dendritic cells to peripheral blood samples, with a ratio of close to 1 (suction: 0.87, blood: 0.70), whereas for cantharidin blisters, the ratio was skewed towards having a higher prevalence of pDC (4.39).

5.3.3 Response to challenges is consistent within individuals, but demonstrate marked heterogeneity between individuals

Not all subjects responded similarly to the equivalent challenges. Figure 29 Panel D (cantharidin) and Panel E (suction) illustrate the main leukocyte constituents of blisters generated on 4 subjects over time, which allow the visualisation of differences between leukocyte composition per subject and the consistency of the outputs at weekly intervals. These subjects were selected as they consistently demonstrate different responses to the blistering agents. For cantharidin-induced blisters, most subjects responded to the blister induction with the extravasation of neutrophils, but others, as demonstrated by Sub A, showed a consistent response characterised by a high monocyte percentage. This cellular response was not maintained between the two different blister modalities, with Sub D showing a consistent neutrophilic response to cantharidin but monocytic to the suction challenge.

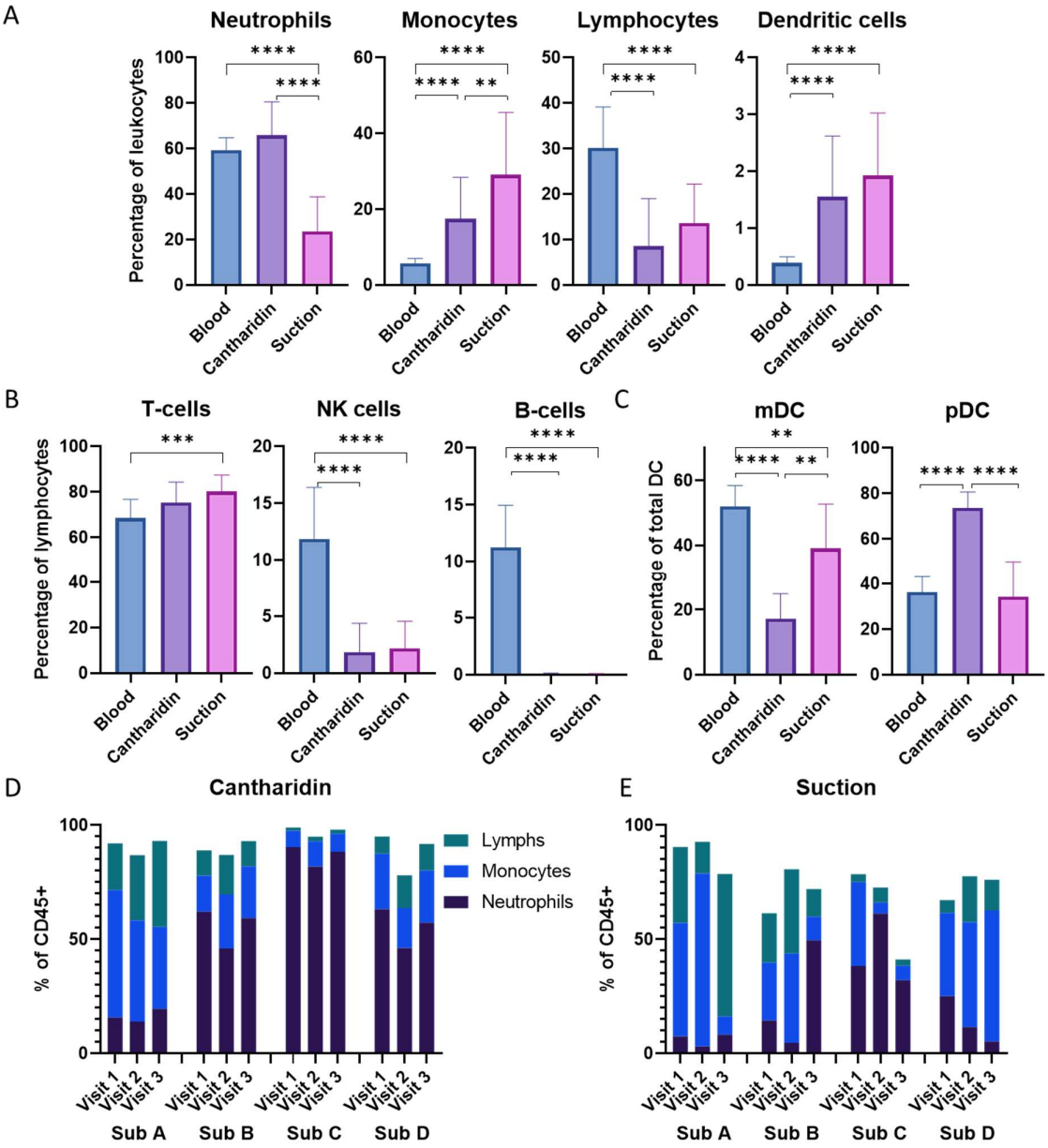


Figure 29: Differences in the median leukocyte composition of the sample types tested. [A]: Main leukocyte populations as percentage of total CD45+ cells; [B]: T, B and NK cells as proportion of lymphocytes; [C]: myeloid and plasmacytoid dendritic cells as percentage of total dendritic cells. Median (with interquartile range) data obtained from 40 suction and cantharidin blisters and 60 whole blood samples from 11 different subjects collected at different visits. Stability of leukocyte response in [D]: cantharidin and [E]: suction blisters in 4 selected subjects

5.3.4 Variance in leukocyte cell surface inflammation marker expression suggests alternative activation dependent on blister induction method

The data presented to date suggests that cantharidin induces a more significant inflammatory response, with a more cellular exudate, greater blister volume and a higher frequency and proportion of neutrophils. Cellular activation data or polarisation status was examined by analysing specific cell surface receptor expression in infiltrating cells, by determining mean fluorescence intensity by flow cytometry, to further explore these findings.

Four leukocyte subsets were tested for activation status in blood and the blister exudates formed by negative pressure and cantharidin: monocytes, lymphocytes, neutrophils and dendritic cells.

Monocytes (defined based on light scatter properties and HLA-DR+ as per Figure 5) can be divided into 3 distinct phenotypes corresponding to different stages of activation. Circulating monocytes were found to be mainly CD14+CD16- (classical, median: 83.5%, 95% CI: 81.5 – 84.8%), whilst blister samples demonstrated a lower percentage of the classical phenotype (Suction: 8.45%, 4.9 - 11.5%; cantharidin: 39.5%, 31.4 - 45.5%). Instead, the intermediate (CD14+, CD16+) population was much highly represented compared to blood and in suction blisters, this phenotype is the dominant (Suction: 77.3%, 64.2 – 81.9%; cantharidin: 39.2%, 32.03 – 50.7%). Very few terminally differentiated CD14-CD16+ cells were observed in the blister samples (Suction: 0.26%, 0.00 - 0.35%; cantharidin: 0.21%, 0.09 - 0.29%).

Significant differences were observed between the two blister induction methods in four separate markers of monocyte activation. Monocytes in the suction blister exudate displayed increased expression of CD163 (suction: median MFI 8071 (95% CI: 6430-10048, cantharidin: 3454 (2997-3838); p=0.001), Siglec-1 (suction: 3206 (1951-9816), cantharidin: 2141 (465.8-8462); p=0.001) and CD64 (suction: 11157 (8096-12684), cantharidin: 8697 (6890-9051); p=0.0049), but decreased expression of HLA-DR (suction: 15599 (11829-22828), cantharidin: 22087 (17751-32818); p=0.001) when compared to contemporaneous cantharidin blisters. Signal stability over the 3 visits (14 days) was maintained for most markers, with the exception of Siglec-1, where clear spikes were observed for two subjects at specific timepoints which may indicate sub-clinical infection episodes and an alternate exogenous influence.

In contrast to monocytes, both lymphocytes (via PD-1 expression) and neutrophils were similar in both blister types with only a minor increase in expression of CD64 on neutrophils being observed in suction blisters (suction: 801.3 (705.0-965.9), cantharidin: 761.7 (686.7-833.0); $p<0.01$) for 40 blister pairs, p=not significant for 11 subject pairs).

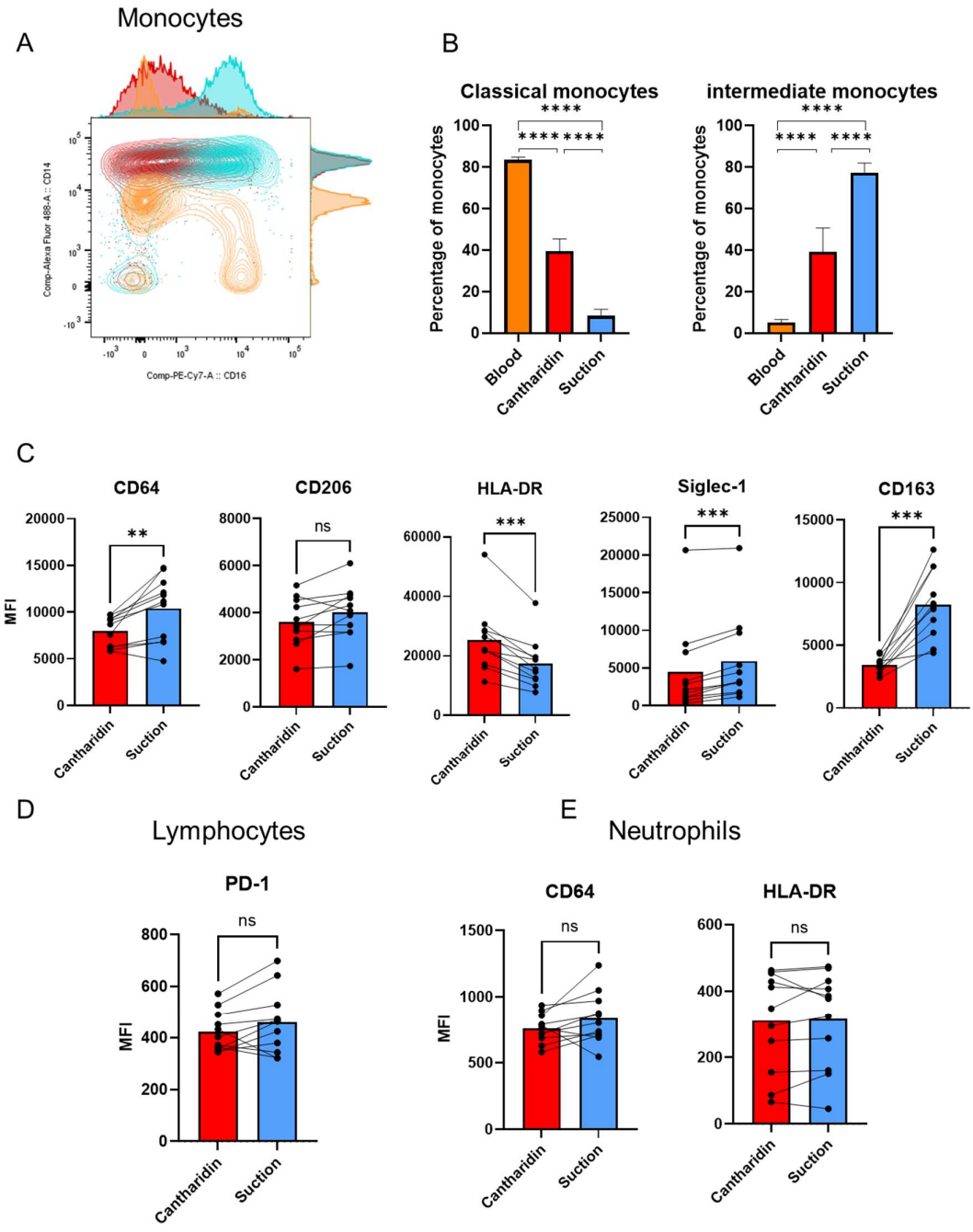


Figure 30: Activation/polarisation leukocyte markers suggest differences between inflammatory status of the two models. (A) Illustrative example for differences in monocyte maturation profile for each of the samples analysed on the same subject – Whole blood (orange), cantharidin (red) and suction (blue); (B) Classical and Intermediate monocyte phenotype percentages of total monocytes for the different samples (data shows median with interquartile ranges); (C) Monocyte, (D) Lymphocyte and (E) Neutrophil activation/polarisation markers presented as mean fluorescence intensity (bars n=40 blisters) and paired data for each subject (n=11 pairs), significance reported for 11 subject pairs

5.3.5 Cantharidin blisters show higher levels of pro-inflammatory cytokines and chemokines

Compared with plasma, cytokine concentration in blister fluid was significantly increased for all cytokines analysed (Summary of data is presented in Figure 31). When comparing between the different blister modalities, IFN-γ, IL-12p70, IL-1β, IL-4, IL-6 and TNF are all significantly increased in cantharidin blisters (all $p<0.0001$), IL-13 is marginally increased ($p<0.05$), IL-10 appears unchanged and IL-2 is significantly increased in suction blisters ($p<0.0001$).

Eotaxin is present at a higher concentration in plasma compared to both blister types ($p<0.0001$ for both) and Eotaxin-3 and MCP-4 have similar values between blister and plasma. For all other chemokines analysed, blister levels are increased in comparison with plasma. IL-8, MCP-1, MIP-1α, MIP-1β (all $p<0.0001$) and TARC ($p=0.0001$) are all significantly increased in cantharidin blisters when compared to suction. Eotaxin, Eotaxin-3, IP-10, MCP-4 and MDC all have similar levels between the two blister modalities.

