



Potential of *in vitro* reconstituted 3D human airway epithelia (MucilAir™) to assess respiratory sensitizers

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ABSTRACT

Respiratory sensitizers are considered as substances of higher risk, at the same level as carcinogens, mutagens and toxic chemicals for reproduction. Presently, there is no validated assay for identifying the respiratory sensitizers. Based on a fully differentiated and functional *in vitro* cell model of the human airway epithelium, MucilAir™, we attempt to develop such assay. To this end, we invented a novel method, using Dextran as carrier, for applying the water insoluble chemicals to the apical surface of the airway epithelia. Using the Dextran carrier method, we successfully tested some reference chemical compounds known to cause respiratory sensitisation in human beings, including MDI, TMA and HCPT. Interestingly, these chemical sensitizers differentially up-regulated the releases of certain cytokines and chemokines involved in allergic responses. We believe that based on MucilAir™ an *in vitro* assay could be developed for identification and characterization of the respiratory sensitizers.

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1. Introduction

Respiratory sensitizers and irritants are considered as substances of higher risk, at the same level as Carcinogens, Mutagens and toxic chemicals for Reproduction. Indeed, in industrialized countries, the prevalence of rhinitis and asthma increased dramatically in the past two decades, especially in infants and children (Hopkin, 1997; Evans et al., 2008). That is why, within the framework of European new legislation on chemicals (so-called REACH), sensitizing effects of the chemicals have to be evaluated. However, until now there is no validated animal model or method, nor *in vitro* cell model for identifying the respiratory chemical sensitizers (Isola et al., 2008; Kimber and Dearman, 2005, 1997a,b; Kimber et al., 2011; Roggen et al., 2006).

Allergic responses are generally considered as immune response, involving different types of leukocytes such as Langerhans cells, dendritic cells, macrophages, T cell, as well as B cells (Verstraelen et al., 2008; Epstein, 2004; Kimber et al., 2011). Therefore, the most appropriate way to study allergy and allergens is to use the animal models endowed with intact immune systems. Indeed, the mouse local lymph node assay (LLNA) has been successfully developed for the identification and characterization of skin sensitization hazards (Kimber et al., 1994, 2001; Kimber et al., 2011).

Now the question is: Is there any possibility to develop an *in vitro* cell model which lacks the complexity of the immune sys-

tems in a whole animal for identification and characterization of respiratory sensitization hazards? We believe that the airway epithelial cells have the potential for this purpose. First of all, as physical barrier, the airway epithelial cells are the first line of defence against the inhaled toxicants including allergens. Second, it is well documented that the airway epithelium plays an essential role in initiating and modulating the airway immune response and inflammation. In fact, the airway epithelial cells are the major, if not predominant, source of cytokine and chemokine production (Chung and Barnes, 1999; Ying and Kay, 2000). For example, some of Th-2 cytokines like IL-13, which play an essential role in allergic response, are also expressed in the airway epithelial cells (Allahverdian et al., 2008; Brochner, 2003; Chung and Barnes, 1999; Ying and Kay, 2000). The chemokines such as Eotaxin, RANTES, responsible for recruiting the leukocytes into the airway lumen during the allergic reactions, are also highly up-regulated in the airway epithelial cells when stimulated by the pro-inflammatory mediators (TNF- α , LPS, etc.) (Brochner, 2003; Chung and Barnes, 1999; Ying and Kay, 2000). In recent years, the importance of the airway epithelium in asthma pathogenesis has been recognized (Holtzman et al., 2002; Holgate, 2008). Therefore, human airway epithelium reconstituted *in vitro* may have the potential to identify and characterize the respiratory chemical sensitizers. Such attempt has been made: using a transcriptomics approach, Verstraelen et al. tried to find genetic markers in A549 cells that are able to distinguish respiratory sensitizers from respiratory non-sensitizing chemicals. Unfortunately, no known canonical signaling pathway was observed to be activated in the A549 cell line after

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the challenge of well-known respiratory chemical sensitizers (Verstraelen et al., 2009).

The aim of this paper is to assess the potential of a ready-to-use and commercially available 3D *in vitro* cell model of human airway epithelium (MucilAir™ developed by Epithelix) to identify and to classify the respiratory chemical sensitizers. To this end, we developed a novel method for applying the water insoluble chemicals onto the apical surface of the airway epithelia. Then, using this method, we tested some reference compounds such as respiratory, dermal sensitizers and respiratory irritants on MucilAir™, using the cytokines and chemokines as readouts. We provided some evidence that MucilAir™ may have the potential to be an *in vitro* cell model for identifying the respiratory sensitizers.

2. Materials and methods

2.1. An *in vitro* cell model of the human airway epithelia

Nasal and airway cells were obtained from patients undergoing surgical nasal polypectomy and lobectomy, respectively. All experimental procedures were explained in full, and all subjects provided informed consent. The study was conducted according to the declaration of Helsinki on biomedical research (Hong Kong amendment, 1989), and received approval from local ethics commission. MucilAir™, made of freshly isolated primary human airway epithelial cells is not only morphologically and functionally fully differentiated; it can also be maintained at a homeostatic state for more than a year. Indeed, while studying cell–cell communication, Epithelix has developed a technology which allows epithelium to reach homeostatic state with a slow turnover. It is a ready-to-use product and commercially available. These features of MucilAir™ allow repeated and chronic exposure to chemical compounds.

2.2. Chemicals

Nine reference chemicals have been selected for the reported study: Methylene diphenyl diisocyanate (MDI), Trimellitic anhydride (TMA) and Ammonium hexachloroplatinate (HCPT) have been shown to be potent respiratory sensitizers; whereas Dinitrochlorobenzene (DNCB), Cinnamic aldehyde and Tetramethyl thiuram disulfid (TMTD) are classified as skin sensitizers; Salicylic acid, Phenol as respiratory irritants and Cinnamic Alcohol as pro-hapten.

2.3. Endpoints

Since MucilAir™ was reconstituted on transparent semi-porous membranes, it allows assessing the toxic effects of chemicals with multi-endpoints, such as morphological inspection, cilia beating monitoring, TEER measurement, as well as the quantification of cell viability, cyto-toxicity and cytokines/chemokines release.

2.3.1. Morphology and Cilia beating monitoring

The Airway epithelium is a pseudo-stratified structure. At normal physiological conditions, the epithelium is uniform and homogenous. Cilia beating are clearly visible under contrast microscope.

2.3.2. Tissue integrity monitoring

Trans-Epithelial Electrical Resistance (TEER) was measured using EVOMX (World Precision Instruments, UK). In order to perform TEER measurement, 200 µl of buffered saline solution (0.9% NaCl – 1.25 mM CaCl₂ and 10 mM Hepes) were added onto the apical surface. The buffer was removed after measurement.

2.3.3. Resazurin test (quantitative)

To measure the cell viability, inserts were transferred in a new 24 wells plate containing 500 µl of 6 µM Resazurin in buffered saline solution (0.9% NaCl – 1.25 mM CaCl₂ and 10 mM Hepes), 200 µl of the Resazurin solution was also applied on the apical surface and the plate was then incubated for 1 h at 37 °C (5% CO₂, 100% humidity). After the incubation, 100 µl of the apical solution was transferred in a 96 wells plate and the fluorescence of the transformed product was measured (excitation filter = 544 nm; emission filter = 590 nm). The inserts were then transferred in a new 24 wells plate containing 700 µl of fresh medium, the remaining apical solution was removed without disturbing the epithelium and the inserts were put back into the incubator.

2.3.4. Elisa measurement of Inflammatory mediators

Medium from each well was collected and stored at –80 °C. IL-8, IL-6, MCP-1, GRO-α and RANTES released in the medium were quantified by ELISA according the instructions of the manufacturer.

3. Results

3.1. Development of a novel method for applying the water insoluble chemical compounds onto the apical surface of the epithelia

Most of the respiratory chemical sensitizers are insoluble in water; therefore it is difficult to apply these chemical compounds in a precise, quantitative manner. It is also difficult to test powders or nanoparticles. In order to solve this problem, a procedure which allows testing all kinds of solid substances was developed (Patent Application Pending: PCT/IB2010/053956). The idea is to use inert and neutral substances as carrier. Dextran is a bacterial by-product; the dextran macromolecule consists of glycan groups linked end to end. It didn't show any harmful effect on MucilAir™. Dextran was successfully used as carrier to deliver the insoluble chemical compounds and nanoparticles onto the apical surfaces of MucilAir™. The following figure describes the procedure and application of the Dextran tablets (Fig. 1). Schematically, the test compound and Dextran carrier are weighed with a precision balance, and then mixed together in a mortar (step 1). The mixture is weighed again and combined with a given amount of pure Dextran carrier, and so on so forth, until all the desired concentrations of test compounds are reached (step 2). For each concentration, the mixture was pressed in a hydraulic press to make a tablet of 1.3 cm² (step 3). Then the smaller tablets are made using a biopsy puncher (step 4). With a forceps, the small tablet is deposited on the apical surface of the epithelium in an insert (step 5).

To validate the method, the big and the small tablets at each solid dilution containing Salicylic acid were dissolved in DMSO and amount of Salicylic acid was quantified by HPLC. The quantity of Salicylic acid was exactly that expected based on calculation (Data not shown). Usually, the Dextran pellet was dissolved within 15 min after being applied onto the apical surface, releasing and exposing the test chemical to the epithelial cells.