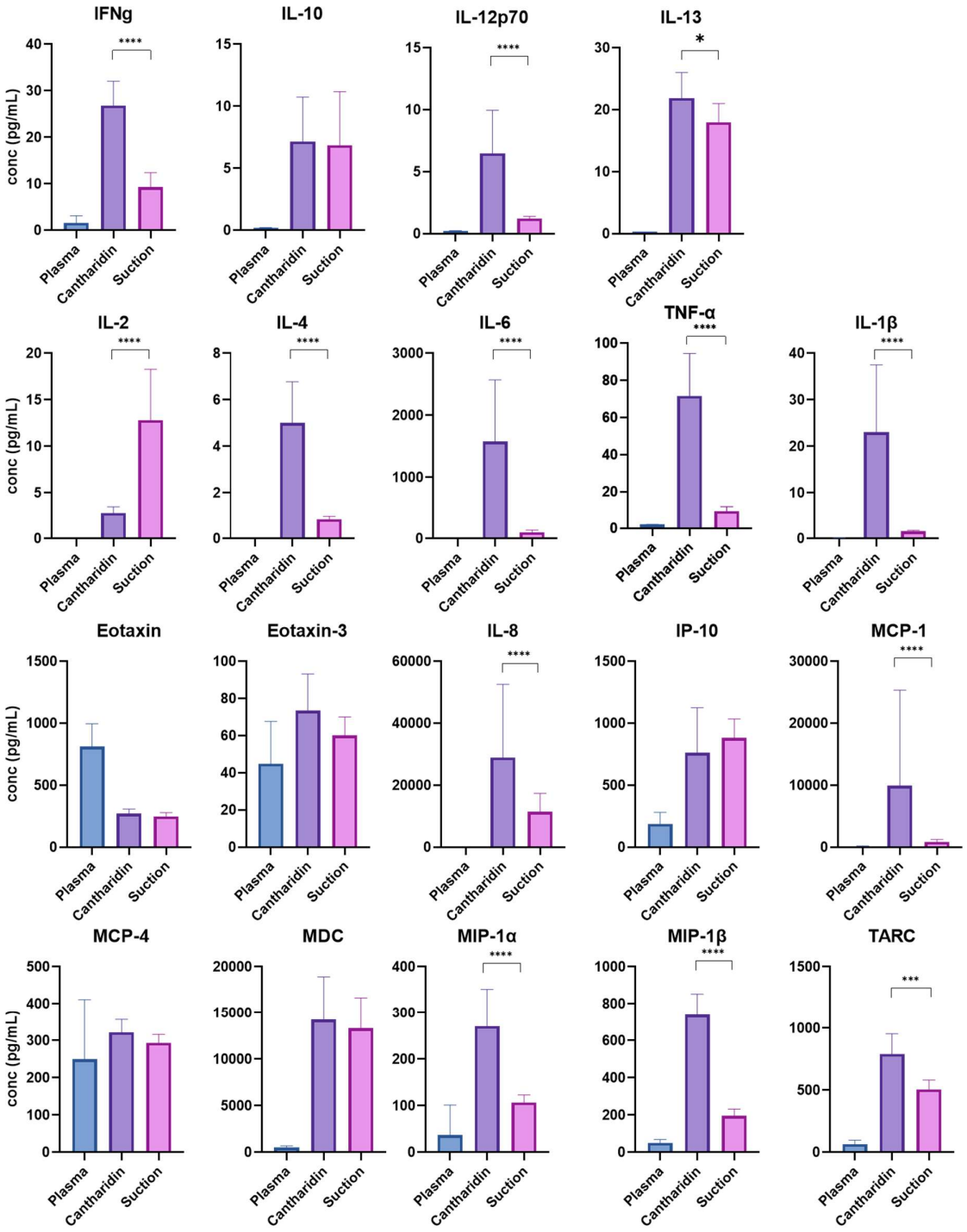


Figure 31: Cytokine and chemokine profiles of plasma, cantharidin and suction blisters. Each panel presents the median value with 95% CI for each cytokine and chemokine analysed (n=60 plasma samples and n=40 for each blister modality). Significance of difference between cantharidin and suction blisters is calculated using a Mann Whitney test

5.3.6 Consistency of the response is highest for within subject comparisons in cellular outputs

As per the experimental design, variability of the blister response can be calculated for simultaneous blisters collected on the same day (intra-day CV), for blisters generated using the same methodology across 3 visits on sequential weeks (intra-individual weekly CV) and between participants, using the same approach (population estimates; inter-subject CV). The data in Figure 32 represent the average CV observed for these measurements for each of the different outputs obtained.

In general terms, for the majority of blister parameters and cellular outputs, most variability estimates are higher for suction blisters when compared with cantharidin blisters. In contrast, for soluble mediators the intra-subject and weekly estimates appear to be higher in the cantharidin model.

Activation and polarisation markers appear to be the most consistent outputs, whereas soluble mediators appear to be the most variable.

		Intra-day CV		Weekly CV		Between subject CV	
		Cantharidin	Suction	Cantharidin	Suction	Cantharidin	Suction
Blister parameters	Volume	19.6%	40.8%	33.8%	48.1%	46.6%	25.8%
	Cellularity	32.4%	52.9%	57.7%	59.9%	71.8%	33.3%
	Cells/blister	37.5%	67.0%	74.0%	81.9%	81.9%	48.3%
% of CD45+	Neutrophils	14.5%	47.2%	33.0%	55.4%	40.1%	47.6%
	monocytes	44.1%	29.2%	42.3%	55.7%	56.8%	33.6%
	DC	58.1%	68.6%	51.6%	57.9%	50.7%	47.6%
	Lymphs	31.1%	63.4%	40.2%	60.6%	89.8%	54.4%
% of Monocytes	Classical	23.1%	60.5%	45.8%	64.8%	31.0%	58.5%
	Intermediate	13.2%	14.3%	37.7%	16.1%	33.0%	15.4%
Monocyte activation/ polarisation	CD64	9.4%	12.1%	21.3%	20.8%	20.2%	32.9%
	CD206	14.3%	18.6%	27.3%	29.8%	28.6%	28.3%
	HLA-DR	10.4%	20.1%	21.3%	20.3%	44.4%	47.2%
	CD163	12.7%	34.1%	28.9%	45.0%	18.3%	32.7%
Other cells activation	CD64 Neutrophils	8.0%	21.5%	12.3%	12.8%	14.3%	23.2%
	PD-1 Lymphocytes	5.4%	30.4%	15.7%	17.4%	18.6%	27.1%
	CD163 mDC	7.4%	12.7%	18.9%	28.9%	15.3%	19.5%
	CD163 pDC	6.8%	11.5%	16.8%	13.7%	16.4%	22.0%
Cytokines	IFN-γ	55.8%	57.3%	61.3%	44.5%	105.5%	71.7%
	IL-10	18.9%	37.6%	26.1%	40.9%	61.5%	72.0%
	IL-1β	43.1%	32.8%	69.8%	48.2%	90.5%	91.1%
	IL-2	33.3%	42.7%	43.6%	45.0%	119.1%	59.1%
	IL-6	46.7%	73.4%	59.6%	66.8%	72.4%	153.8%
	TNF	25.2%	30.8%	42.0%	21.2%	49.7%	55.9%

Figure 32: Differences in variability between same day blisters and blisters raised over 3 weekly sessions. Areas highlighted to represent high (red) and low (green) variability.

5.3.7 Comparison between contemporaneous blister and whole blood samples

If blister exudates represented passive extravasation from blood to tissue, we would expect a consistency in the cellular composition between the two sample types. Further, activation status, as determined by cell surface marker expression, would be expected to be consistent. A change in magnitude would be expected if extravasation changed the activation profile and correlation between blood and blister expected if basal levels in circulation influence the activation state observed in tissue. Figure 33A shows the lack of correlation between the leukocyte composition of blood and blister exudates for the main subsets.

In addition, heterogeneity in the relationship between blood and blister monocyte markers is demonstrated (Figure 33B). In some cases, statistical correlation between expression of markers in blood and blister cells was identified, as exemplified by the

monocyte markers CD206 ($p<0.01$ for both suction $r=0.793$ and cantharidin $r=0.844$) and HLA-DR (suction: $p<0.001$, $r=0.850$; Cantharidin: $p<0.05$, $r=0.707$). In monocytes, Siglec-1 also demonstrated correlation, but this was not observed for either CD64 or CD163.

Similar heterogeneity was also seen in other leukocyte subsets. Whilst lymphocyte (PD-1) and neutrophil (CD64) activation did not correlate between blood and blister exudate, in the dendritic cell population relationships between blood and blister-derived mDC CD206 ($p<0.01$ for NP and $p<0.05$ for cantharidin) and Siglec-1 ($p<0.05$ for both NP and cantharidin) were found. Markers in the pDC population again displayed similarity whether derived from blood or blister.

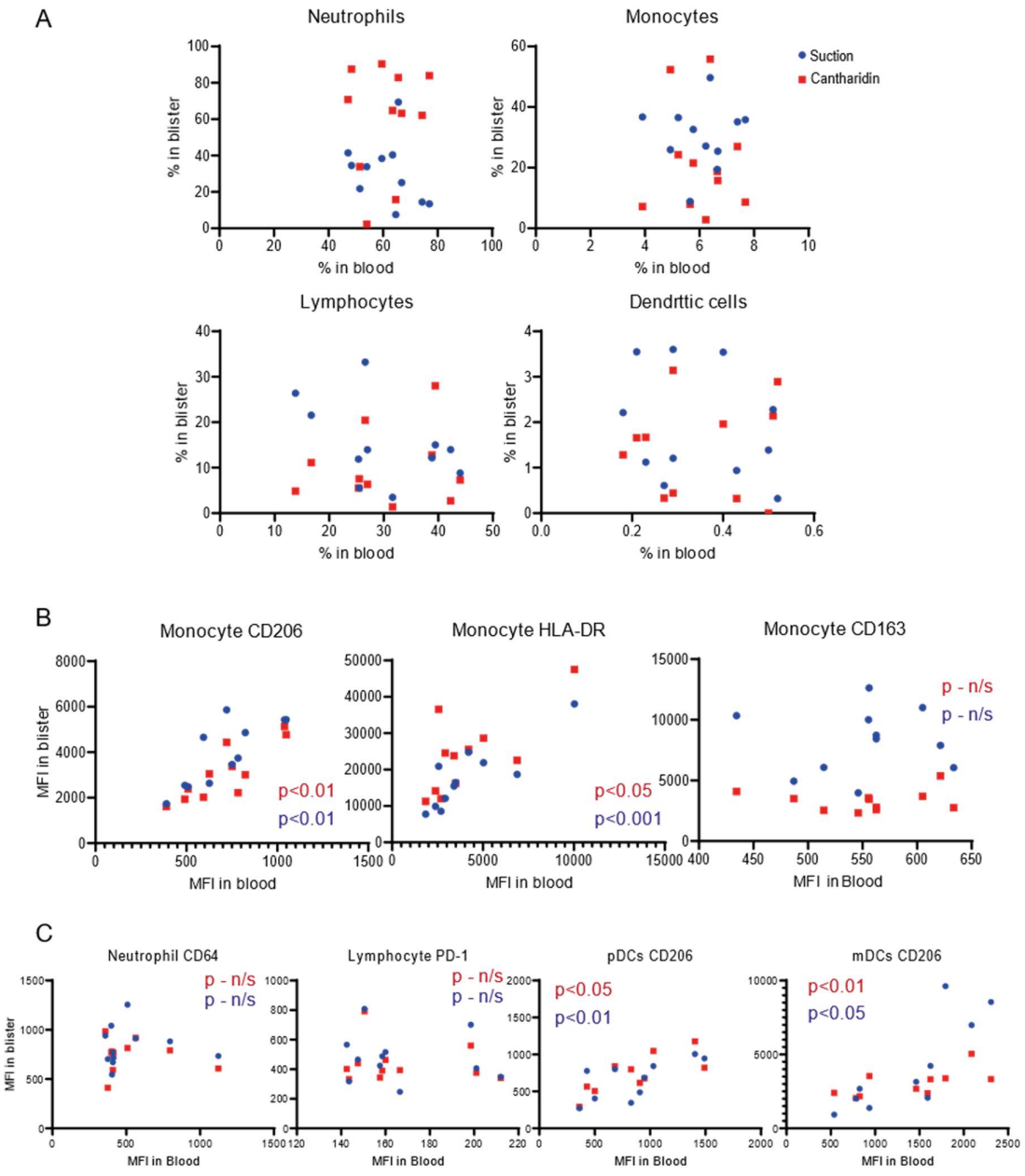


Figure 33: Association in the activation status of blood and blister sample populations. Correlation between blood and blister contents for [A] percentage of main leukocyte populations, [B] selected activation markers in monocytes and [C] selected activation markers for other cell subsets. Data presented correspond to visit 1 where replicate blister outputs were averaged and compared with contemporaneous blood sample.

5.3.8 Cellular outputs correlate with levels of mediators in blister fluid

Cytokines and chemokines mediate the inflammatory response and are responsible for the recruitment of leukocytes to the tissues. Significant correlations between concentration of these cellular mediators and total leukocyte numbers were observed.

The strongest correlations to total cell numbers were MIP-1α, IL-10 and TNF for cantharidin blisters and TNF, MCP-1 and IL-6 for suction.

Specific cell subsets were also analysed, and all mediators listed above also correlate significantly with neutrophil total numbers in both blister methodologies, with mediators being specific to either method (IL-2, IL-12p70 and IL-8 correlate with neutrophils for cantharidin model whereas MDC correlates with neutrophil numbers for suction).

Monocytes and lymphocytes correlate positively with TARC levels and have an inverse correlation with IL-13, IL-12p70 and IL-6 in cantharidin blisters. For suction blisters, monocytes and lymphocytes also correlate with TNF.

Volume also correlates with some of these soluble mediators. In cantharidin blisters, volume correlates with MCP-1, IL-2, MIP-1α, IL-1 and TNF whereas in suction blisters, volume correlates with IL-6, MCP-1, IL-10, IL-13 and TNF.

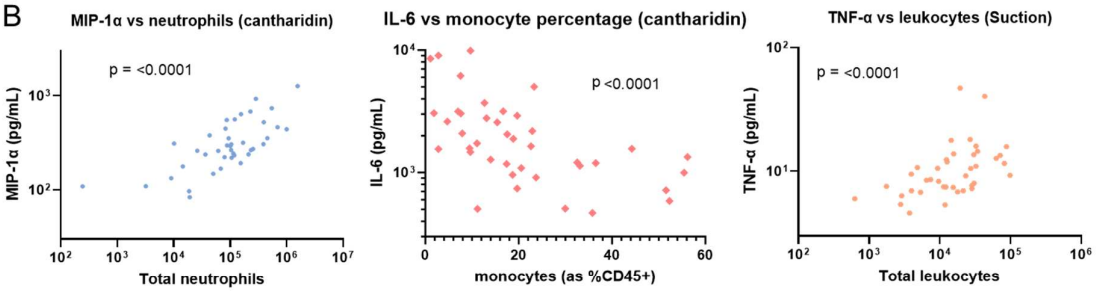
Cytokine and chemokine levels also appear to correlate with some activation/polarisation markers in monocytes and neutrophils, but without common links between the two models as could be expected.

A summary of the correlations observed between soluble mediators studied and cellular/soluble outputs with a selection of the graphical outputs is displayed in Figure 34.

A

	Level of correlation	Cantharidin		Suction	
		Absolute number	% of total CD45+	Absolute number	% of total CD45+
Total cells	****			TNF	
	***	MIP-1a			
	**	IL-10, TNF		MCP-1, IL-6	
Neutrophils	****	MCP-1, MIP-1a, IL-10	IL-10, IL-12p70, IL-4	MCP-1, TNF	
	***	IL-2, TNF	MCP-1, MIP-1a, IL-6	MDC, IL-10	
	**	MIP-1b, IL-8	MIP-1b, IFN-γ, IL-1b, IL-2, TNF	MIP-1a, IL-6	MCP-1, MDC, MIP-1a, IL-10
Monocytes	****		IL-12p70, IL-4, IL-6		
	***			TNF	
	**	TARC, IL-13	IFN-γ, IL-10, IL-13, IL-1b	IL-6	
Lymphocytes	****				
	***		MCP-1, MIP-1a, IL-10, IL-12p70, IL-4		
	**	TARC, IL-12p70	IL-2, IL-6		

B



C

	Level of correlation	Cantharidin	Suction
Volume	****		IL-6
	***	MCP-1, IL-2	MCP-1, IL-10
	**	MIP-1a, IL-10, TNF	IL-13, TNF
CD163 (monos)	****		
	***	MCP-1	
	**	IL-8, TNF	
CD16+ Monos	****		
	***		
	**	IL-10	
CD206 (monos)	****		
	***		
	**	MDC	IP-10
HLA-DR	****		
	***		IL-10, IL-13
	**	Eotaxin-3, MIP-1b, IL-10	
CD64 Neutrophils	****		
	***		
	**	IP-10	

D

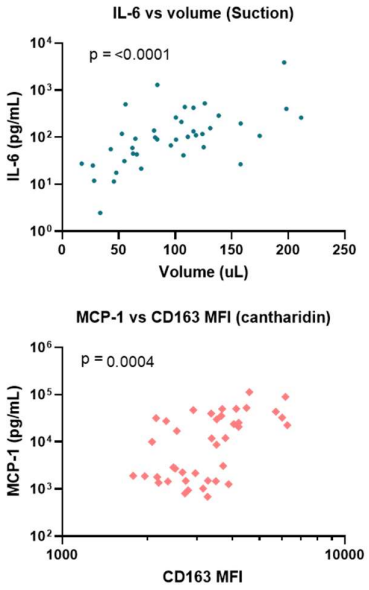


Figure 34: Correlations between cellular outputs and concentration of soluble mediators for both blister methodologies. Summaries for correlations found between mediator levels and leukocyte levels [A] or other blister outputs [C]. Positive correlations displayed in black whereas negative in red. Correlation levels analysed (** p<0.01, *** p<0.001, **** p<0.0001); [B, D] examples of significant correlations

5.3.9 Volunteer perception of blistering techniques

Public and patient perception of blistering techniques is an important consideration when seeking to employ these methods in clinical or basic research. The nine subjects

that completed both negative pressure and cantharidin blisters participated in a survey-based questionnaire to inform future study design and applications for ethical approval.

In terms of apprehension prior to undertaking blistering, subjects reported median values of 4/100 to the suction and 20/100 to the cantharidin methods. When asked to describe their greatest concerns about these techniques, responses included potential for scarring and time commitment for the suction technique, compared with pain or adverse reactions to the cantharidin along with risk of scarring.

After undergoing blistering, induction pain was evaluated on a scale of 0 - no pain/discomfort to 100 - worst pain/discomfort imaginable. Volunteers reported median values below 10/100 for all stages of the procedures, with the highest median value of 7/100 arising during harvesting of the cantharidin blister. For overall pain/discomfort levels, whilst both methods were very well tolerated, volunteers reported slightly lower levels of discomfort on the suction blister methodology (2/100) compared to cantharidin (5/100). The main issues described were discomfort during the lengthy suction procedure and slight pain and tenderness on collection of the cantharidin blisters exudate.

The largest difference between the two methods was the subjects' perception of inconvenience, rated 0 - no disruption to 100 - major disruption to daily activities, with reported median values of 30 for suction, compared to 5 for the cantharidin blisters. The time for blister formation was the main negative aspect of the suction blister protocol, identified by 8 out of 9 participants.

Subjects demonstrated willingness to partake in a future studies involving blisters, with median responses of 75 to undertake a study with suction blisters and 90 to repeat the cantharidin methodology on a scale of 0 – never again to 100 – without question. This was also reflected on the binary question for method preference, where all subjects indicated that they would prefer to enrol in a study employing cantharidin over suction blistering.

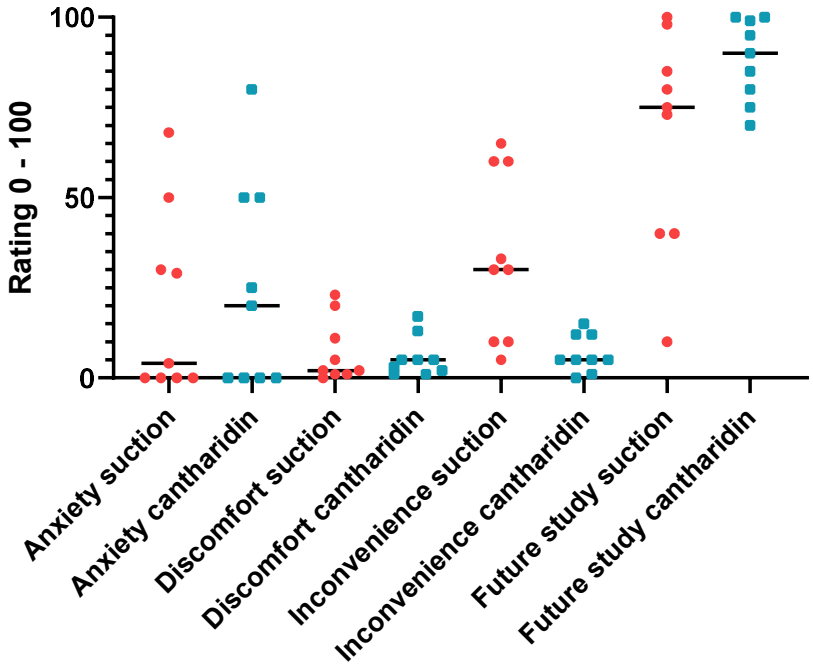


Figure 35: Responses to volunteer feedback questionnaire. Volunteers were asked to rate (1-100) the levels of anxiety prior to participation, discomfort and inconvenience during the procedures and willingness to participate in future studies for both blistering methodologies

5.4 Discussion

5.4.1 Limited available data hinders use of blister models in research

Blister exudates are used in academia and early phase clinical research, classically being employed as standardised approaches to eliciting and measuring the inflammatory response. Despite their prevalence, the methodology used to create the blister varies between published studies. Currently there is no clear scientific rationale for the choice of method used, with the main decision factors likely relating to availability of equipment, reagents or local expertise. This overlooks the potential impact of the blister induction process on the phenotype of the cellular and humoral composition of the blister, which could have major implications on the comparability of data between studies. Moreover, if distinct cell and humoral profiles exist between the two methods, this could provide an opportunity for selection of the most appropriate blister model for a particular application.

In addition to understanding the differences in the biological response to the two blister induction methods, there is a need to address uncertainty regarding the consistency of the formed blisters both within and between subjects. As described earlier, variability is a feature of blister models but has neither been formally quantified, nor built into clinical study design to date. A number of factors might be involved, some of which may be controlled, but if not, accounted or adjusted for. The importance of understanding the level of variance between blisters should not be underestimated when ensuring there is provision of adequate sample sizes and sufficient replicates to robustly detect observable effects.

5.4.2 Rationale for blister model selection and use in clinical research

This body of work provides researchers with data that allows for direct comparison of expected data and variability estimates between the two blister methodologies for traditional cellular and humoral outputs, allowing selection of methods based on expected biological data, rather than practical reasons.

There is a published abstract where the authors used suction and cantharidin blisters in the same participants, but the data is limited to mean peak neutrophil number and percentage for each of the methods and a description of increased inflammatory profile from a selection of mediators (VEGF-A, CXCL1, 2, and 5, IL-1a and b, TNF, IL-36, and IL-8) (Connell, Greenhalgh et al. 2020). To our knowledge, the work presented here is the first detailed report of side-by-side cellular and humoral data obtained from these methods.

By running these two models side-by-side, it is possible to compare aspects of their biology and address some of the gaps identified in the literature. Considering that the force needed to separate the skin layers will generate some tissue damage in the suction blister model, and that cantharidin is described as a slower process, which indirectly targets the desmosomes, and thus slowly detaches epithelial cells, without causing cell death, one could hypothesise that suction blisters cause a more inflammatory reaction than cantharidin.

This contradicts the observations in terms of leukocyte extravasation into blisters and cytokine and chemokine levels in blister supernatant, obtained when comparing

suction and cantharidin blisters in the same subjects. In that, the leukocyte counts for cantharidin blisters is more than 10 times higher than for suction (327,000 vs 24,000) and pro-inflammatory cytokines are also elevated in cantharidin blisters when compared to suction: IFN-γ (3x), IL-12p70 (5x), IL-1β (15x), IL-6 (10x), TNF (8x). Some of the measured chemokines were also elevated in cantharidin blisters when compared to suction: IL-8 (3x), MCP-1 (10x), MIP-1α (3x), MIP-1 β (3x). The data obtained appears to be consistent with other reference values from literature (Kiistala 1968, Day, Harbord et al. 2001, Akbar, Reed et al. 2013), at least for cell counts as cytokine measurements in the 2 types of blisters were obtained via different analytical methods and thus more difficult to compare accurately.

More frequent reports of pain and tenderness when harvesting were also identified, which may be connected to alterations in local humoral mediators mediating nociception (Di Maio, Villano et al. 2023). These data, alongside the conclusions from Chapter 3, points again to the role of cantharidin directly initiating an immune response via the stromal cells. If seeking to explore a de-novo immune response, the cantharidin model should be preferred.

Interestingly, there is a strong correlation between volume of blister and number of cells in the exudate for the suction model, whereas in the cantharidin blister this is not observed at all. It may be that volume in case of suction correlates with force needed to disrupt the skin barrier and thus, more damage equates to a bigger blister and higher level of inflammation. A very strong correlation was also observed between volume and IL-6 in the suction blisters. IL-6 is promptly synthesised in local lesions in the initial states of inflammation (Tanaka, Narazaki and Kishimoto 2014) and this also links level of inflammation to the size/volume of the blister.

In cantharidin, the size of the blister and the degree of inflammation appear to be independent. This appears to indicate that the acantholytic effect and the initiation of an inflammatory signal are indeed two distinct processes initiated by the same chemical.

Differences observed in the volume of exudate and number of cells migrating into the inflammatory site can also have practical consequences for downstream applications. Immunophenotyping of rarer populations or isolation of cells for ex-vivo functional assays can be impacted by the limited number of cells observed in the stand-alone

negative pressure methodology. Also, the differences observed between blister leukocyte populations (e.g. monocyte/neutrophil or mDC/pDC ratios), may inform selection of the model, based on population of interest. The knowledge of differential expected levels of specific cytokines and chemokines may be relevant for studying drugs or mechanisms targeting specific pathways.

In addition to comparing the biological read-out of blisters induced via the two techniques, the project deliberately incorporated feedback from participants as an element of patient and public involvement (PPI) activity. Recruitment of volunteers for blister studies may be hampered by anxiety associated with perceived consequences of blistering, such as pain, discomfort, or cosmetic problems. Questionnaire data suggests that moderate levels of pre-study apprehension in study volunteers were transformed into willingness to accept repeating performance of the methodologies with participation in future studies. The survey also revealed that time commitment required by volunteers for the suction blister methodology was the main negative factor reported. These findings, together with negative pressure induction method being a more technically demanding technique for investigators to apply (i.e. need to control the pressure gradient during the raising procedure), are additional factors to consider when selecting the choice of blister induction methodology.

The amount of time required to induce the blister is notably different between the two methodologies. Whereas the negative pressure methodology elicits a fully raised blister typically within 120-180 minutes, which can be immediately sampled, the temporal effect of cantharidin on blister formation is not clearly defined, resulting in most studies to date opting for a minimum 24-hour time-point for sampling. The negative pressure method thus allows the exploration of a more complete time course, as exemplified by the temporal changes in inflammatory response to E.coli challenge (Motwani, Newson et al. 2017), from acute inflammation at 4 hours to resolution after more than three days. The use of the suction as a method to obtain cells from a secondary challenge, as observed in the example above, should not overlook the fact that this procedure could be an inflammation-inducing challenge in itself and some of the effects observed may result from the application of negative pressure and the resulting tissue damage with alarmin release.

5.4.3 Inter-subject heterogeneity is consistently observed in these inflammatory models

The response to cantharidin challenge was consistently characterised by a higher proportion of neutrophils in the blisters for the majority of volunteers. However, this response was not universal. Two subjects had a consistently low percentage of neutrophils in their blisters implying a phenotypically different inflammatory response. The cause of this different 'cellular signature' is unknown at present, but may originate from genetic or demographic differences (one point to note was that these subjects were the oldest of the cohort), and suggests the existence of discrete immunotypes which could influence response to a pathogenic challenge or drug (Kennedy, Simon et al. 2016, Kaczorowski, Shekhar et al. 2017). Interestingly, these observations were only detected after immune challenge and in the tissue, and were not apparent from the analysis of circulating cells.

5.4.4 Understanding variability of outputs for each model allows rational study size estimations

The current clinical study was designed to assess biological replicates (in two blisters formed simultaneously), consistency over time (analysis over three visits, each separated by one week) and inter-subject variability of the two techniques. The data suggest that the cantharidin method affords a higher consistency, both between subjects and in replicate blisters. With regards to specific parameters, variability estimates are highest for oedema whilst cell activation markers are more consistent than cell trafficking parameters. The highest inter-subject and temporal variability for activation markers came from siglec-1. This can be pinpointed to two clear spikes (20-50 fold) of expression of this marker on single visits from two subjects. Siglec-1 expression in monocytes is a Type I IFN biomarker (Oliveira, Karrar et al. 2018, Graf, von Stuckrad et al. 2022) and therefore these spikes could be indicative of a viral event at these specific timepoints.

Variability estimates are required for power calculations in clinical studies to determine the number of subjects needed to observe expected drug effect. The skin blister models investigated so far in this study seem to be most suited to detect

pharmacological endpoints from drugs where activation status represents an accurate biomarker of mechanistic effect. Due to low variability of intra-subject replicates, the current study suggests that using a pre/post drug, within subject design may be optimal to detect drug effect, rather than seeking population (placebo vs. active) values in separate cohorts, where higher number of volunteers would be needed to observe an effect.

5.4.5 Soluble mediators drive active recruitment of cells from periphery

When assessing the composition of contemporaneous blood and blister samples, the relative proportion of leukocytes in these two matrices does not correlate. This suggests that blister exudates represent neither a direct sampling from local vasculature nor the result of passive accumulation from the circulation where similar subset proportions would be expected.

There appear to be active processes, different for each of the models, that pull cells from circulation into the blister compartment. In some cases, as exemplified by monocyte expression of CD206 and HLA-DR, there appears to be a consistent increase of these putative markers of activation upon migration to tissue, showing that these monocytes descend from the peripheral compartment. Other markers such as CD163 show an expression pattern which is independent from original baseline, possibly indicating a specific response to local milieu.