3.2. Assessment of the potential of MucilAir™ as a tool to discriminate and to classify the respiratory sensitizers

Using this novel Dextran carrier method, we tried to develop an *in vitro* assay for identifying the respiratory chemical sensitizers. The test compounds at a range of concentrations were applied onto the apical surface of MucilAir™. After 24 h of exposure, the apical surface was washed with saline solution to remove the test compounds. Then several endpoints were simultaneously assessed at 24, 48 and 72 h, week 1, week 3 and week 6. This multi-endpoint

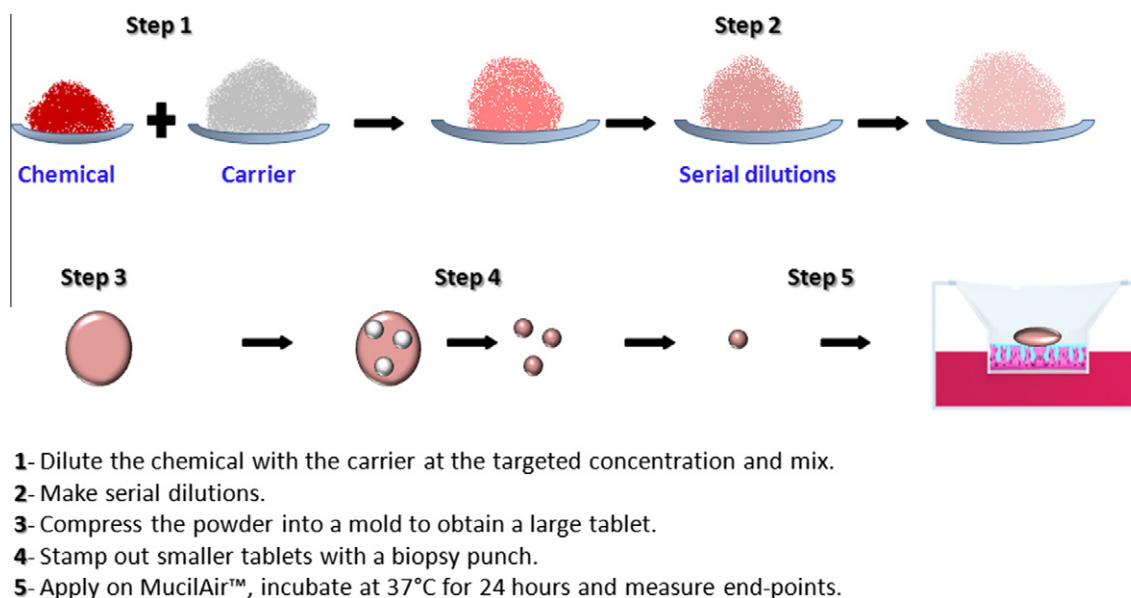


Fig. 1. Schematic representation of the Dextran tablet preparation.

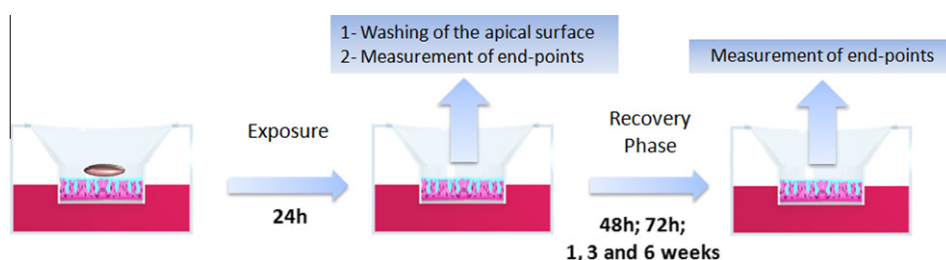


Fig. 2. Schematic representation of the experimental procedure: 2 days before the experiments, the apical surface of the epithelium was washed to remove the accumulated mucus. Then the Dextran tablets containing the desired amount of test chemicals were deposited on top of the epithelium with a pair of forceps. After 24 h of exposure, the apical surface was washed with saline solution to remove the test compounds. The endpoints were simultaneously assessed at 24 h, 48 h and 72 h, week 1, week 3 and week 6.

test strategy greatly enhanced the sensitivity and reliability of the test system (Fig. 2).

To assess the potential of MucilAir™ as a tool to discriminate and to classify the respiratory sensitizers, some well known reference chemicals are used as training compounds. For example, based both on epidermological and animal studies, Methylene diphenyl diisocyanate (MDI), Trimellitic anhydride (TMA) and Ammonium hexachloroplatinate (HCPT) have been shown to be potent respiratory sensitizers; whereas Dinitrochlorobenzene (DNCB), Cinnamic aldehyde and Tetramethyl thiuram disulfid (TMTD) are classified as skin sensitizers; Salicylic acid, Phenol as respiratory irritants and Cinnamic Alcohol as pro- hapten. These substances are often used in academic as well as industrial researches and abundant literatures exist (For reviews, see Kimber and Dearman, 2005; Roggen et al., 2006; Isola et al., 2008). For these reasons, we also chose these chemical compounds for our initial tests. Dose-finding experiments were performed using reduction in TEER as read-out and presented in the Supporting information as well as data on IL-8, IL-6, Gro- α and MCP-1 release. In the following graphics, the effects of several different chemicals on the releases of RANTES are presented. Interestingly, two days after removal of the compound (e.g. 72 h time point, Fig. 3), RANTES is highly unregulated upon exposure to respiratory sensitizers. This up-regulation is not observed for respiratory irritants and dermal sensitizers. Similar results were obtained for other cytokines/chemokines (Supporting data).

The effects of MDI, TMA and HCPT on cytokine release are not attributable to their cyto-toxicity on the epithelial cells: MDI didn't show obvious toxicity at all the concentrations tested; TMA stimulated the cytokine release already at $0.5 \mu\text{g}/0.33 \text{ cm}^2$ but showed toxic effects only starting from $96.1 \mu\text{g}/0.33 \text{ cm}^2$; Similarly, HCPT enhanced the cytokine release at concentrations 10 folds lower than its toxic effects. These observations suggest that the chemical sensitizers such as TMA, HCPT and MDI activate the intrinsic and specific signaling pathway(s) in the airway epithelial cells, leading to the increased cytokine/chemokine releases.

4. Discussion and conclusion

In this article, we described a novel method for applying the water insoluble chemicals onto the apical surface of the airway epithelia reconstituted *in vitro*. We also used this method to test the respiratory chemical sensitizers like MDI, TMA, HCPT on MucilAir™, in order to assess the potential of this *in vitro* cell system for identifying the respiratory sensitizers.

Most of the chemical sensitizers like MDI, TMA are strong electrophiles and react rapidly in water, so they are difficult to test as solution. Using the Dextran carrier method, such obstacle can easily be overcome. This method is relatively cheap compared to the dynamic airflow systems. This Dextran carrier method can also

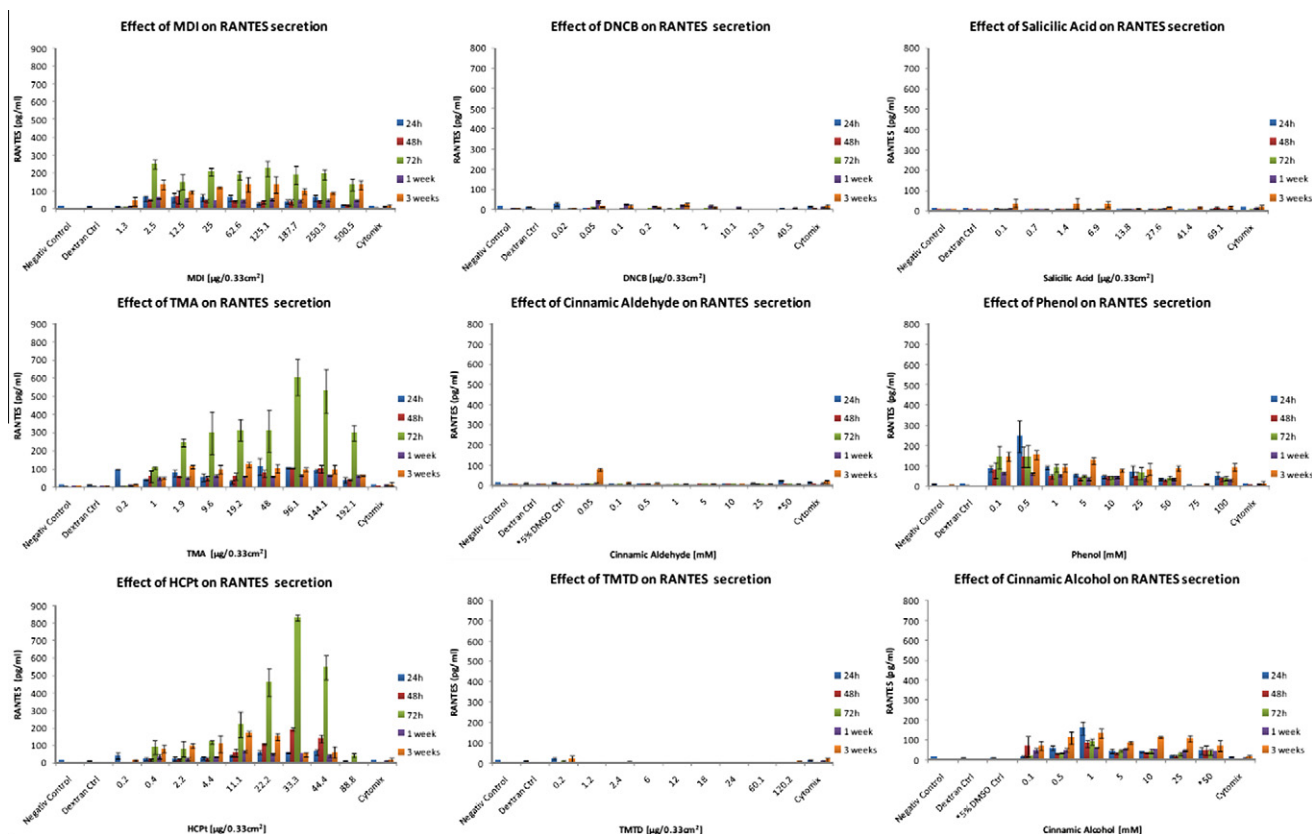


Fig. 3. Dose response curve – RANTES secretion upon exposure to selected chemicals ($N = 3$).

be used for delivering other insoluble materials such as nanoparticles.