Many correlations are found between soluble mediators and different blister and cellular phenotypes. Correlation does not mean causation, and in this case it is difficult to interpret whether cytokine or chemokine levels drive cellular ingress or if extravasated cells regulate secretion of these mediators in response to the internal milieu of the blister fluid.

Examining some of these values and adding the context of the observed time course, it is interesting to observe that TNF appears to correlate with total number of cells, particularly neutrophils, and with blister fluid volume in both models. As discussed in previous chapter, this cytokine peaks at a very early timepoint in the cantharidin blisters and appears to be highest at 4h in suction blisters. These timepoints are before initiation of cellular migration, suggesting enhanced TNF secretion by stromal cells.

The correlation between TNF and volume in both cases may also point to a link between this cytokine and the total area of the insult, pointing to the origin of this cytokine being the local cellular environment. Production of TNF by the local environment appears to be the trigger for the initial influx of neutrophils; the correlation between these two outputs helps to build the story that TNF is the first signal that triggers the inflammatory response initiated by the influx of neutrophils. In fact, in the report of the validation of the cantharidin methodology, Dinh et al show that the primary result of an anti-TNF dose pre-blistering was the inhibition of neutrophil trafficking to the blisters.

5.5 Chapter conclusions

- Cantharidin blisters have a higher inflammatory profile when compared to parallel suction blisters.
- Blister overall responses are heterogeneous, but remarkably consistent for each individual for most outputs.
- To optimise the use of blister methods in clinical research, studies should be designed (where possible) to measure intra-subject changes with repeat blisters pre/post drug challenge.

Chapter 6: Utility of in vivo skin blister models to detect inflammatory modulation by pharmacological agents

6.1 Introduction

6.1.1 Glucocorticoid physiology and therapy

Insults to the body such as infection, inflammation, pain or stress will trigger activation of the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases corticotropin releasing hormone (CRH) which acts on the anterior pituitary to induce synthesis and release of adrenocorticotropic hormone (ACTH). This hormone will induce the release of glucocorticoids (GCs) such as cortisol from the adrenal cortex.(Newton 2000). Cortisol is the main endogenous glucocorticoid and has a multitude of physiological targets in humans, affecting nearly every organ and metabolic process in the body (Kadmiel and Cidlowski 2013).

To exert their effects, GCs diffuse through cell membranes and bind to glucocorticoid receptors. The glucocorticoid/receptor complex formed will then move into the cell nucleus where it will bind to glucocorticoid response elements which will either suppress (transrepression) or activate (transactivation) gene transcription. These effects are characterised by a relatively slow onset, with start of, or alteration in, mRNA synthesis from about 15 minutes post dose (Colbert and Young 1986), and a slow dissipation. As most of the effects of GCs occur in the nucleus during transcription, the main effects of GCs are not immediate and will occur over many hours to days, even after dissipation of GCs from plasma.

Therapeutically, synthetic analogues of GCs are used almost universally in the treatment of inflammatory, allergic and autoimmune diseases both acute and chronic due to their wide-ranging anti-inflammatory and immunosuppressive effects. Indeed, it is estimated that the expression of several hundred genes are directly impacted by glucocorticoid treatment (Phuc Le, Friedman et al. 2005).

Systemic GCs are usually only prescribed for short-term dosing, to curtail inflammatory or immune responses, or to contain acute exacerbations of diseases like asthma or COPD. GCs are generally not prescribed for long-term treatment of chronic conditions since they have pleiotropic effects in different organs, and their desired action can then be overshadowed by multiple off-target effects, giving rise to a multitude of unwanted adverse effects. However, direct and limited dosing, including by topical administration

or inhalation, can be used in a more targeted way to elicit specific effects in desired organs, avoiding severe systemic effects (Williams 2018).

6.1.2 Molecular mechanisms of glucocorticoids action

As mentioned before, most of the effects of GCs are exerted through the GCR; however, there are some effects that appear independent of transcription and are observed on a much quicker timescale. These non-genomic effects may be due to the interaction between the GCR and components of signalling pathways. GCs have been described to influence the MAPK pathway (Abraham, Lawrence et al. 2006), T cell receptor signalling (Ghosh, Baatar et al. 2009), and cellular cytoskeleton and structure (Muller, Fischer et al. 2013).

The GC receptor is maintained in the cytoplasm as an inactive multi-protein complex. It contains a ligand binding domain, a DNA binding domain and 2 activation domains. After entering the cell, via passive diffusion, GC will bind to the GCR, leading to conformational changes that will cause the dissociation of the multi-protein complex to allow migration to the nucleus (DeFranco 2002).

In the nucleus, it will act in 2 major ways. First, GC/GCR will form homodimers which will bind to Glucocorticoid Response Elements – DNA sequences, characterised by common 5′-XXTACA XXXTGTTCT-3′ regions, containing two binding sites for the glucocorticoid receptor homodimer. The binding between GCR/GC homodimer (with or without other cofactors) and DNA recruits the transcription apparatus and initiates gene transcription (Czock, Keller et al. 2005).

Secondly, The GCR/GC complex interacts with pro-inflammatory transcription factors, inhibiting their control in gene expression. Nuclear Factor κB (NF-κB) and Activator Protein 1 (AP-1) are critical to the inflammatory response, as initiated for example by TNF or IL-1β, and their action in initiating pro-inflammatory gene transcription is repressed by the glucocorticoid receptor (Kagoshima, Ito et al. 2003).

6.1.3 Effects of GC in host response

The GC/GCR are at the centre of a network that regulates different inflammatory pathways. They counter inflammation and inhibit prostaglandin production through 3 independent mechanisms: the induction and activation of annexin I, which inhibits cytosolic phospholipase A2α (cPLA2α) and thus the release of arachidonic acid and conversion to eicosanoids; the induction of MAPK phosphatase 1 which dephosphorylates and inactivates Jun N-terminal kinase, thereby inhibiting c-Jun–mediated transcription of pro-inflammatory mediators; and the blockage of NF-κB transcription, a major transcription factor in the synthesis of pro-inflammatory cytokines and cyclooxygenase 2 (COX2), an enzyme essential for prostaglandin production (Rhen and Cidlowski 2005).

The selective effect on cytokine (suppression of pro-inflammatory IFN-α and IL-1β and induction of anti-inflammatory cytokines TGF-β and IL-10) and chemokine (suppression of MCP-1 and IL-8) levels (Almawi, Beyhum et al. 1996), will impact the immune cell response. Both the ingression of new leukocytes to circulation and the trafficking of these to the tissues are dependent on activation of blood cells by cytokines and chemokines and mediated by cellular adhesion molecules. Glucocorticoids can reduce leukocyte trafficking by directly inhibiting cellular adhesion molecules (Ince, Weber and Scheiermann 2018).

Glucocorticoids also have an effect in the cell cycle of leukocytes (proliferation and apoptosis) and their polarisation state. By inhibiting co-stimulatory receptors in lymphocytes (Galon, Franchimont et al. 2002) and dendritic cells (Kalthoff, Chung et al. 2003), GC induce tolerogenic Treg phenotypes and stop differentiation of Th1 cells.

The inflammatory process is initiated by trafficking of neutrophils to the injured tissue. Glucocorticoids also affect the microcirculation in the site of inflammation by inhibiting the vasodilation of capillaries, limiting blood access, reducing permeability of these vessels, reducing exudate formation and suppressing leukocyte transmigration (Perretti and Ahluwalia 2000). Neutrophil access is thus restricted, but also the function of these cells is impacted by GC, as phagocytosis and then apoptosis is also controlled by inflammatory mediators derived from arachidonic acid, and by cytokines. As observed in chapter 4, the cantharidin blister cellular response is initiated by the trafficking of neutrophils to the site and then by the recruitment of further immune cells

from cytokine signals secreted by these. By impacting the microcirculation and the trafficking and function of neutrophils, it is expected that GC dosing will affect the blister formation.

6.1.4 Chapter aims:

Compare the effects of topical and systemic applications of a similar immunomodulator in tissue (skin) and circulating immune cells.

Perform a proof-of-concept study to identify whether blister models of inflammation can be used to detect pharmacological properties of topically applied drugs.

6.2 Additional methods:

6.2.1 Clinical study design

The study was designed to compare the capacity of both blister models to be modulated by a pharmacological challenge and to compare topical and oral administration of the same class of drug on local (blister) and systemic (whole blood) inflammo-immune biomarkers.

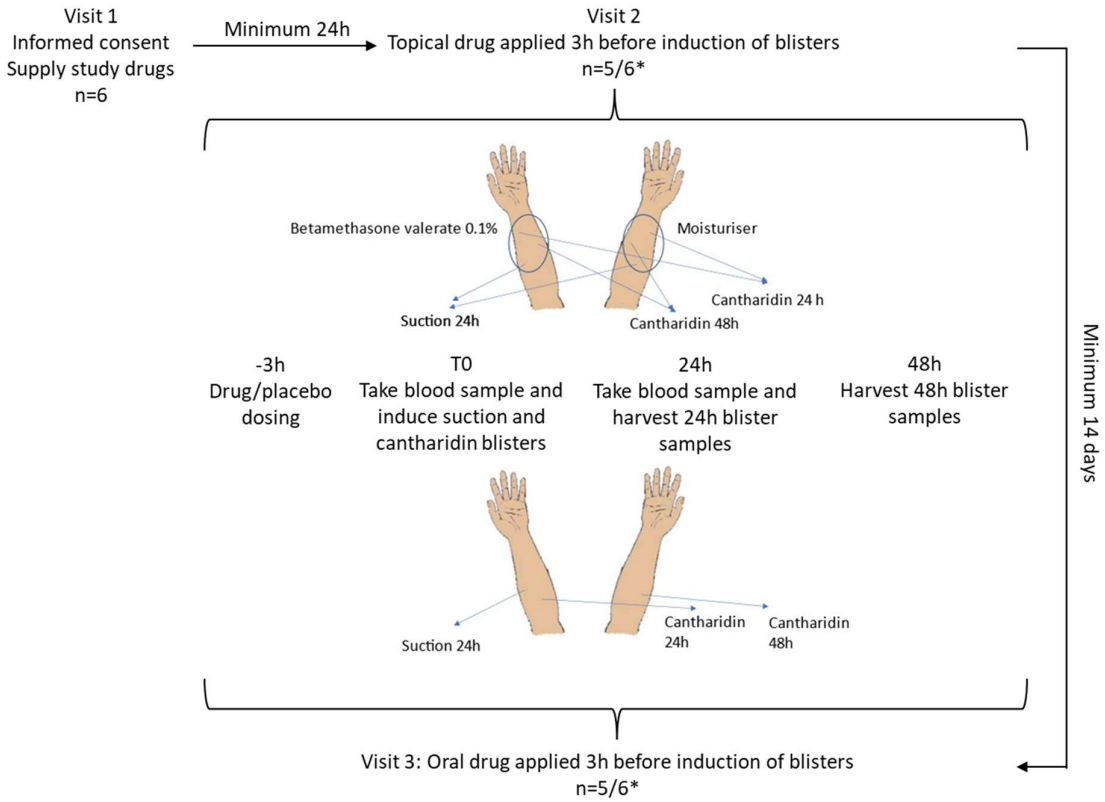
A study schematic is presented in Figure 36 and the full methodology, as documented in ethics approved protocol and Participant Information Sheet, is attached as supplementary data (Appendix 2). The study employed an un-blinded, within-subject design, participants being exposed to two interventions sequentially and their effect on both local and systemic immune responses multi-modally evaluated and directly compared. Direct comparison of drug effect on the two different blister paradigms was facilitated by contemporaneous and parallel formation post-exposure.

In short, after obtaining informed consent, subjects received a pack with the topical drug (betamethasone valerate 0.1%) for home application. In the morning, 3 hours prior to coming to the unit to initiate blister raising procedures, participants applied one fingertip unit of betamethasone valerate 0.1% to the volar (hairless) surface of one forearm and one fingertip unit of moisturiser to the other forearm. When at the clinical unit, a blood sample was collected, and to each arm, cantharidin was applied to two sites and one suction blister was raised. 24h later, participants returned to clinic to

have one suction and one cantharidin blister harvested in each arm and a blood sample taken. At 48h after raising, participants returned to have a final pair of cantharidin blisters harvested.

A similar process was repeated at least 2 weeks later (to allow wash-out), with participants taking an oral dose of Prednisolone (30 mg) in the morning, before attending the unit 3 hours later to collect a sample of blood and having a total of one suction blister raised and two cantharidin applications in the forearms. At 24h post blistering, participants returned for collection of one cantharidin and one suction blister and returned at 48h for the final collection of the last cantharidin blister.

One subject applied Betamethasone cream to the hairy part (dorsal aspect) of the arm and so his blister data was excluded, a per protocol approach to analysis being adopted given the small number of participants in the study.



* 1 participant applied cantharidin on hairy side of arm and blister data was excluded (blood data not affected)

Figure 36: Study schematic. After obtaining consent, participants applied topical Betamethasone to left and moisturiser to right arm 3 hours prior to arriving on site. At that point, a blood sample was collected and suction and cantharidin blisters were raised in both arms. 24 hours later, one suction and one cantharidin blister were harvested in each arm after obtaining a second blood sample. 48 hours after raising, final cantharidin blisters (one in each arm) are collected. A minimum of 14 days later, subjects took one oral dose of Prednisolone 30 mg and a similar schedule of blistering was followed.

6.2.2 Blood collection and Whole Blood Stimulation with LPS

Blood samples were collected from the antecubital fossa into BD Vacutainer® sodium heparin tubes. 50 µL of the sample were reserved for flow cytometry analysis and 2 mL for whole blood stimulation.

Remaining blood sample was centrifuged at 1500 g for 10 minutes and aliquots of plasma stored at -80 °C.

The LPS used for stimulation was prepared from a lyophilised sample (10 mg – Lipopolysaccharides from *Salmonella enterica* serotype abortus equi, L5886-10MG) by diluting with 10 mL of RPMI 1640 (Gibco) and aliquoted into 1 mL aliquots. These were kept at -20 °C and when thawed, had 1 month shelf life when kept at +4 °C. For each experimental day, this 1 mg/mL stock was serially diluted in RPMI 1960 (Gibco) to 1 µg/mL, 12.5 ng/mL before a final dilution to 1.25 ng/mL.

In 15 mL centrifuge (Falcon) tubes, 2 mL of LPS 1.25 ng/mL or RPMI 1960 (negative control), were added to 500 µL of whole blood and incubated for 4 hours at 37 °C.

After incubation, samples were centrifuged at 1200 g for 10 minutes and aliquots stored at -80 °C.

6.3 Results:

6 subjects completed the trial, but 1 subject had a major treatment non-compliance. On the first session, the topical treatment was applied to the dorsal (hairy) side of each forearm. As this was not the site where the blistering process was due to take place, the blister data for this subject was excluded.