As validation, we quantified by HPLC the amount of salicylic acid contained within each tablet dissolving them in DMSO and found the exact values as calculated. Judging by the small standard deviations of each data point, in terms of cytokine release as well as the toxicity, the experimental results obtained further validated the Dextran carrier method.

Using this method, we successfully tested the effects of MDI, TMI and HCPT on cytokine releases on MucilAir™. As control reference chemicals, dermal sensitizers (DNFB, Cinnamic Aldehyde, TMTD) as well as respiratory irritants (phenol, salicylic acid, and pro-hapten (Cinnamic Alcohol) were tested in the same sets of experiments. Several cytokines/chemokines (IL-6, IL-8, Gro- α , RANTES, MCP-1) were used as biomarker to assess the effects of these chemical compounds.

The results of the quantification of the inflammatory mediators, before and after the exposure to the 9 chemicals, provided a rich data set. It looks like that each compound had very specific pharmacodynamics on the different cytokines.

- Regarding respiratory sensitizers, even if the profile of IL-8, IL-6 and MCP-1 secreted after 24 h exposure is not the same for MDI, TMA and HCPT, it is clear that the secretion increases with time. GRO- α and RANTES seem to react differently. While the first cytokine increases after 24 h, then decreases to a normal level and then increases again after 2 weeks, the second one only increases after 72 h.
- In contrast, the three dermal sensitizers, DNFB, Cinnamic aldehyde and TMTD, don't show significant effects on any cytokines secretion, even though at certain concentrations the levels are slightly higher than control values.

- The three irritants tested, Salicylic acid, Phenol and Cinnamic alcohol, don't show the same profile of cytokines release. Phenol and Cinnamic alcohol strongly stimulate IL-8, IL-6, MCP-1, GRO- α and RANTES release, whereas Salicylic acid has small effects. Moreover, GRO- α and RANTES secretion seem to be able to discriminate respiratory sensitizers from irritants. GRO- α secretion is stimulated at later time points, after 1 and 3 weeks. RANTES secretion is stimulated at all time points but with moderate amplitude.

For the moment, we don't know the underlying molecular and cellular mechanisms of the increased releases of these cytokines upon exposure to the chemical sensitizers. We speculate that, like the skin sensitizers (Aptula and Roberts, 2006; Roberts and Aptula, 2008; Roberts et al., 2007), the respiratory sensitizers must have the ability to react covalently, usually as an electrophile, with protein or peptide in the airway epithelial cells. Indeed, respiratory chemical sensitizers like TMA, TDI, HCPT, are strong electrophiles and highly reactive. Since there are no immune cells in MucilAir™ model, the effect of respiratory sensitizers on cytokine release must be direct, without the help of leukocytes. Their targets reside in epithelial cells. But it remains to uncover what and where their targets are. Whatever the targets are, it looks like that they are specifically, abnormally, persistently activated by the respiratory sensitizers. Since the expression of most, if not all, the cytokines and chemokines requires the activation of NF- κ B signaling pathway (Smale, 2011), we hypothesize that the chemical respiratory sensitizers activate somehow the NF- κ B pathway. In contrast, the dermal sensitizers seem to be unable to activate the NF- κ B signaling pathway, at least in this series of experiment (Fig. 3). Consistent with this idea, it has been shown that NF- κ B is highly and persistently expressed in the asthmatic patients (Hart et al., 1998) and a

master-regulator of NF- κ B signaling, IRAK-M, is involved in early-onset persistent asthma (Balaci et al., 2007). Interestingly, to explain the molecular mechanism of the skin sensitization, (Natsch, 2010) proposed that a covalent modification of cysteine residues on Keap1 by cystine-reactive chemical allergens will stimulate, among other changes, the production of IFN- γ that will promote Th1 cells and the acquisition of skin sensitization. He also speculated that the respiratory chemical allergens may preferentially or exclusively bind to lysine residues of an unknown target to stimulate IL-4 production, leading to Th2-type responses favoring sensitization of the respiratory tract. The validity of this theory need to be tested further.

Respiratory allergic diseases such as asthma are as often characterized as a condition that depends on over-expression of TH-2 and under-expression of TH-1 cytokines by immune cells. Interestingly, the TH-2 cytokines are also integral parts of airway epithelium: For example, IL-4 and IL-13 signaling pathways are essential for the differentiation and regeneration of the airway epithelium, especially for the goblet cell differentiation (Allahverdian et al., 2008; Atherton et al., 2003; Booth et al., 2007). Furthermore, it has been shown that IL-13 is expressed by human airway epithelial cells itself (Allahverdian et al., 2008). Taken together, the TH-2 response seems to be “hardwired” in the airway epithelial cells. The combination of the genetic predisposition and environmental stimuli may cause over-expression of the TH-2 cytokines in the airway epithelial cells, leading the allergic reactions.

In fact, exposed to the same allergen, not everyone will develop respiratory diseases like asthma. So, the genetic background is particularly important in allergic responses (Barnes and Marsh, 1998). Indeed, asthma and atopic diseases tend to cluster in families (Gerrard et al., 1976). Furthermore, linkage studies show a relationship between asthma and the chromosome 5q within which several genes coding for TH-2 cytokines (IL-3, 4, 5, and IL-13) are located (Postma et al., 1995; Marsh et al., 1994; Holgate, 1999). In light of a recent publication, showing that 76 percent of disease-associated variants in non-gene regions are actually located within or are tightly linked to regulatory DNA (Maurano et al., 2012), the relationship between asthma and 5q is particularly significant: This suggests that allergic diseases may result from changes in when, where, and how genes are turned on rather than changes to the gene itself. As discussed before, the NF- κ B signaling pathway is central to immune responses, both innate and adaptive (Barnes and Karin, 1997; Caamano and Hunter, 2002; Baltimore, 2011; Ruland, 2011; Ben-Neriah and Karin, 2011), we postulate that the respiratory sensitizers must, one way another, activate the NF- κ B signaling pathway. In the normal individuals, the expression of the TH-2 cytokines in the airway epithelial cells may be low or moderate. Alternatively, activation of NF- κ B signaling pathway may also lead to the expression of the antagonists of TH-2 cytokines. In this regard, IL-12, a TH-1 cytokine which suppresses the expression of the TH-2 cytokines, could be an ideal candidate. Indeed, expression of IL-12p40 is also driven by NF- κ B transcription factors and its expression in the airway epithelial cells has been reported (Murphy et al., 1995; Walter et al., 2001; Holtzman et al., 2002). In contrast, in atopic individuals, activation of NF- κ B signaling pathway may be abnormal and persistent, causing excessive expression of the TH-2 cytokines and allergic responses (Fig. 4). This hypothetical mechanism may operate not only in the airway epithelial cells but also in the leukocytes, whenever the NF- κ B signaling pathway is activated. Our hypothesis may appear to be, perhaps is, over-simplified, but it provides us with a theoretical framework as well as some insights for the future assay development.

If this hypothesis was correct, one would expect that the airway epithelial cells from only a subpopulations of individuals respond to the respiratory sensitizers by secreting increased

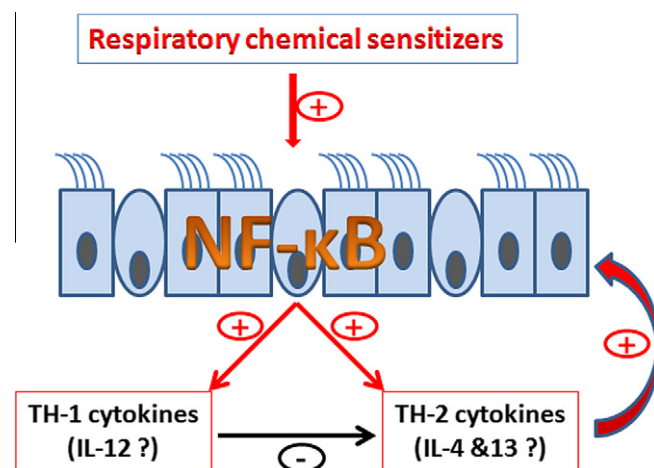


Fig. 4. Schematic illustration of the underlying cellular and molecular mechanism of the effects of the respiratory chemical sensitizers on the airway epithelial cells: We postulate that the TH-1 and TH-2 cytokines are integral components of the airway epithelial cells; even though they are mainly expressed by the T cells *in vivo* situations. NF- κ B signaling pathway plays a central role in this allergic reaction. We speculate that the respiratory chemical sensitizers have the capacity to activate NF- κ B signaling pathway. Once activated, NF- κ B will in turn activate the gene expression of both TH-1 and TH-2 cytokines. In the non-allergic individuals, these two antagonist pathways are in equilibrium. However, in individuals with certain genetic predispositions, activation of NF- κ B by allergens will lead to over-expression of the TH-2 cytokines, which could be further enhanced by a cascade of inflammatory reactions, with several positive feedback loops, involving a plethora of down-stream effectors cells (eosinophil, basophils, mast cells, etc.). The end result is the allergic responses exemplified by diseases like asthma. This hypothetical mechanism may operate not only in the airway epithelial cells but also the leukocytes, whenever the NF- κ B signaling pathway is activated. + means stimulation; – means inhibition.

Table 1
Suitable cytokine for the discrimination of respiratory sensitizers.

Discrimination potential	Recommended cytokine and recovery time
Respiratory sensitizers vs dermal sensitizers	RANTES (72 h) IL-6 (24, 48 and 72 h; 1, 3 and 6 weeks) MCP-1 (3 and 6 weeks)
Respiratory sensitizers vs respiratory irritants	RANTES (72 h) Gro- α (24 h)

amount of cytokines/chemokines. It seems to be the case (unpublished data).

In conclusion, the reported results, also preliminary, show that the *in vitro* cell model of the human airway epithelia, MucilAir™, has probably the potential to discriminate the respiratory sensitizers from dermal sensitizers or respiratory irritants (Table 1). Further investigations are needed to develop and validate *in vitro* assay based on MucilAir™ for identifying the respiratory sensitizers.

Conflict of Interest

The authors state no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tiv.2012.10.010>.

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Title: Potential of *in vitro* reconstituted 3D human airway epithelia (MucilAir™) to assess respiratory sensitizers

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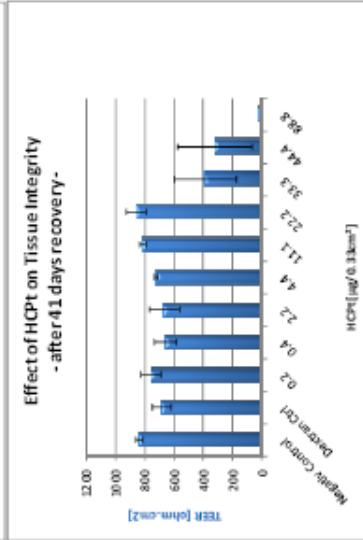
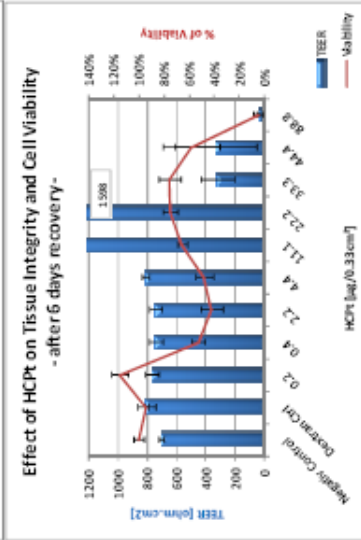
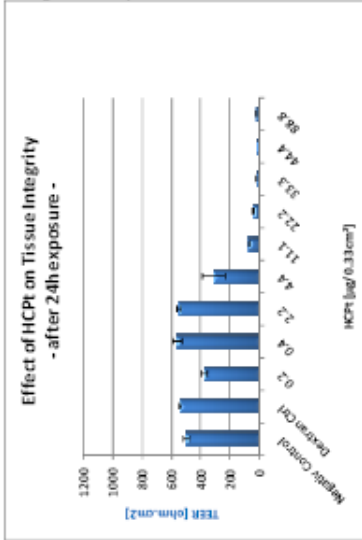
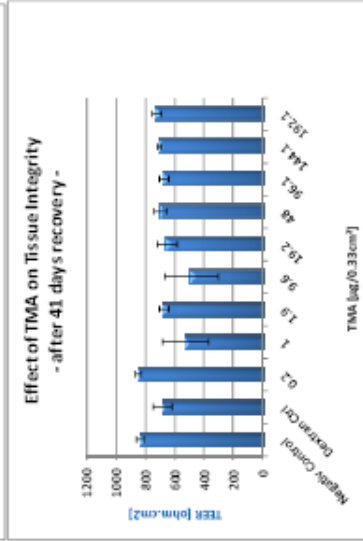
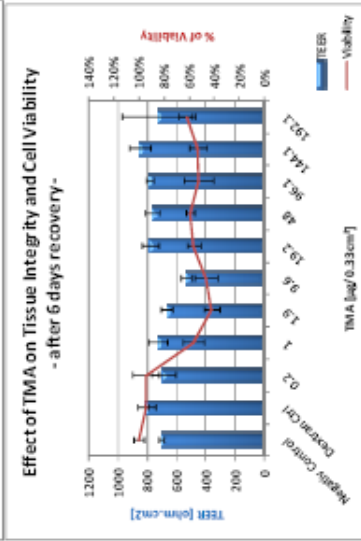
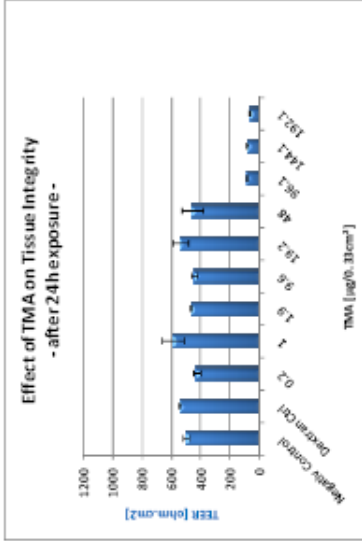
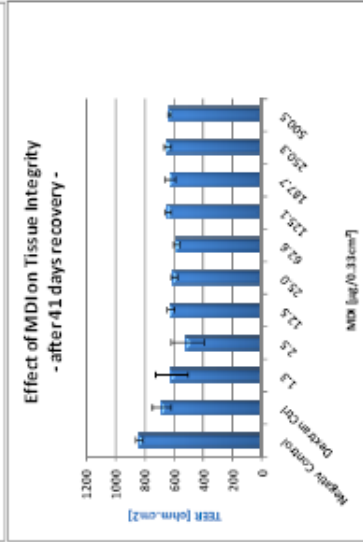
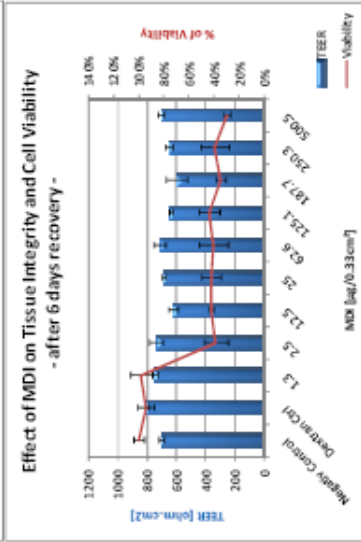
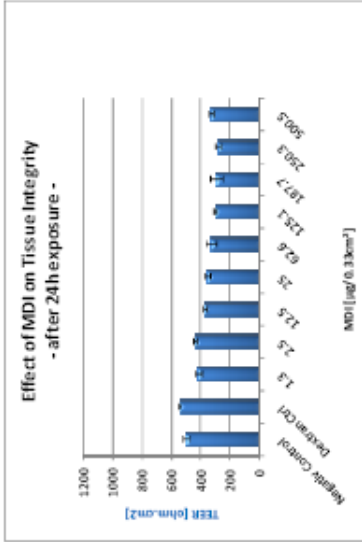
² Novozymes, Krogshoejvej 36, 2880 Bagsvaerd Denmark