For each subject, a set of 9 blisters was obtained: Cantharidin 24h, Suction 24h and Cantharidin 48h for each of the 3 treatments: Placebo, Topical and Oral dose. A blood

sample was obtained at start of blistering procedure on first day and before blister harvest at 24h for both sessions.

6.3.1 Oral steroid treatment impacts blood composition

Whole blood samples collected were used for immunophenotyping by flow cytometry and for 6h LPS stimulation. The cytometry panel was identical to the one used in previous chapters and was designed to identify major leukocyte subsets and a selection of activation markers.

Using the gating strategy defined in Figure 5, the main leukocyte populations (CD16+ Neutrophils, HLA-DR+ Monocytes and FSC^{lo}/SSC^{lo} Lymphocytes) were identified and proportions of these tracked over the 4 collections (Figure 37 A).

The proportion of neutrophils was consistent over the 2 sampling points at 3h and 27h post topical steroid application (mean 61.9%) and increased in all subjects at 3h after systemic application to an average 81.8%, before dropping back to 60.3% 27h post systemic steroid dose. Changes in circulating neutrophils contrast with reductions in the percentage of monocytes and lymphocytes in response to the systemic steroid dose. The changes are only observed as differences in percentage of total leukocytes as absolute number determination was not carried out. It is unclear if the contemporaneous reduction in lymphocyte and monocyte proportion is due to increased circulating neutrophil numbers or a reduction in other cell types.

Oral steroids were also associated with an alteration in the relative proportion of Classical, Intermediate and non-classical subsets, determined based on the surface expression of CD14 and CD16 (Kapellos, Bonaguro et al. 2019). A reduction on the percentage of classical monocytes, compensated by elevation of intermediate and non-classical phenotypes is observed at the same timepoint post oral steroid dose (Figure 37C).

In the lymphocyte populations we see a similar temporal effect with CD3+ T cells reduced and CD3-/CD16+ NK cells increased at the 3h post-oral dose. Relative B cell population proportions were unchanged (Figure 37B).

No consistent alterations were observed between samples taken at 3h and 27h post topical steroid application for any of the populations studied.

Activation status of whole blood leukocytes was also assessed, by measuring markers such as CD163 and CD208 on monocytes and CD64 on neutrophils, which are commonly used as activation or polarisation biomarkers in these leukocytes. No consistent differences between the time points studied for either oral or topical GC for all markers analysed were observed. (Figure 37 D,E),

As a functional pharmacodynamic marker, whole blood samples were stimulated with LPS, freshly after collection, for 6h. Culture supernatants were then assayed for TNF production. Unstimulated samples present similar baseline levels throughout (median 0.54 pg/mL), whereas stimulated samples show a major increase in TNF production (median 821.1 pg/mL for all timepoints except 3h post treatment). Treatment with oral steroid significantly decreased TNF production 10-fold to near unstimulated levels at 3h post dose (median 65.3 pg/mL; $p<0.0001$), however no effect was seen 27h post dose indicating offset of systemic effect. Topical treatment had no effect on TNF production.

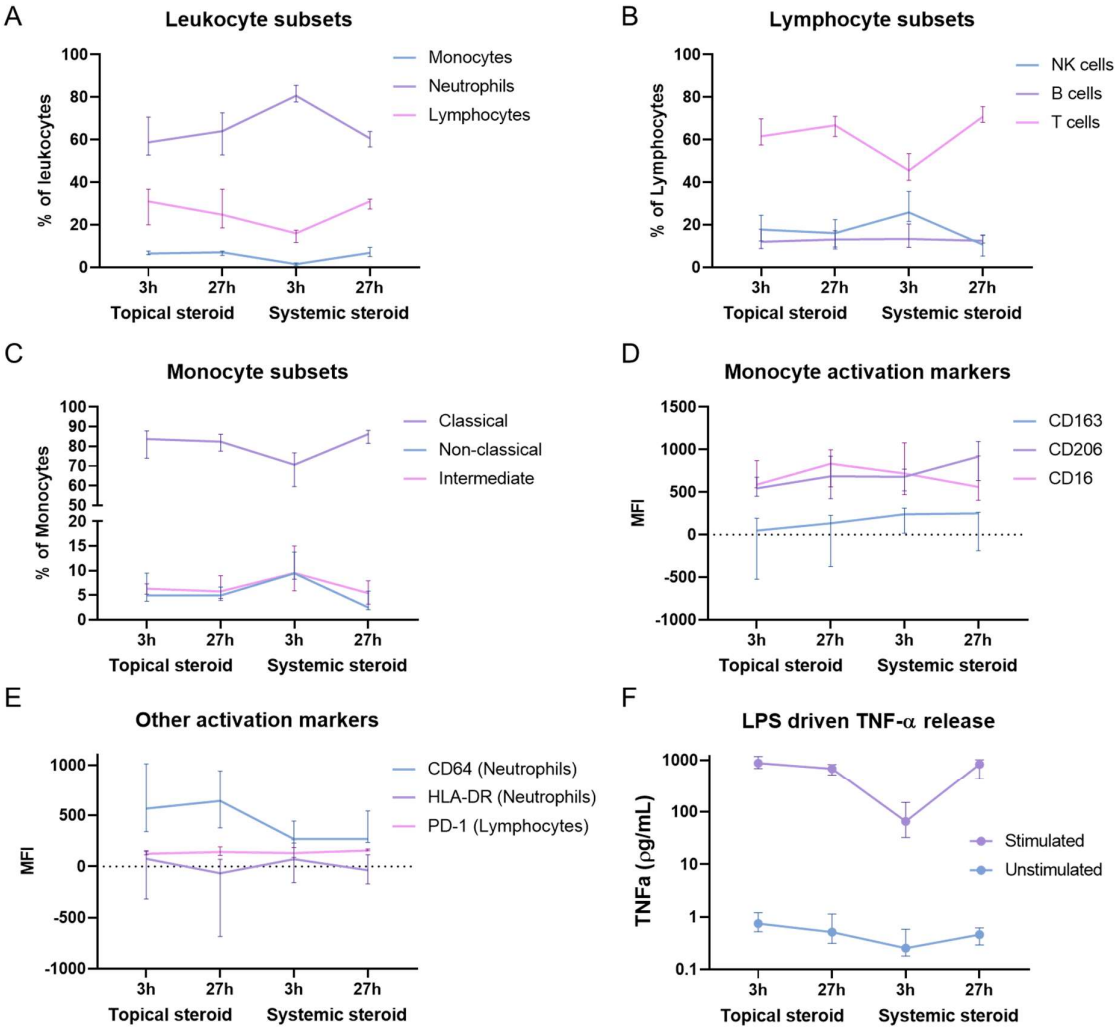


Figure 37: Effect of GC dosing in whole blood cellular profile. Major leukocyte subset changes over the timepoints analysed in the study: 3 and 27h post Topical dose and 3h and 27h post systemic dose. Median and interquartile ranges for n=6. [A] Monocytes, neutrophils and lymphocytes as percentages of all CD45+ leukocytes; [B] NK cells, B cells and T cells as percentage of total Lymphocytes; [C] Monocyte subsets as percentage of total HLA-DR+ monocytes; Activation or polarisation markers for Monocytes [D] and other cell types [F]; TNF release after LPS ex-vivo stimulation for 6h

6.3.2 Suction blister cellular outputs do not appear to be influenced by steroid treatment

Suction blisters were raised following a pre-defined stepwise pressure gradient until a bleb was apparent and then pressure was kept or slightly reduced until full blister formation. The time until blister formation is usually different between different participants (Figure 38A displays pressure used per participant for each of their placebo blisters), but more consistent for different blisters in each subject. Median time

of full blister formation (Placebo: 01:36; Topical: 01:36; Oral: 01:46) was not different between conditions and looking into each subject separately, time of blistering was not consistently impacted by treatment, nor were any operational observations (e.g. blister leakages).

The resulting blisters were covered overnight and harvested 24h later. Volume was assessed from mass, assuming a fluid density of 1.025 mg/mL: Placebo median volume was 130 µL (range 74 – 196 µL), Topical: 117 µL (27 – 211 µL) p=0.8125, Oral: 92 µL (70 – 124 µL) p=0.1875; and cellularity was measured by haemocytometer counts (Placebo: 50,000 cells/mL (10,000 – 210,000 cells/mL), Topical: 60,000 cells/mL (17,500 – 140,000 cells/mL) p=0.8125, Oral: 55,000 cells/mL (10,000 – 282,500 cells/mL) p=0.3750). Derived numbers of cells/blister were obtained by multiplying volume (in mL) by the cellularity (Figure 38B).

Data for volume and cellularity was normalised to placebo by subtracting the value observed in placebo from the observed on the treatment arm. After normalising the data for each participant, the difference observed for each blister pair (value placebo – treatment) was not consistently above or below 0, indicating that there were no consistent trends observed for treatment effect in the volume or cellularity estimates (Figure 38 C-E).

The median composition of the suction blisters, grouped by treatment, is shown in Figure 38F. As previously observed in the initial blister characterisation study (Figure 29), a wide inter-subject variability of cell subset ratios was observed, with different subjects showing a predominance of monocytes/neutrophils/lymphocytes on their placebo blisters. Assessment of pharmacological modulation of leukocyte subsets via normalising data from treatment to placebo revealed no consistent treatment effects. The same lack of apparent effect was observed both when looking at the difference between treatment and placebo for percentages of leukocytes (Figure 38G) and for absolute numbers of each of the cell subsets (Figure 38H). These data suggest GC administration had no impact on the migration of specific leukocyte subsets.

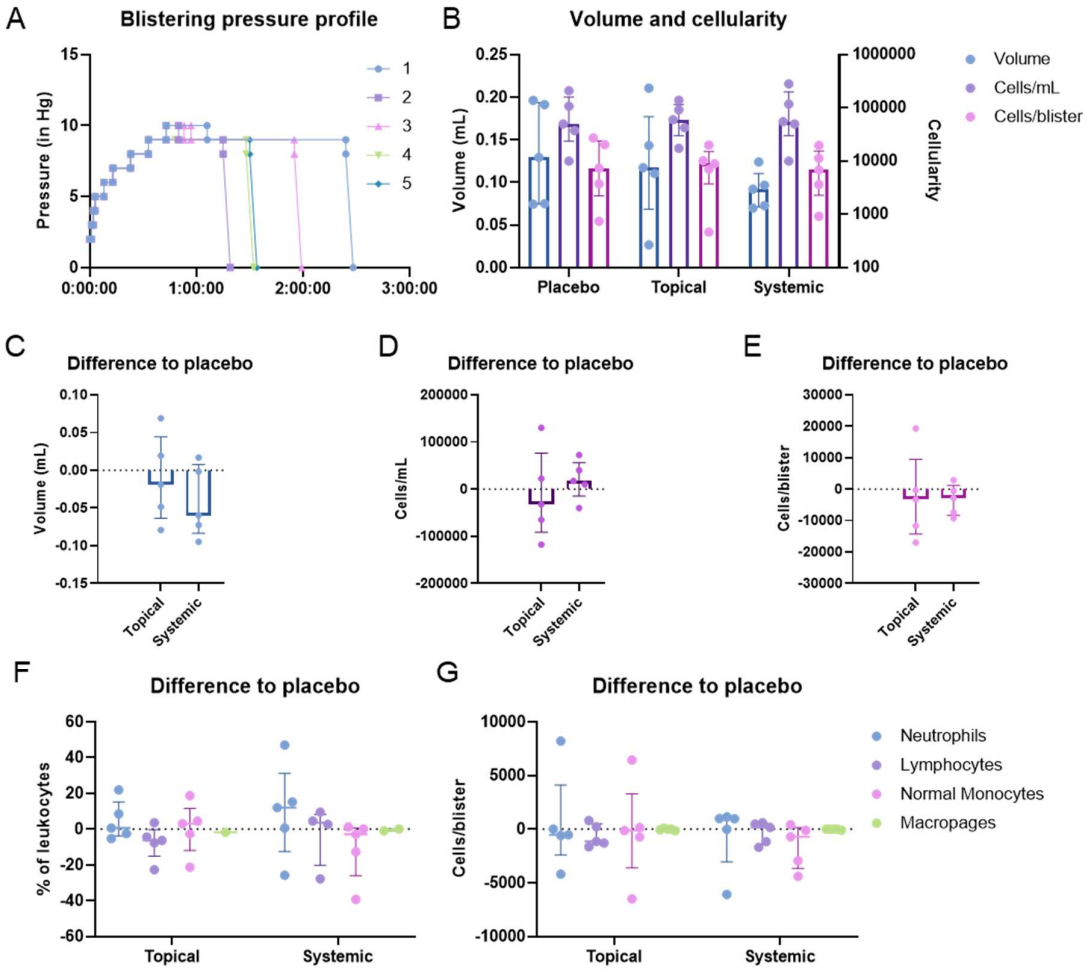


Figure 38: GC dosing, both oral and topical, have no consistent effect in suction blister outputs. Overall, bars and error bars represent median and interquartile range of each population. Data for n=5. [A] Individual pressure gradient used over time until the complete formation of the placebo blister for each of the subjects; [B] Volume and cellularity observed for each suction blister; normalised volume [C] and cellularity [D, E] data, calculated by subtracting placebo from value observed in treated sample; Difference in leukocyte makeup between active and placebo blisters expressed as percentages of CD45+ leukocytes [F] and as absolute differences in cells/blister [G]

6.3.3 Inflammatory profile of suction blisters is unchanged with treatment

Cell activation was determined by flow cytometry by analysing different monocyte (CD206, CD163, CD64, and CD16), neutrophil (HLA-DR, CD64) and lymphocyte (PD-1) activation or polarisation markers (Figure 39A). The data was extracted as MFI (mean fluorescence intensity) for each parameter/population and then normalised as difference to corresponding placebo sample. There were no consistent trends in

activation levels on any cell type, again indicating that both topical and systemic GCs did not impact cells migrating into the suction blister.

Mediator analysis was carried out with cytokine (Figure 39B) and chemokine (Figure 39C) panels analysed using MSD. Samples were stored at -80 °C and analysed in one batch using commercially available multiplex kits for inflammatory cytokines and chemokines. IL-8 data was below the lower limit of quantification for all suction blister samples and is not present in the figures, but other analytes were mostly within assay range. No significant trends for change due to treatment on any of the mediators analysed were evident.