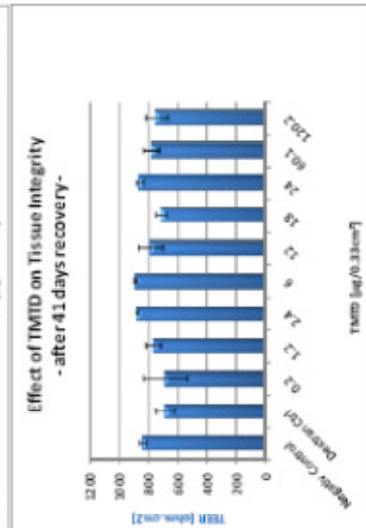
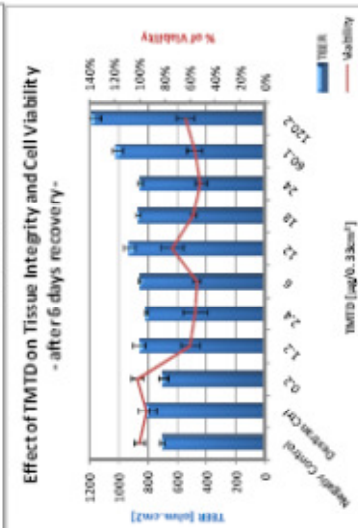
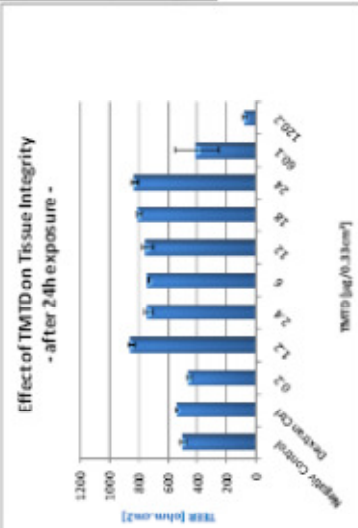
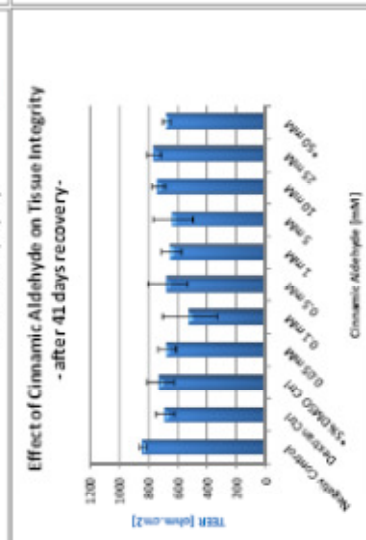
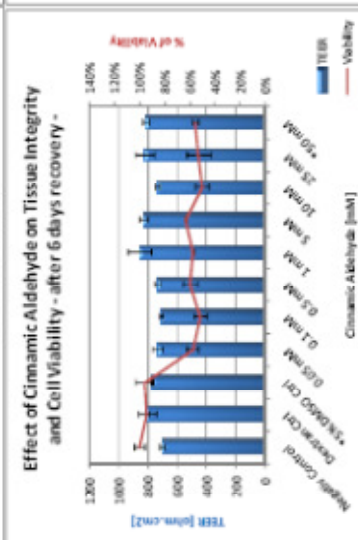
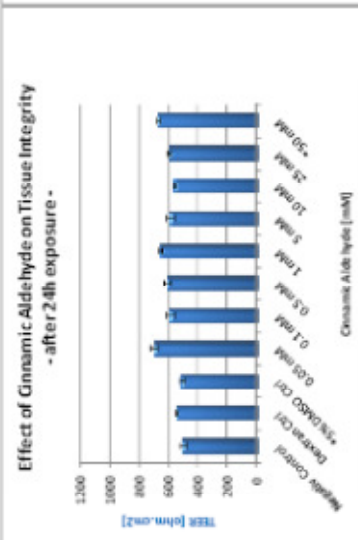
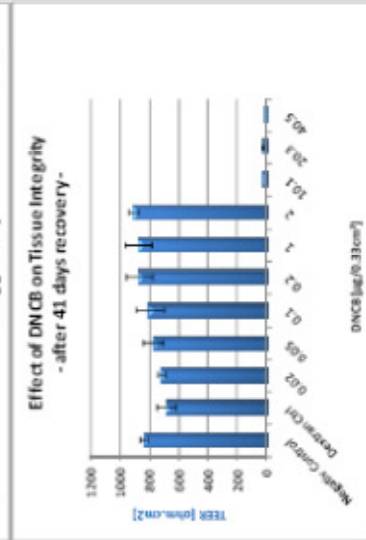
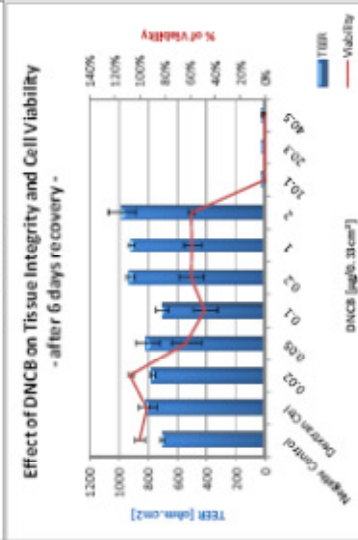
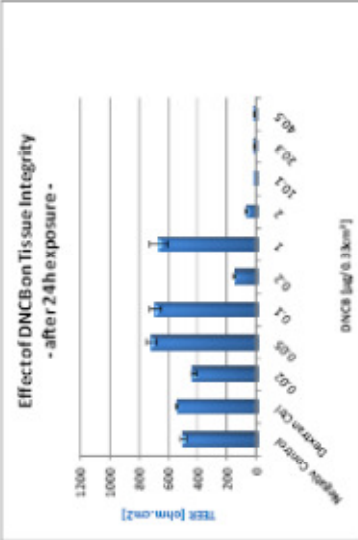
SUPPORTING INFORMATION

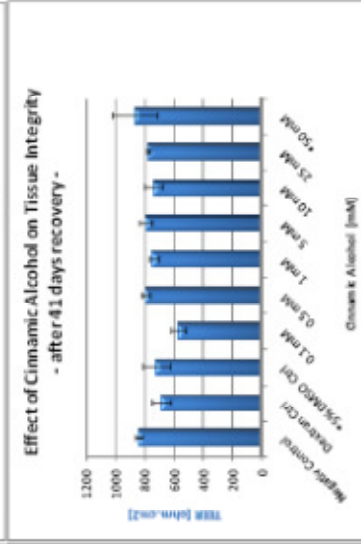
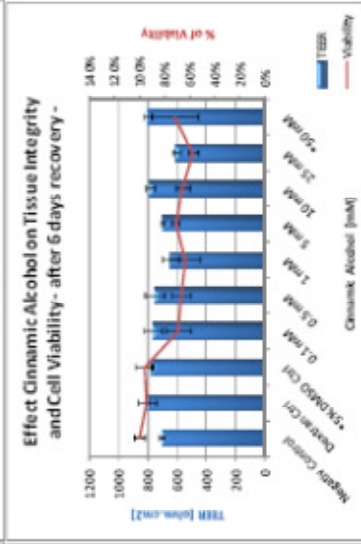
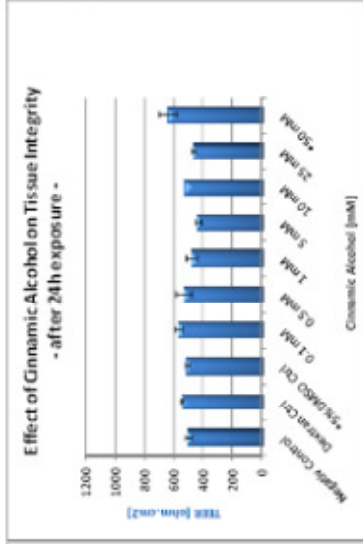
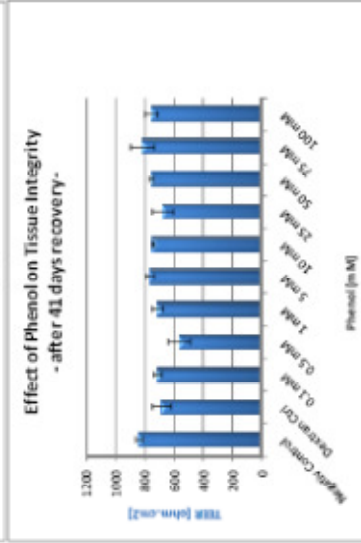
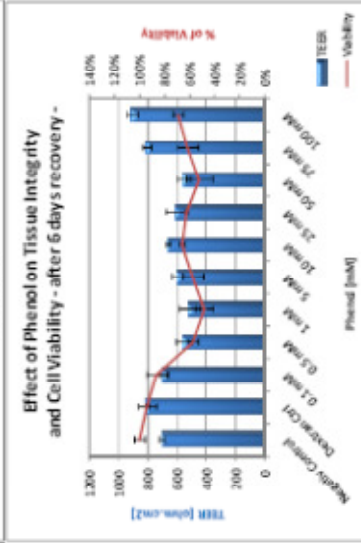
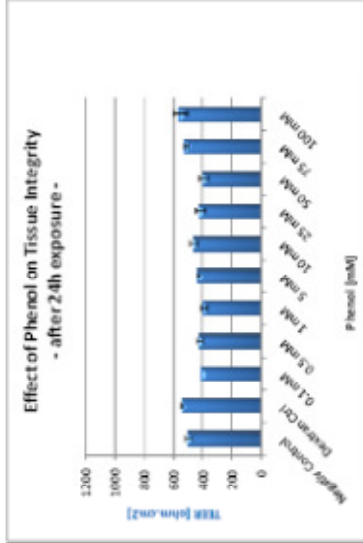
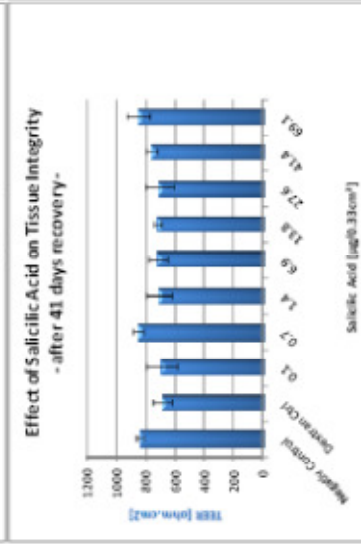
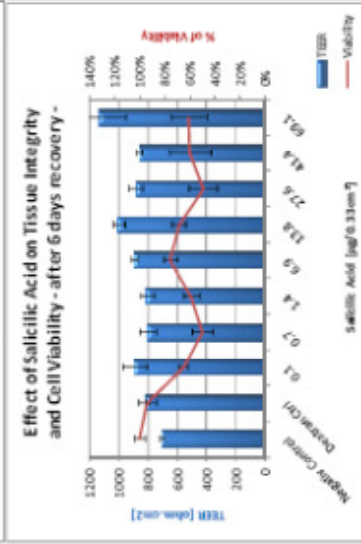
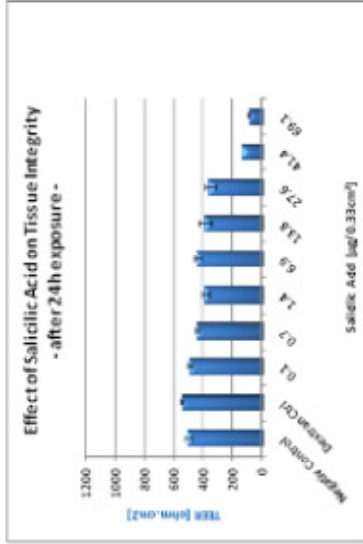
Tissue Integrity and Cell Viability assessment

The following graphics represent the result of the toxicity test for all compounds. The change of TEER reflects the integrity of epithelia. For example if holes were present or if cellular junctions were broken, the TEER values would be generally below 100 $\Omega\cdot\text{cm}^2$. In contrast when epithelia are not damaged, the TEER values are typically comprised between 200 to 600 $\Omega\cdot\text{cm}^2$. A notable decrease of the TEER value (but > 100 $\Omega\cdot\text{cm}^2$) could be observed in certain cases, it generally reflects an activation of the ion channels. A drastic increase of the TEER value reflects a blocked ion channel activity or a destruction of the ciliated cells. The result of the percentage of viability (value of the fluorescence of Resazurin transformed after one hour incubation with cells) reflects the metabolic activity of cells and consequently the cell viability. However, variation in the percentage of viability, without any effect on TEER, reflects an effect on cell metabolism and not an effect on cell viability. When the cell viability is affected, the TEER value of the corresponding insert would be inferior to 100 $\Omega\cdot\text{cm}^2$.