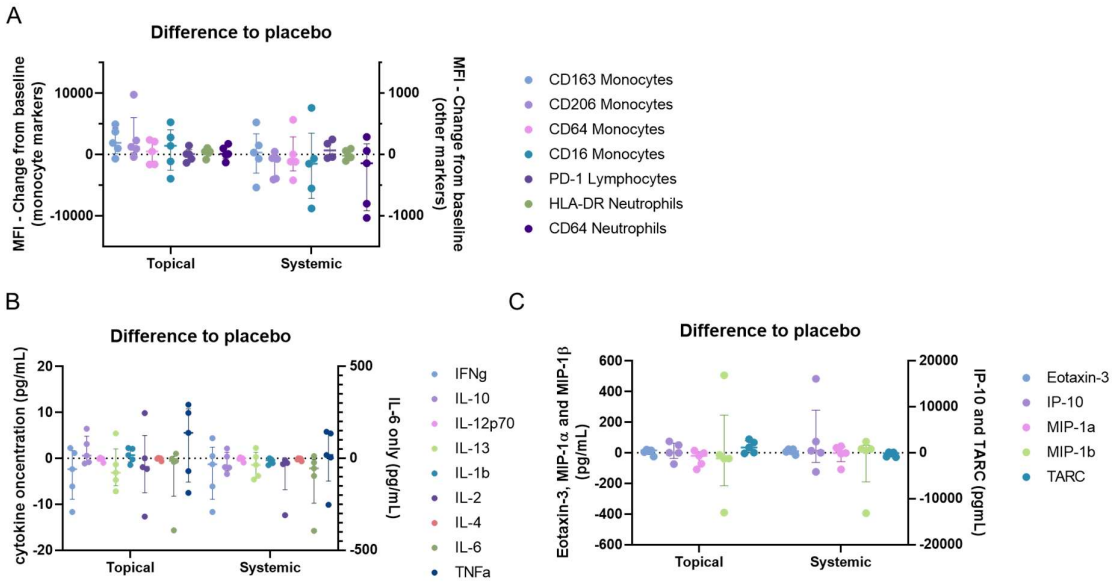


Figure 39: Cell activation and soluble mediator profile of suction blisters are not affected by steroid treatment. All data presented correspond to difference between treated and placebo samples in n=5 expressed as median with interquartile range. Difference to placebo in cellular activation markers for different cell subsets [A], and cytokine [B] and chemokine [C] levels in blister fluid.

6.3.4 Volume and cellularity of cantharidin blisters appear to be impacted by both oral and topical GC drugs

The effect of treatment in size and cellularity of cantharidin blisters is summarised in Figure 40. Median placebo blister volume at 24h was 164.8 µL (range 84.7 – 441.3 µL) and that was slightly decreased by treatment to 126.1 µL (54.3 – 235.8 µL) with topical steroid and 104.6 µL (27.3 – 306.5 µL) with oral steroids (Figure 40A). A similar trend to reduction is also seen at 48h, but statistically, the effect of both topical and oral steroid dosage in volume is not significant when looked at the group level (Figure 40 A). Cellularity, both expressed as cells/mL and cells/blister, is also not impacted by GC treatment when looking at group effect. The inter-subject variability is high for both these measurements, as previously observed in the first blister characterisation study, and within-subject analysis reveals more evident treatment effects (Figure 40 D-E).

By tracking individual responses, we can see that all 5 participants had a smaller volume at 24h in the arm treated with topical steroid, when compared to placebo (all 5 values after baseline subtraction are below 0). In total, there are clear trends for reduction of volume and possibly also for a decrease in cellularity (measured as cells/blister) by steroid treatment in the cantharidin blisters. Due to low cell numbers, paired non-parametric analysis on n=5 cannot be statistically significant, but when merging 24h and 48h data, reduction of volume is significant for topical (mean reduction of 96 µL, 95% CI 9 – 193 µL reduction; p=0.0273) and oral (mean reduction of 121 µL, 95% CI 29 – 214 µL reduction p=0.0273) treatment, and reduction of cellularity (mean reduction of 2.8×10^5 cells, 95% CI 0 – 5.7×10^5 cells reduction; p=0.0039) is observed for topical treatment.

6.3.5 Neutrophil migration into cantharidin blisters is impacted by steroid treatment

24h cantharidin placebo blisters in this study were very similar to those observed in the comparative blister study (Chapter 5), as can be observed in Figure 40G. The median 48h placebo cantharidin blister has a composition similar to that observed at 24h, with a trend towards an increased proportion of monocytes. In all subjects, an increase in monocytes at 48h is observed, with 4/5 subjects showing a reduction of the percentage of neutrophils from 24 to 48h (Figure 40H).

A consistent treatment effect on the distribution of leukocyte subsets was observed, with topical GC dosing reducing blister neutrophil percentages at both 24h (4/5) and 48h (5/5) relative to placebo, whereas monocyte percentages are increased at both timepoints (4/5 at 24h and 5/5 at 48h). Lymphocyte numbers are consistently reduced at 48h only (5/5). Oral GC dosing appears to only consistently effect the proportion of monocytes, with all subjects studied showing an increase in the proportion of this subset at both timepoints (Figure 40I).

When analysing total cells numbers, the biggest effect of the topical steroid application appears to be a reduction of the number of neutrophils infiltrating the blister at both 24h and 48h (mean reduction of 1.7×10^5 at 24h and 3.0×10^5 for 48h; p=0.0625 for either timepoint separately; reduction of 2.3×10^5 with p=0.002 when analysing the combined 24 and 48h pairs). Lymphocyte numbers were also markedly reduced at 48h (p=0.0625) and overall (p=0.0039), whereas the number of monocytes was not consistently altered. Oral GC treatment did not have any statistically significant effect in terms of changes to absolute counts of leukocyte subsets.

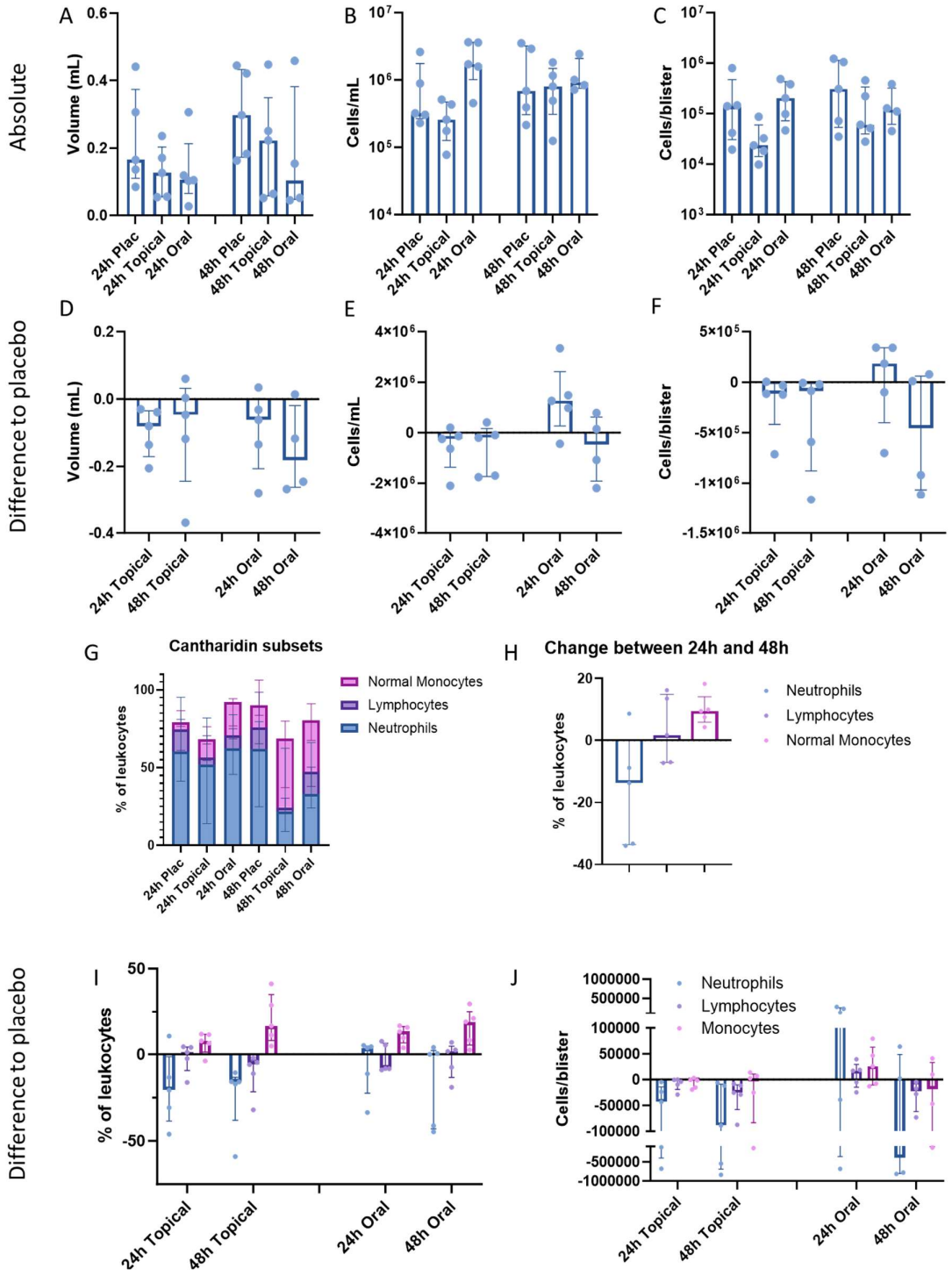


Figure 40: Impact of GC dosing in cantharidin blister outputs. Overall, bars and error bars represent median and interquartile range of each population. Data for n=5. Absolute values for Volume [A], cells/mL [B] and cells/blister [C] observed in cantharidin blisters for each timepoint analysed; normalised data, by subtracting placebo, for the same outputs of volume [D], cells/mL [E] and cells/blister [F]; [G] Median blister composition per treatment; [H] difference between 24h and 48h for placebo blisters; [I, J] Difference in leukocyte makeup between active and placebo blisters expressed as percentages of CD45+ leukocytes [I] and as absolute differences in cells/blister [J]; [I] MFI (mean fluorescence intensity) for activation and polarisation markers observed in different cell subsets;

6.3.6 Phenotypic changes to neutrophils and monocytes are driven by treatment

Most cells collected from blister samples have profiles similar to the ones encountered in the periphery, although some of the CD45+ blister cells found do not fit in with classical gating of the main leukocyte subsets. When trying to characterise all leukocyte populations, $CD45^{+}$ cells that present low/medium expression of CD16, but high SSC (side scatter) were categorised as 'other granulocytes'. Also, the 'monocyte like' ($HLA\text{-}DR^{+}/SSC^{mid/hi}$) population was divided into low, normal and high FSC (Gating example in Figure 41A). Although not singular cell subsets, clearly characterised in all samples, the proportion of these cells is considerable in most blisters analysed. The median values of both 'small' monocytes (FSC^{low}) and 'other granulocytes' appear increased in topical steroid treated samples at both 24h (Figure 41B): median percentage of small monocytes: placebo 8.0 % (range 0.1 – 17 %) vs topical 22.3 % (0.9 – 37.7 %); percentage of 'other granulocytes': placebo 1.1 % (0.9 – 4.6 %) vs topical 5.4 % (2.0 – 10.4 %) and 48h (Figure 41C): median percentage of small monocytes: placebo 4.3 % (range 2.3 – 9.3 %) vs topical 8.43 % (3.93 – 16.8 %); percentage of 'other granulocytes': placebo 3.2 % (1.3 – 5.5 %) vs topical 9.1 % (2.4 – 11.9 %).

The normalised data after subtracting the placebo baseline from the corresponding treated samples is displayed in Figure 41D. The observed percentage of 'other granulocytes' is increased in blisters raised on topical GC applied arms compared to placebo for all subjects at both timepoints ($p=0.002$ when analysing both timepoints) and the levels of small monocytes appear also increased (5/5 at 24h and 3/5 at 48h, $p= 0.0371$).

As discussed in the timecourse chapter (Chapter 4), there are significant differences in the expression of activation/polarisation markers between 24 and 48h blisters, notably an increase in monocyte MFI for CD163 and CD206 (Figure 41E). GC administration (topical or systemic) however had no effect on cellular markers of activation or polarisation at 24h, either as a group change or after normalising the data by subtracting placebo and exploring within-subject effects (Figure 41F). In contrast, at 48h a reduction in CD163, CD206 and CD16 MFI between treated and placebo blisters are apparent for both treatments (Figure 41G).

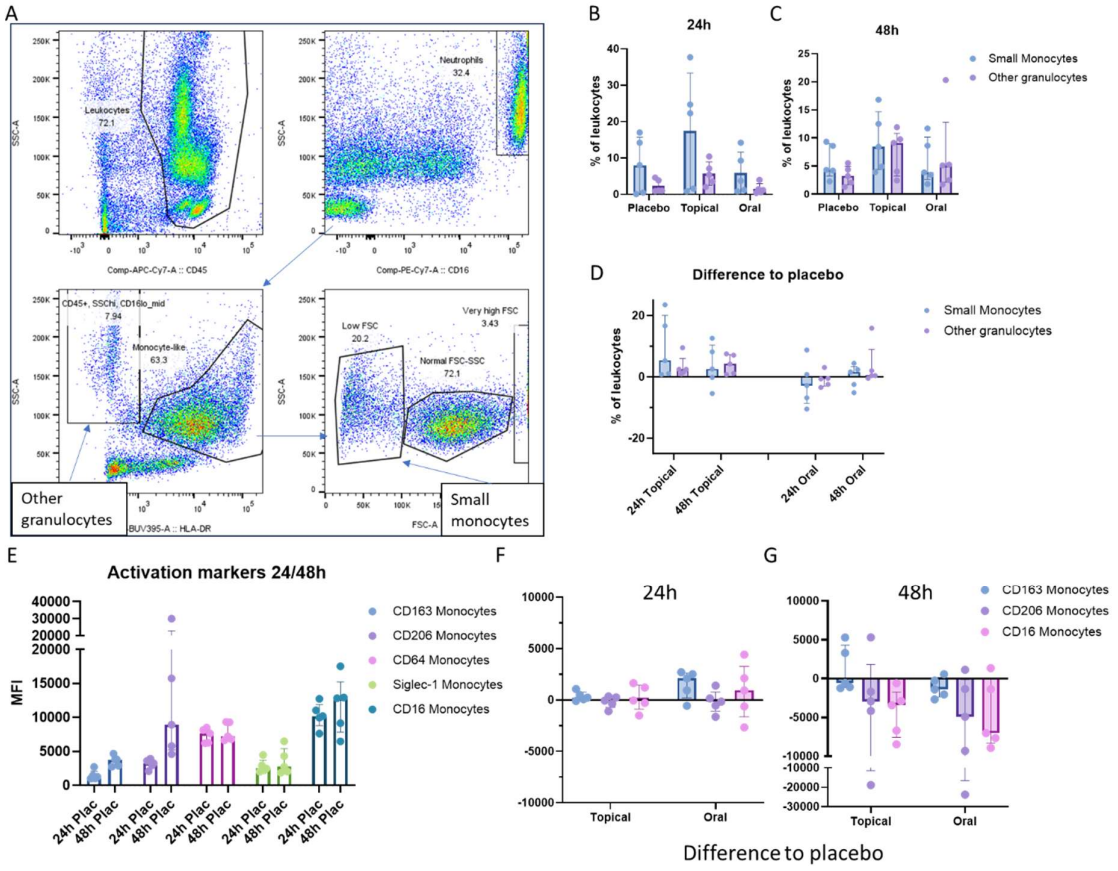


Figure 41: Phenotypic changes in cantharidin blister leukocytes. Overall, bars and error bars represent median and interquartile range of each population. Data for n=5. [A] Gating identifying presence of 'small monocytes' (HLA-DR^{+}/FSC^{lo}) and 'other granulocytes' (SSC^{hi}, CD16$^{mid/lo}$); percentage of these subsets in cantharidin blisters at 24h [B] and 48h [C]; [D] Normalised data to placebo for these two subsets; [E] Comparison between 24h and 48h for different monocyte activation markers in placebo blisters expressed as mean fluorescence intensity (MFI); GC elicited change on monocyte markers at 24h [F] and 48h [G]

6.3.7 Chemokine, but not cytokine levels are impacted by GC treatment

Cytokine and chemokine levels were quantified by MSD in all cantharidin blister samples for the 5 subjects who completed the study.

No convincing changes were observed for any cytokines, but a trend towards increased TNF after topical GC treatment was observed at both timepoints (4/5 for 24h and 5/5 for 48h) although, in direct contrast, systemic GC reduced TNF in 4/5 blisters at 24h (Figure 42 A,C,E).

GC treatment had a clearer effect on chemokines concentrations. MIP-1α and MIP-1β were reduced in 5/5 samples at 24h with topical GC with a median reduction of 74% and 78% respectively, whereas systemic treatment did not consistently impact these chemokines. At 48h, although there is a reduction in absolute levels of these molecules to close to baseline levels, MIP-1α levels appear still reduced by topical steroid in 4/5 samples and MIP-1β in all subjects. Systemic dosing still makes no impact on these at 48h (Figure 42 B,F).

At 24h, IL-8 was modulated by both GC treatments. Topical treatment reduced IL-8 levels in all 5 subjects (median reduction of 37%) whereas systemic treatment lowered IL-8 in 4/5 subjects (median reduction of 74%) (Figure 42 B,F). TARC levels increased significantly at 48h compared to 24h, and at this timepoint topical steroid reduces TARC levels in 4/5 subjects and systemic steroid was able to reduce TARC for all evaluated blisters. Topical steroid also appears to reduce the levels of Eotaxin-3 at 24h in all 5 subjects (Figure 42 D,F).

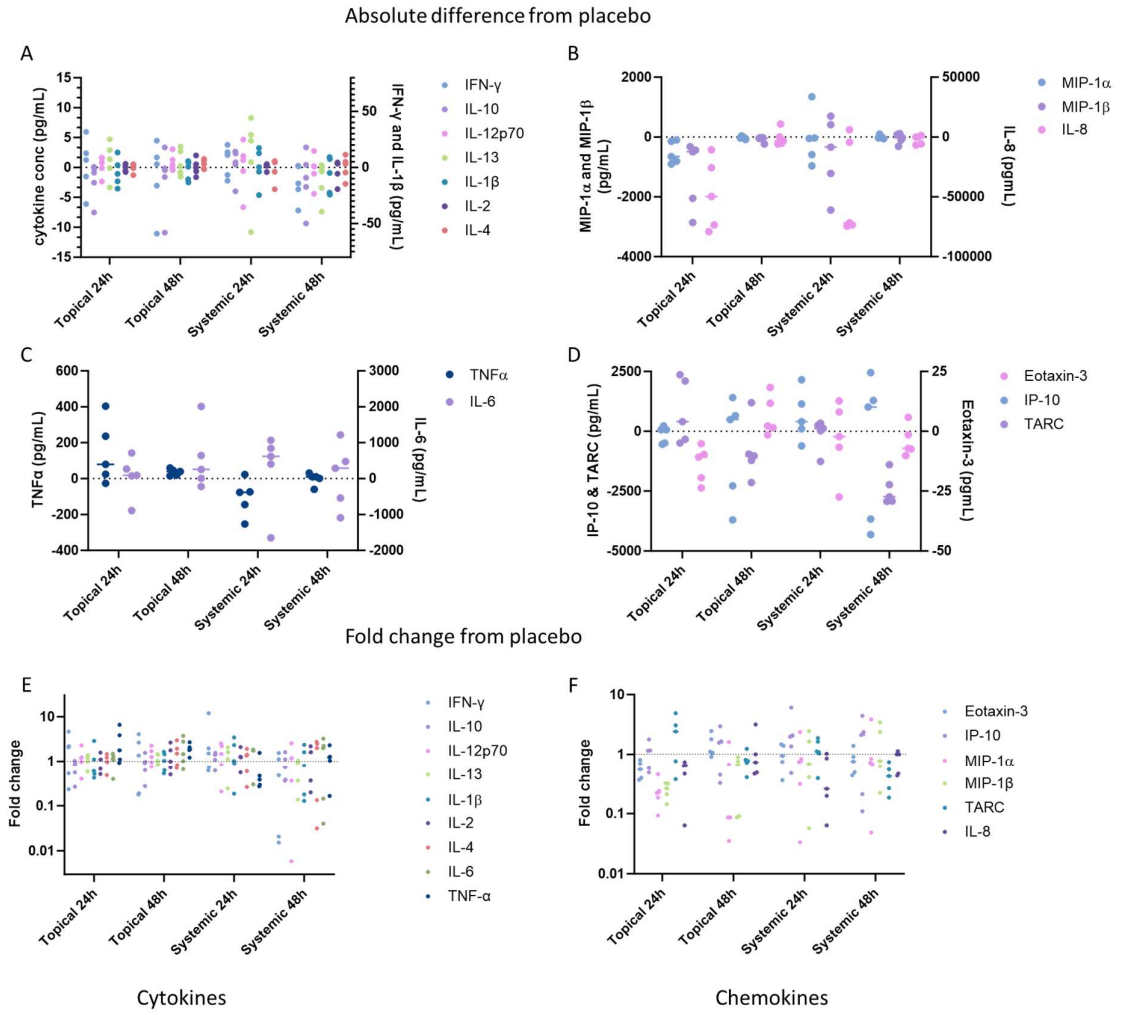


Figure 42: Steroid treatment appears to consistently impact selected chemokines. Absolute difference from corresponding (24/48h) placebo was calculated for all cytokines (left) and chemokines (right) and each value (placebo/active pair) is represented for all 5 treated subjects. Cytokines with lower absolute values are represented in [A] and higher concentration in [C]. Absolute difference to placebo for chemokines is also separated between panels [B] and [D]. Fold change from placebo (ratio active/placebo) is represented in panel [E] for cytokines and [F] for chemokines

6.4 Discussion:

6.4.1 Considerations over study design

This study was primarily designed to compare and evaluate the use of different blister models for demonstrating anti-inflammatory drug proof-of-concept in experimental medicine and/or early phase clinical studies.

To elicit an anti-inflammatory effect, tool compounds were selected based on known pharmacology and safety profiles. GCs are some of the most widely used and well characterised anti-inflammatory drugs and are available in different formulations and dosages. Oral glucocorticoids have been successfully used in previous blister studies, with oral prednisolone reducing monocyte, DC and NK cell migration to imiquimod challenged skin while also reducing concentration of TNF, IL-6 and IL-8 in the blister fluid (Assil, Buters et al. 2023). Limited treatment effects was also observed in eosinophil cell recruitment to blisters in a cantharidin blister model (Dinh, Corraza et al. 2011) and in trafficking of monocyte subsets, dendritic cells, natural killer cells and T cells to suction blisters raised on top of an LPS challenge (Buters, Hameeteman et al. 2022).

As blistering methodologies are specifically designed to interrogate skin inflammation, the difference between topical and oral delivery was investigated to ascertain their relative sensitivity to each route. This design also allows exploration of whether it is drug effect on circulating cells (where systemic dosing would be expected to have a greater effect vs. topical) or on the resident immune and stromal cells (where topical would be expected to have the greater effect due to exposure) that drives observed differences in blistering responses (Brown, Martin et al. 2006). Further, the effect of topical GCs have never been studied using cantharidin blister derived biomarkers.

We selected a moderately potent topical GC – Betamethasone valerate 0.1%, and a dose of oral steroid used in the clinical setting to treat active immune-mediated inflammatory pathology (e.g. flairs of ulcerative colitis and Crohn's disease) – Prednisolone 30 mg, which have similar pharmacological properties.

Although the percutaneous absorption of topically applied steroids is low, and low systemic effects were expected from topical drugs (Dhar, Seth and Parikh 2014), a single dose regimen was selected to minimise any potential systemic effect of the topical drug, enabling the contemporaneous use of the 2 arms of the participant, one for the placebo and one as the active. Using different samples collected from the same subject at the same time allows for minimisation of any batch effect or inter-experiment variability. As a single dose of the topical drug was used, we chose to also use a single dose of oral Prednisolone, although this is a shift from what normally is seen when tool compounds are used to observe pharmacological effects. Most reports usually dose

drugs to steady state in order to expect a maximum effect, as, for example, was the case for the uses of GC drugs to evaluate the blister models cited above (Dinh, Corraza et al. 2011, Buters, Hameeteman et al. 2022). Given the mechanism of action of GC's however, it was felt reasonable to assume a single-dose would have immunomodulatory effects.

The timepoint selection was based on the PK/PD profiles of both oral and topical drugs (Goa 1988, Fleishaker, Mukherjee et al. 2016). At 3 hours post dose, the maximum PK has just been achieved, and some of the relevant expected downstream effects of the drug are starting to occur, such as changes in neutrophil and lymphocyte trafficking, which will then last for many hours: the period in which the blisters will be formed, and early inflammatory events will occur in those sites. A substantial PD effect, the almost complete suppression of LPS-induced TNF release, was in fact observed at the blistering time for the oral drug, demonstrating that systemic effect of steroids were present in all subjects and thus validating the use of this timepoint and a single-dose regimen.

GC's, both oral and topical, induced very noticeable changes in the trafficking of cells into cantharidin blisters, demonstrating the rationale for the study design. This was however only observed after normalisation of outputs to placebo, a practice that is not always followed. As the blister response is, by its nature, very distinct between different subjects - reflecting inter-individual immune variation more generally - significant group effects are inherently difficult to demonstrate with low subject numbers and a parallel group design. One clear example is the volume of the blisters; this study achieved a similar population level decrease to that observed by Dinh et al. (Dinh, Corraza et al. 2011), with blister size being reduced by a similar magnitude, with both not reaching significance as any trends for a reduction of volume with GCs are masked by high inter-individual variability, whereas the fact that in this study 16 out of 19 blisters raised under GC treatment are smaller than placebo (using intra-subject comparisons) reveals an extremely consistent treatment effect.

Along similar principles to those described above, a decision was made to concurrently employ both blister models to directly compare the capacity of each model to be affected by drugs was made. This intra-individual, within-timepoint comparison is novel and will be informative in the design of future clinical trials. In this case, we have

observed very different outcomes in blisters from the two methodologies carried out side-by side and these observations can strongly suggest that the two methods for creating blisters are not interchangeable.

6.4.2 Steroid doses elicited expected pharmacology in whole blood leukocytes

Glucocorticoids are important therapeutic agents which modulate cellular transcription in multiple tissues and their cellular constituents.

One of the more immediate effects of glucocorticoid action is the inhibition of cytokine production (Schwiebert, Beck et al. 1996) via leucocytes. The suppression of LPS-induced TNF is a key bioassay of immune competence and has been used both after in-vivo LPS challenge (de Kruif, Lemaire et al. 2007) and as an ex-vivo model to detect pharmacodynamic effects of glucocorticoids (Wirtz, von Kanel et al. 2004). In this study we observed that systemic, but not locally applied GC, significantly reduced the ability of ex-vivo monocytes to produce TNF in the presence of LPS only at 3 hours post oral dose, with no effect observed on the next day. This occurred in all 6 subjects dosed with oral prednisolone and demonstrates the expected pharmacology of the drug.

For blood cells, endogenous glucocorticoids are one of the main promoters of maturation of neutrophils, controlling the release of neutrophils into circulation through shedding of L-Selectin (Cavalcanti, Lotufo et al. 2007). This effect was evidenced in our data, the percentage of neutrophils, which was relatively constant during topical dosing (3h: 58.8%, 24h: 64.1%) and on the 24h post prednisolone (60.6 %) was increased in all subjects to a median of 80.7% at 3h post prednisolone oral dose. The shedding of L-selectin and the downregulation of other cell adhesion molecules (Pitzalis, Pipitone and Perretti 2002) also diminish neutrophil recruitment to tissue. Consistent with this known biological effect, a reduction of neutrophil numbers in cantharidin blister samples at 48h post oral and systemic prednisolone was observed when compared to placebo.

The opposite effect is expected in lymphocytes. In this population, glucocorticoid treatment is described to induce circulating lymphocytopenia, with a maximum effect at 4h and a return to baseline after 24h (Fauci and Dale 1975), as a result of

redistribution of lymphocytes into other lymphoid compartments (lymph nodes, spleen and bone marrow) (Fauci and Dale 1974). This aligns with our data, lymphocyte relative proportion being reduced in blood in response to prednisolone treatment (median of 16.0% at 3h post prednisolone compared to 29.5% over all other timepoints).

A similar reduction at the same timepoint was also observed with monocytes. These were reduced after prednisolone treatment (1.52% compared with 6.9% over all other timepoints). In our study, the reduction appears to be mostly due to decrease in classical monocytes, although glucocorticoid therapy has previously been described as preferentially depleting intermediate and non-classical CD16+ monocytes (Fingerle-Rowson, Angstwurm et al. 1998, Dayyani, Belge et al. 2003), at least after repeated dosing. Given the lack of absolute counts, it is impossible to accurately attribute the shifts in cellular proportion observed following prednisolone administration however these are likely due to both relative increases (neutrophils) and decreases (lymphocytes, monocytes) in number.

In contrast, no prednisolone-induced change in circulating leukocyte activation markers in blood were observed. This may be due to a lack of concurrent inflammatory stimuli and, in future studies, flow cytometric analysis of cell activation after ex-vivo LPS stimulation should be conducted, where the effect of prednisolone may be more apparent.

In summary, GC-induced alterations recapitulated what is described in the literature, serving to confirm the oral prednisolone dose was administered in all participants, and that immunomodulatory effects were witnessed in circulating leukocytes at the same time as the formation of the blisters with associated initial leukocyte trafficking. As such, pharmacological modulation of the blister contents should be observed if they are truly sensitive to drug effect.

6.4.3 Comparing outcomes from similar glucocorticoids given via different routes of drug delivery

In this study, circulating leukocytes were impacted at 3h by the effect of one dose of oral Prednisolone 30 mg in the morning. In contrast, there were no obvious

observations in whole blood leukocytes after topical application of betamethasone valerate 0.1% demonstrating the expected higher potency of peripheral effects by a systemic rather than a topical GC drug.