Note: For TEER the error bars represent the standard error of the mean (SEM) and for Resazurin, the error bars represent the standard deviation (N=3).







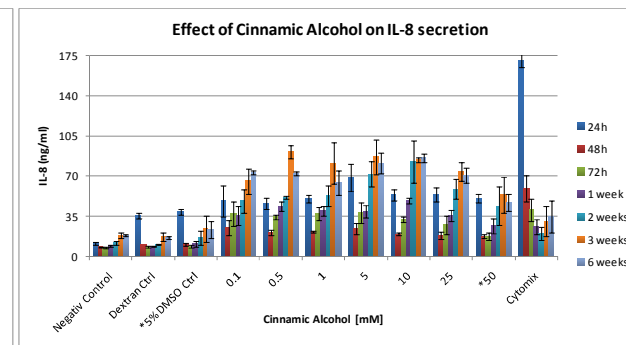
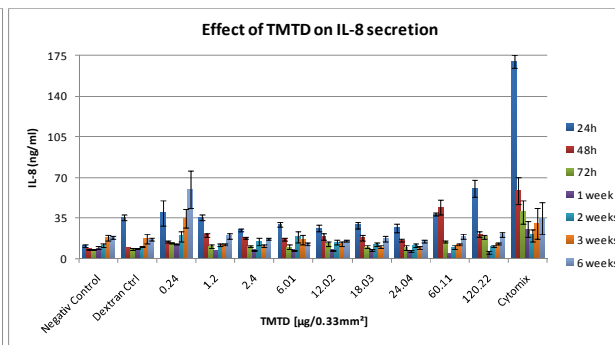
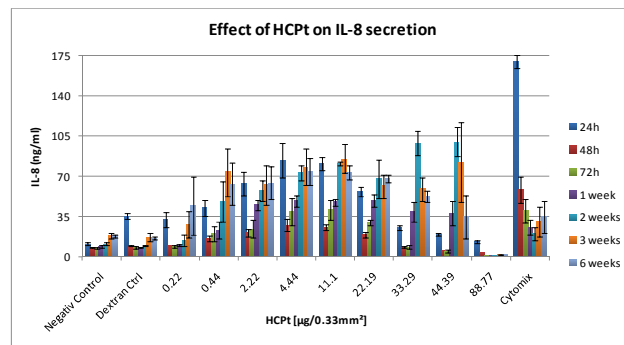
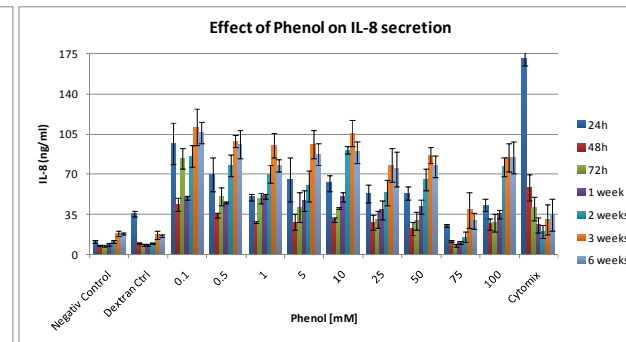
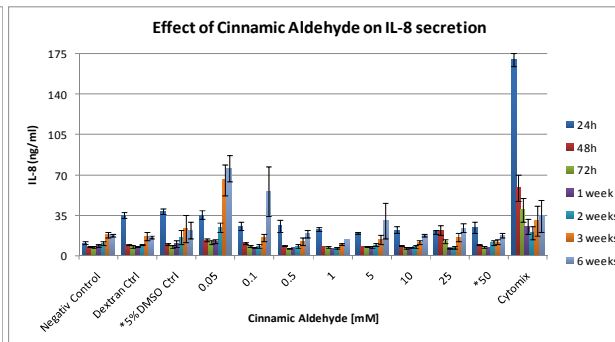
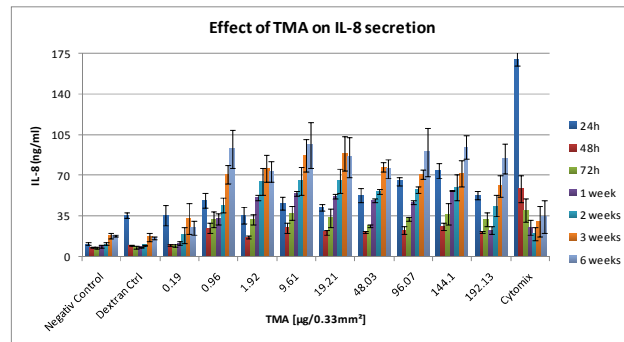
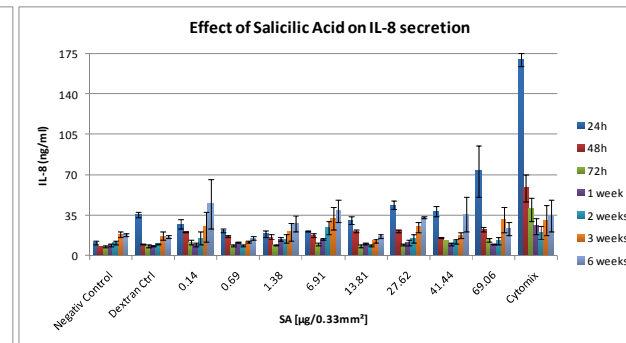
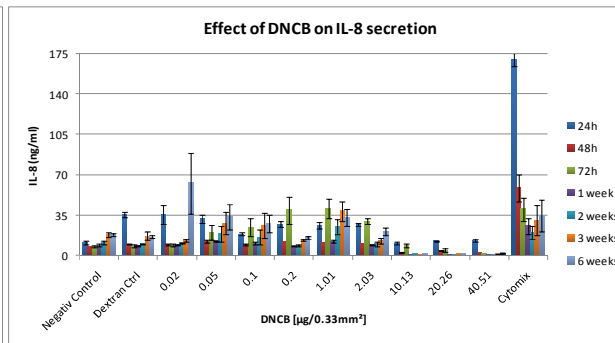
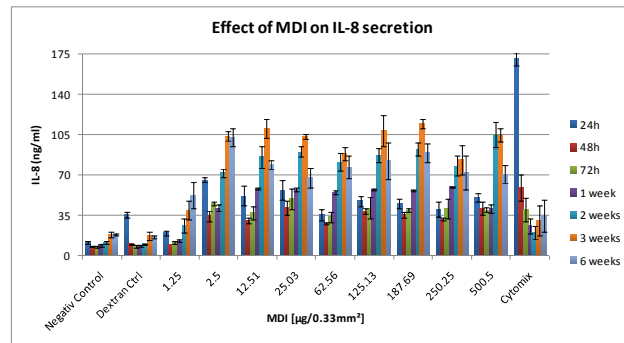
Quantification of soluble factor release

Cytokines release in the culture medium were quantified by ELISA using the following commercially available detections kits:

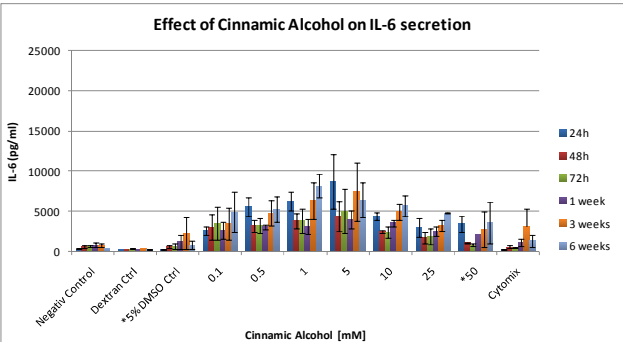
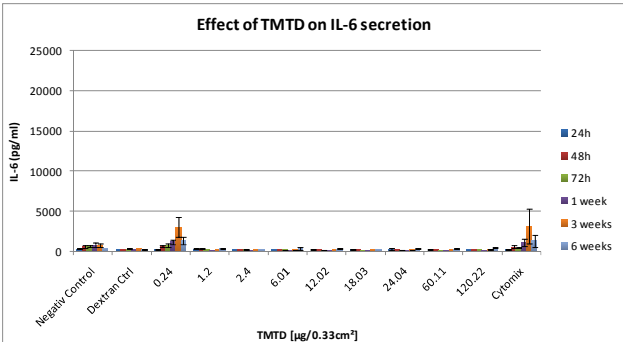
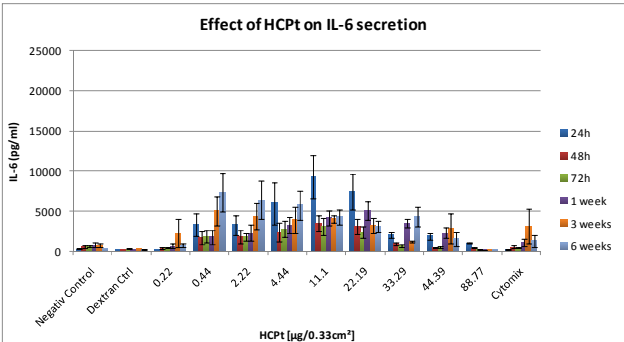
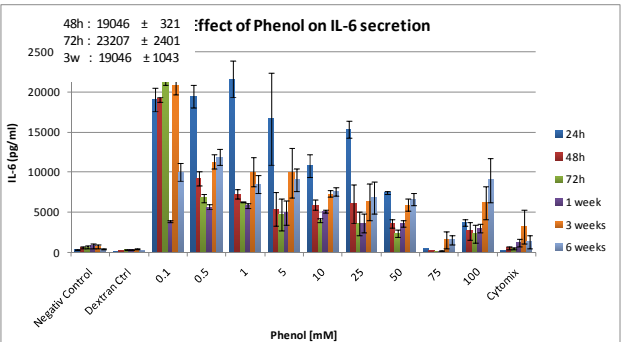
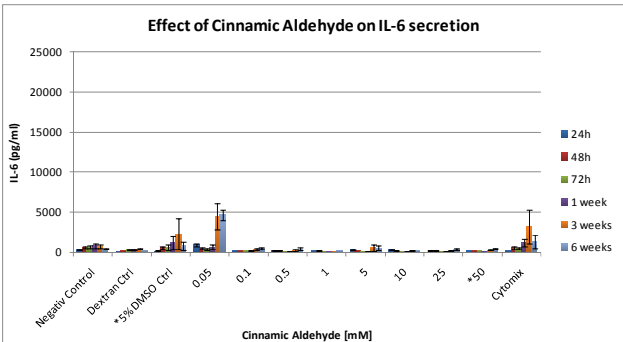
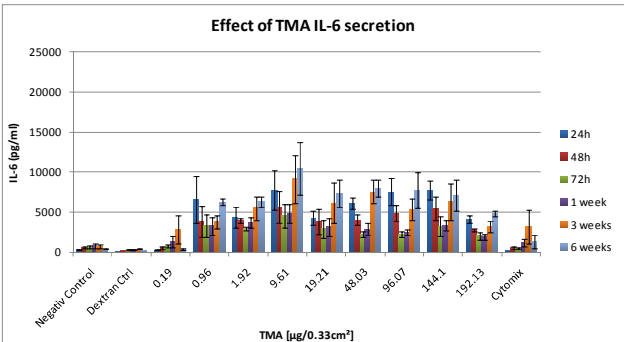
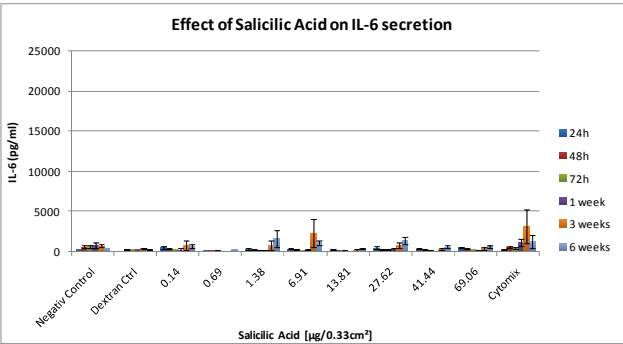
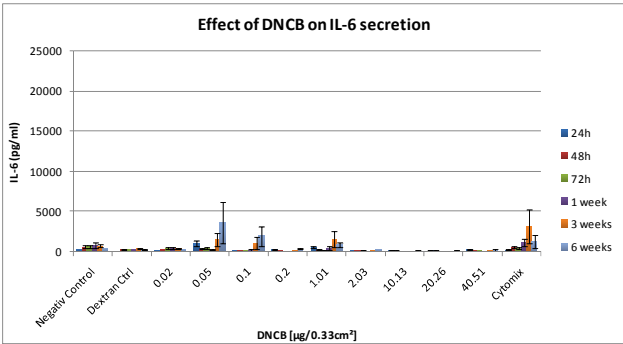
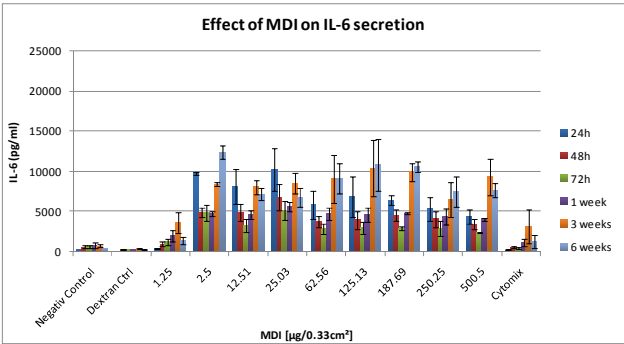
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- ✓ IL-6 : Becton Dickinson – OptEIA™ Human – IL8 set # 555220
- ✓ MCP-1 : Becton Dickinson – OptEIA™ Human – MCP-1 set # 555179
- ✓ GRO- α : R&D Systems® – Human GRO- α DuoSet – # 555244
- ✓ RANTES : R&D Systems® – Human RANTES DuoSet – # 555244

Note: For soluble factor release the error bars represent the standard error of the mean (SEM - N=3).

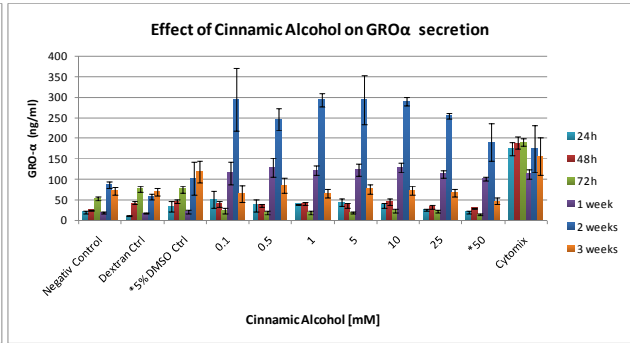
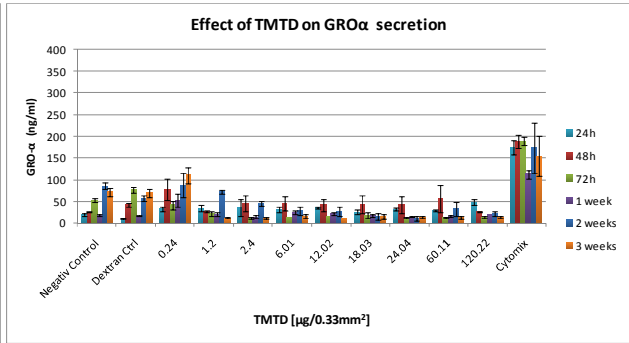
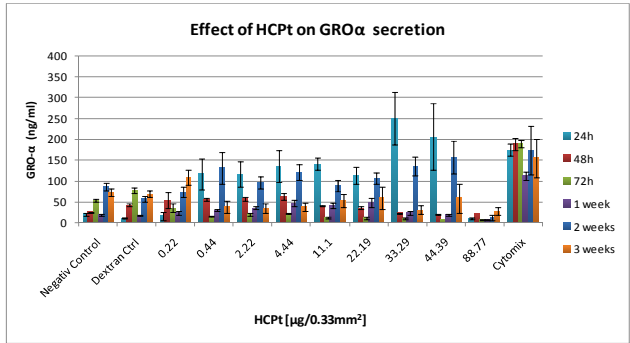
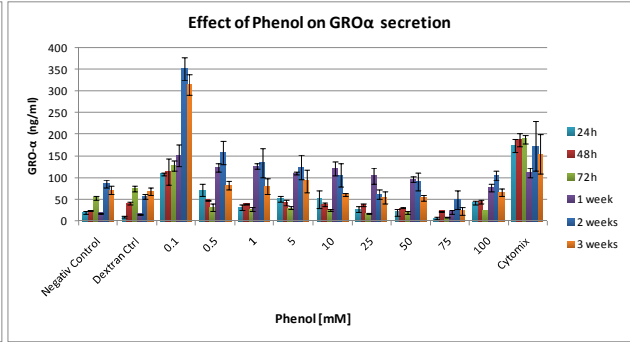
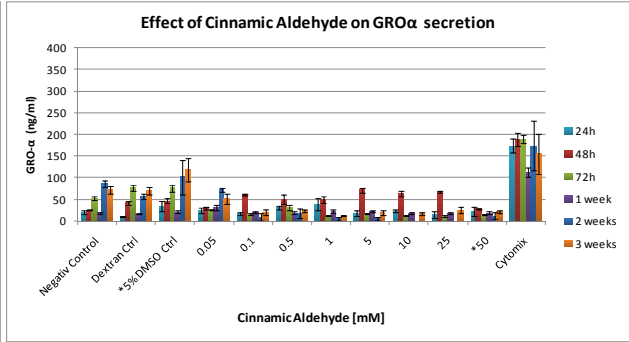
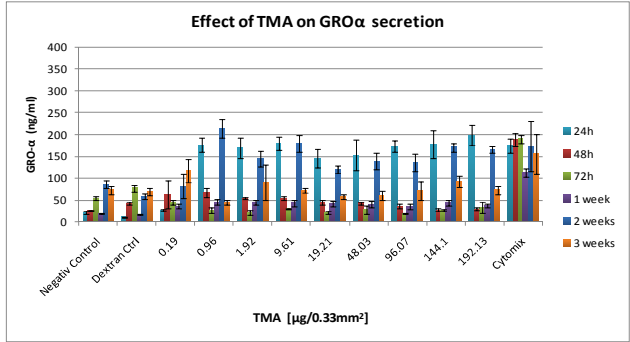
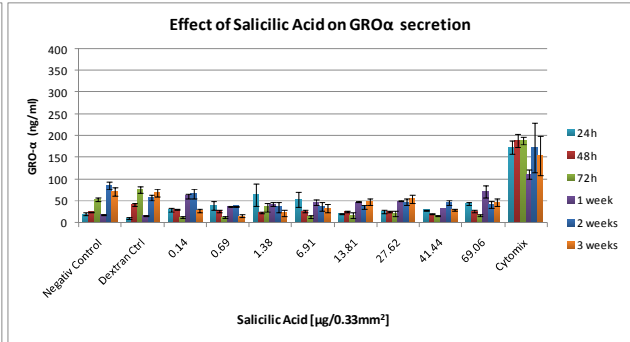
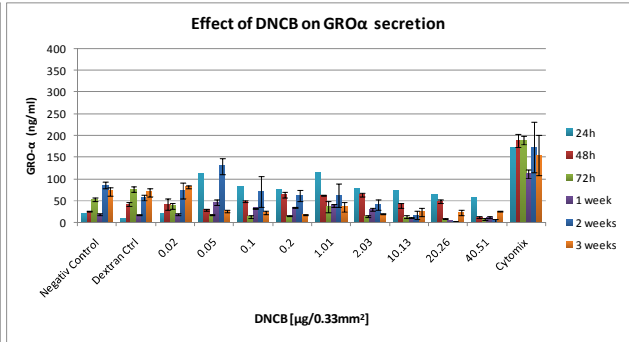
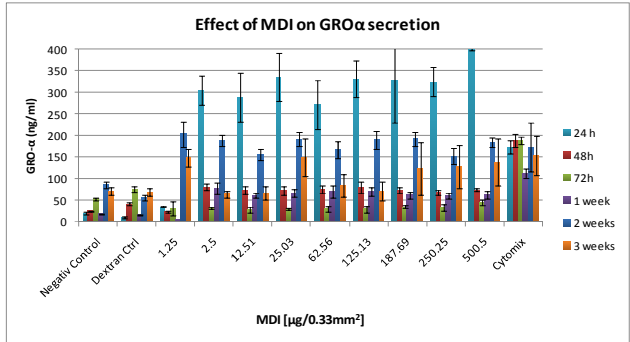
IL-8