When investigating the effects in local tissue, there are changes in the efficacy of the GCs derived from the different application routes.

Oedema formation is one of the characteristics of the inflammatory response and occurs secondary to the movement of fluid and plasma proteins through permeable blood vessels into the extra-vascular space. Steroids (including endogenous), are known to reduce blood flow (Ahluwalia and Flower 1993). This may be a consequence of different mechanisms, including sensitization to vasoconstrictors or inhibition of the synthesis of vasodilators. By controlling the synthesis of inflammatory mediators such as prostaglandins and nitric oxide, GCs also impact the permeability of vessels (Williams and Morley 1973, Appleton, Tomlinson and Willoughby 1996) which is dependent on vasodilation and endothelial cell permeability. GC have been shown to act directly on endothelial cells, blocking the formation of intercellular gaps, needed for the extravasation of fluid (Bjork, Goldschmidt et al. 1985). Oedema may thus be considered a valid biomarker for determining the pharmacological effect of these drugs and has been employed previously to identify candidates for topically applied GCs for skin conditions.

The effect of both topical and oral steroids on limiting the formation of the blister, an exaggerated form of oedema, appears to be consistent with the expected pharmacology. In this case, both drugs appear to have a similar outcome in terms of direction and magnitude of change, when compared to placebo.

It has been long postulated that GC effects on leukocyte migration may be central to the clinical effects observed (Ishikawa, Mori and Tsurufuji 1969). Several molecular mechanisms appear to be involved, and the effects depend on the initial inflammatory stimulus and the dose of GC used (Perretti and Ahluwalia 2000). Through the inhibition of NF-kB pro-inflammatory gene expression, GCs reduce expression of adhesion molecules in the vascular epithelium, like ICAM-1, which is critical for the capture of rolling leukocytes in the vasculature on the site of inflammation, the first step for cell trafficking (Wheller and Perretti 1997). GCs also have an impact on the expression of adhesion molecules in leukocytes. GC treatment reduces expression of CD62-L (L-

selectin) and CD11b in human neutrophils (Filep, Delalandre et al. 1997), which are anchor molecules that allow for cell adhesion to the vascular epithelium. Not only neutrophils can be affected by GCs, but also monocytes – through reduction in ICAM-1 expression (van de Stolpe, Caldenhoven et al. 1994) and lymphocytes – by downregulation of CD11a and CD2 (Pitzalis, Pipitone et al. 1997).

Cell trafficking in this study appears to be more strongly impacted by topical when compared to systemic treatment. Topical betamethasone significantly reduced total leukocyte migration over both timepoints (p=0.003). In contrast, oral steroids did not exert a consistent effect. Differences in cell recruitment appear to be predominantly driven by a reduction in neutrophil migration when compared to placebo. With topical steroid treatment, a reduction in total number of neutrophils in blisters was observed at 24h and 48h for all 10 Topical/Placebo pairs (p = 0.002). Lymphocyte recruitment was also inhibited by topical steroid at both timepoints (p=0.039), but interestingly, monocyte total numbers are maintained with both treatments. These alterations result in a change in relative leukocyte composition in blisters with a significant increase in the monocyte relative proportion for both treatments (p=0.0039 for topical and p=0.0020 for oral dosing).

These differences between treatments may be driven by the impact of GC in the production of chemotactic proteins. In this study, topical steroids reduced blister levels of IL-8, MIP-1α, MIP-1β and eotaxin-3 at 24h in the cantharidin model. This reduction is accompanied by the decrease in the number of leukocytes, especially neutrophils in those samples, probably driven by the reduction of IL-8, one of the most potent chemoattractants for neutrophils (Russo, Garcia et al. 2014), although its ligands (CXCR1 and CXCR2) are also present on other leukocytes. In contrast, systemic prednisolone did not achieve a similar consistent reduction, consistent with the prior observation of no significant differences in cell trafficking.

Differences between GC application routes can also be observed in the modulation of cell phenotypes. Although monocyte polarisation markers appear to be equally impacted on 48h for both topical and systemic GCs, the different treatments may also differentially impact the life cycle of monocytes and neutrophils. Publications looking at later blister phenotypes describe a large proportion of apoptotic neutrophils, as determined by uptake of Annexin V and propidium iodide (Evans, Haskard et al. 2013),

which are characterised by a lower CD16 staining and lower FSC properties. We have also observed the presence of these neutrophils in both suction and cantharidin blisters and can also postulate the presence of another population of apoptotic cells, although not able to fully characterise these cells as apoptosis or viability markers were not included in the flow panel. A population of cells with side scatter properties in between those of normal lymphocytes and neutrophils, and high expression of HLA-DR (identical to normal monocytes), but with a reduced forward scatter, indicating a smaller size of cell was observed in most blisters.

Topical treatment with GC appears to result in an increase of the proportion of these apoptotic phenotypes at both timepoints (10/10 for apoptotic neutrophils, 8/10 for apoptotic monocytes), which is not observed for oral treatment. GCs have profound effects in inducing apoptosis via its classical action on regulation of gene transcription after GR binding, but interestingly, the effect may differ depending on cell type (Gruver-Yates and Cidlowski 2013).

Interestingly, but as observed in an earlier GSK internal study, cytokine levels at 24h and 48h appear to be refractive to anti-inflammatory treatment. In this study, no single cytokine was consistently impacted by either topical or oral GC. The main trend was for TNF expression at 24h, but with a differential treatment effect whereby systemic prednisolone appeared to reduce TNF (4/5 subjects) whereas topical steroid application increased this cytokine's concentration in 4/5 subjects. Steroids have a selective effect on cytokine levels, usually suppressing pro-inflammatory and inducing anti-inflammatory cytokines (Almawi, Beyhum et al. 1996) and will inhibit secretion of TNF for activated leukocytes, regardless of stimuli (Debets, Ruers et al. 1989). As observed in chapter 4, TNF levels peak early in cantharidin blisters (8-16h) and are at near baseline levels by 24h. It is thus likely that data obtained at 24h may not be truly indicative of GC effect on TNF synthesis/release over the course of blister formation.

In summary, although similar trends were observed for both GC applications tested, topical betamethasone was more efficacious in producing significant changes in this study.

6.4.4 Use of 48h blisters reinforces 24h observations and provides insights on effect of GC on limiting inflammation

In contrast with other sections of this project, cantharidin blisters were designed to be collected also at 48h. This derived from observations from an unpublished GSK internal study report, that revealed alterations in blister inflammatory biomarkers at 48h or 72h, but not at 24h following 4 days GC oral treatment.

Previous studies have evaluated cantharidin blisters of different ages to study the inflammatory process, from acute inflammation to resolution. Different timepoints were analysed, and differences observed between early (16-24h) and late (40-72h) blisters. In most published data, blister volume appears to evolve, with later blisters being slightly larger and more voluminous, but with no major differences noted in cellularity (Jenner, Motwani et al. 2014, De Maeyer, van de Merwe et al. 2020). Despite the consistent number of cells, the makeup of later blisters alters with lower numbers of total neutrophils, and proportionally lower neutrophils and higher monocyte ratios (Evans, Haskard et al. 2013, Jenner, Motwani et al. 2014, De Maeyer, van de Merwe et al. 2020). The decrease in total neutrophils is accompanied by an increase in a population of neutrophils, characterised by lower forward scatter, and staining with Annexin V and Propidium Iodide (Evans, Haskard et al. 2013), indicating that the neutrophils that initially extravasated to the blister have been marked for apoptosis. As for other differences in cell phenotypes, Philippidis et al described upregulation on expression of the haemoglobin scavenger receptor CD163 (Philippidis, Mason et al. 2004). Blister soluble mediators also evolve. TNF is reduced at 40 and 72 hours when compared with 24h blisters (Evans, Haskard et al. 2013, Jenner, Motwani et al. 2014) along with MCP-1, MIP-1β and eotaxin, whereas IP-10 is elevated (Evans, Haskard et al. 2013).

Although not designed or powered to characterise differences between 24 and 48h blisters, there are interesting comparisons between timepoints that can be derived from the data in this study. Our data appear to concur with previous published data, showing a trend for an increase in volume and for a change to a more monocytic and less neutrophilic blister at 48h. Differences in monocyte polarisation markers CD163 and CD206 were also noted with substantial increases in both these markers for all subjects.

Analysing the effect of CG dosing, the 48h timepoint delivered similar trends to what was observed at 24h for most outputs. The main difference observed between the 2

timepoints studied appears to be around the monocyte polarisation markers. At an early stage after blister formation, level of activation appears independent of dosing. Monocytes recently extravasated are phenotypically different to ones found in periphery, as noted in the first characterisation study, and both oral and topical application of GC drugs do not affect these changes observed after extravasation. The effect is then observed during the maturation of these cells in the tissue. At 48h, GC dosing appears to reduce the expected activation observed in the late blisters. CD206 and CD16 are consistently reduced by both GC drugs when compared to placebo, with CD163 also appearing impacted.

6.4.5 Glucocorticoid treatment impacts blister biomarkers in cantharidin but not suction blisters

One of the main objectives of this study was to evaluate the level of response to a known anti-inflammatory exposure on both suction and cantharidin blisters. Although created using different methodologies, both types of blisters allow for the enumeration and characterisation of cells migrating to the site of inflammation (Maini, George et al. 2016) and the analysis of the mediator rich fluid (Kool, Reubsaet et al. 2007). Both methods have also been successfully used to characterise biological events in the inflammatory pathways and to support pharmaceutical development both for analysing PK and drug compartmentalisation (Nicolau, Sun et al. 2007) and to document pharmaceutical control of immune cell migration to site of inflammation (Bouma, Zamuner et al. 2017). In this study we used the two methodologies in parallel to compare the effect of a topical and systemic steroid dosage on blister contents.

One of the main observations to be taken from this study appears to be a lack of response to either topical or oral steroids in the suction blister outputs:

- Oedema and overall cellularity appear unaffected in this model. Although a change in volume of suction blister fluid might not be expected, as this parameter is mostly determined by the operator's decision of when to apply and stop pressure, a trend to a decrease in overall cellularity was observed in the cantharidin blister but not in the suction model.

- More obvious were effects of glucocorticoids in the cellular composition of cantharidin blisters, that were not observed in suction blisters. A preferential selection for absolute and relative reductions of neutrophils and an increase of monocytes is observed in cantharidin blisters, whereas no trends were observed for suction.
- There were also no trends observed in leukocyte activation markers between active and placebo in suction blisters. In cantharidin blisters, any consistent trends were only apparent at 48h, a timepoint not used for suction.
- Cytokine and chemokine levels in suction blister fluid are not impacted by either oral or systemic GC treatment.

In previous chapters it was observed that suction blisters contain fewer cells, with a lower inflammatory profile. They also do not show clear time profiles in pro-inflammatory cytokines or chemokines suggestive of a response to a clear challenge. Finally, treatment with either topical or systemic GC does not influence cellular and humoral outputs as it is observed on parallel cantharidin blisters, suggesting that suction blisters are refractory to anti-inflammatory drug effects.

When observed in its entirety, these data appear to suggest that although macroscopically identical and microscopically very similar, the contents of the two different blister models are determined by different pathways, with recruitment to blisters originated by suction being a more passive, and not an inflammatory driven migration.

These data clearly support the use of suction blisters as an easy, safe and well tolerated way of accessing tissue, that can be applied to a primary immune challenge such as DTH (Akbar, Reed et al. 2013), imiquimod (Assil, Buters et al. 2023) or E. coli (Motwani, Flint et al. 2016), with little extraneous effect on that primary challenge, and not by itself a model of inflammation as sometimes suggested (Davidsson, Bjorkman et al. 2013), contrasting to the application of cantharidin.

6.5 Chapter conclusion

- Effects of glucocorticoid dosing on cantharidin blister contents can be observed when normalising to individual response, but not when comparing placebo vs active populations. Within subject protocols are likely superior for detecting pharmacodynamic effect than parallel groups.
- Single topical application of GC showed potent anti-inflammatory effects on the cellular and soluble outputs in the cantharidin model of inflammation in this proof-of-concept study.
- Suction blister contents appear to be refractory to both topical and systemic glucocorticoid drugs, likely reflecting the lack of induction of local inflammation. They are thus useful as a sampling method of other inflammatory lesions but should not be considered an inflammatory model themselves.

Chapter 7: Concluding remarks

Skin blister models are relevant for translational immunology and clinical pharmacology, yet there has been little direct comparison of the immunological processes each model elicits or their responsiveness to pharmacological intervention.

Suction blisters have been characterised as producing an inflammatory response characterised by influx of primed neutrophils and presence of inflammatory mediators. Although we see cells migrating from periphery to tissue over time and observe maturation of monocytes in suction blisters, our data suggest a lack of sustained inflammatory response in this model. The cellular events are also not modulated by the use of steroids. Primary utility of this model should be to access cells and fluid in an area subjected to a local inflammatory challenge.

Immune response to cantharidin challenge is not well characterised in literature. In-vivo, after diffusing through epidermis, cantharidin not only breaks up desmosomes to create extracellular spaces that fill with interstitial fluid (as is the current view), but also initiates an inflammatory response. By stimulating stromal cells to release TNF and initiating a pro-inflammatory cascade, the immune response to cantharidin is ongoing prior to visual signs of blistering. By the time epidermal cell structure is compromised, extracellular spaces are already full of pro-inflammatory cytokines, and immune cell recruitment has been initiated with neutrophils migrating to the epidermis from the vasculature. Clinical blistering occurs between 8 and 16 hours post-challenge, with a first wave of signalling from TNF and IL-8 already having peaked, and with neutrophils already present in the inflammatory site.

The cellular and humoral time course that follows the cantharidin challenge, first described in this report, will allow for precise targeting of critical steps in the formation of the blister at the most relevant time. Also identified are activated pathways and their biomarkers, which are probably the most likely to be modified in response to specific therapy. Cantharidin functions as a stand-alone inflammatory challenge, which contrasts with the suction method.

Unique patterns of cellular and humoral responses are observed in the population for both methods, making it impossible to perfectly describe a universal blister reaction. In contrast, the inflammatory events observed for both suction and cantharidin challenges are consistently observed in the same individual upon rechallenge. The inter-subject variability observed prevents these models to be used in a typical clinical

or experimental medicine trial design, where conclusions are drawn from dosed vs placebo cohorts. In contrast, the use of blisters at baseline followed by rechallenge post dosing, with data normalised by participant, appears to be a rational methodology for observing tissue pharmacodynamics in a context of a small early phase clinical trial.

These insights will inform the next generation of early-phase clinical trials and mechanistic studies, maximizing both scientific rigor and clinical relevance in immunopharmacology.

Chapter 8: References

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