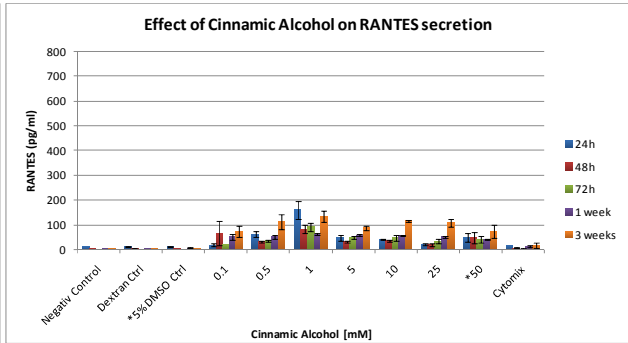
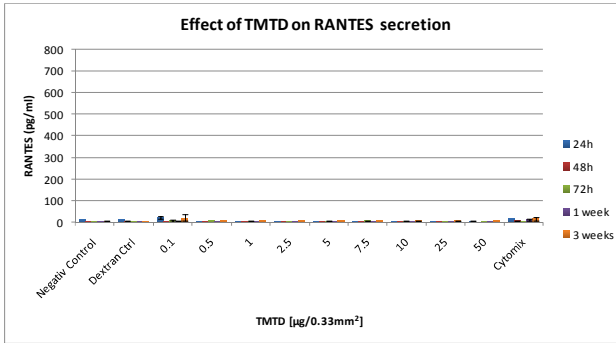
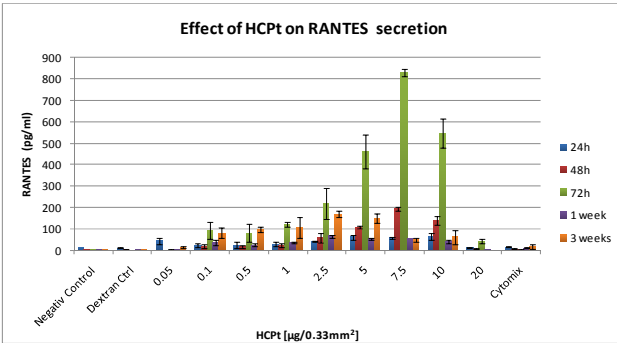
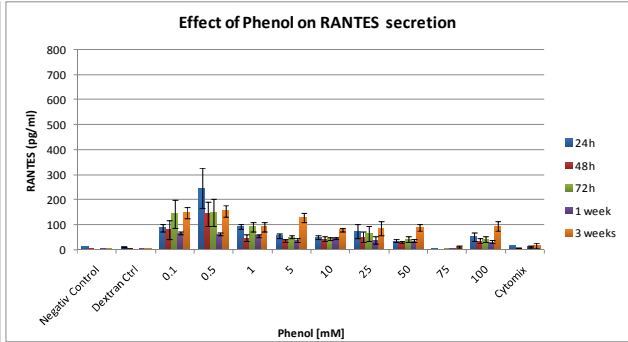
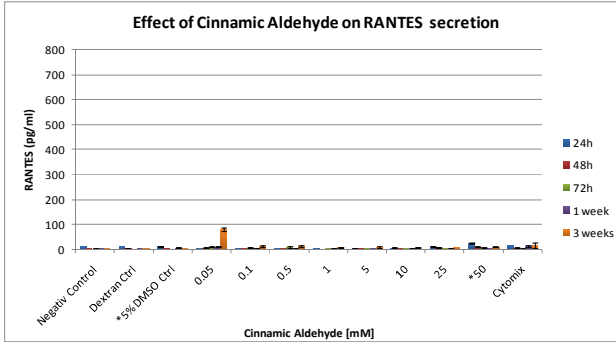
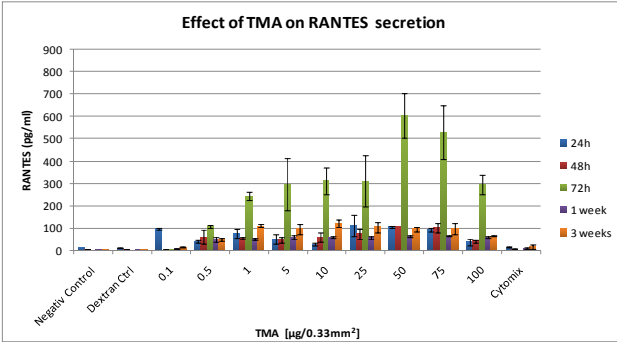
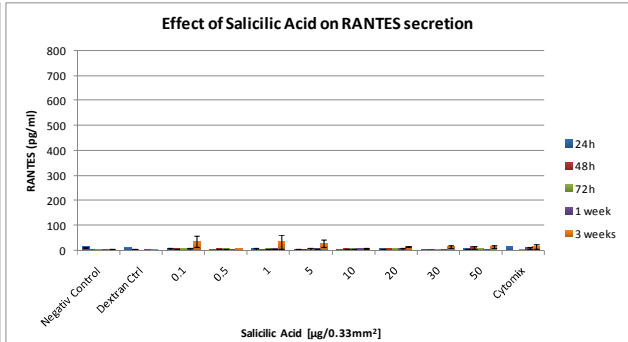
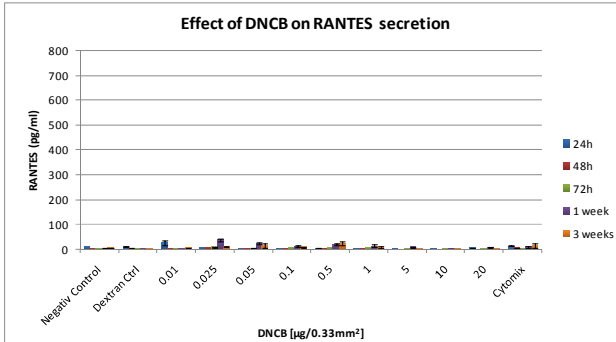
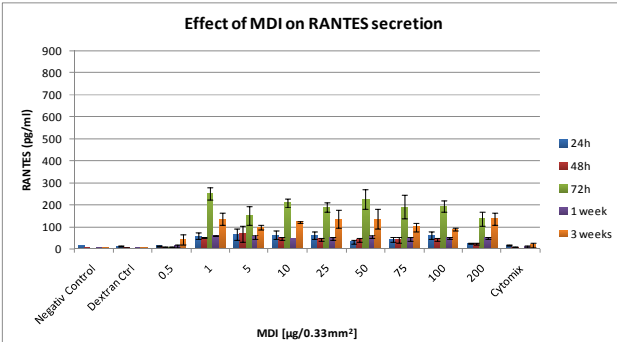
IL-6



GRO-α



RANTES



MCP-1

