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The Effects of Electronic Cigarettes on Human Monocyte-derived Dendritic Cells

Submitted by

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Abstract

Electronic cigarettes (E-cigarettes) are electronic devices producing vapour for inhalation to simulate the experience of smoking conventional cigarettes. They first became publically available in 2004-2006 and gained rapidly in popularity around the world. Although E-cigarettes are promoted as a safe alternative to conventional cigarettes and a tool for smoking cessation, there is growing evidence for adverse effects of E-cigarette use and the effects of E-cigarettes on the human immune system are not unclear. Due to the important role of DCs in the immune system of linking innate and additive immunity, the aim of this study was to investigate the effects of E-cigarette vapour extract (ECVE) on DCs in vitro at a variety of levels and using various techniques.

Human DCs were generated from fresh blood of healthy, never-smoker donors using positively selected CD14+ monocytes. The monocyte-derived DCs (MoDCs) were then exposed to ECVE. The viability of DCs under ECVE exposure was firstly investigated using flow cytometry. The signalling pathways of DCs were also assessed using reverse phase protein array (RPPA), followed by analysis of surface marker expression using flow cytometry. Moreover, cytokine production by DCs was determined using enzyme-linked immunosorbent assay (ELISA). Finally, the ability of DCs to communicate with T cells and their influence on T cell proliferation was examined using allogenic mixed lymphocyte reaction (MLR). In addition, a clinical case of a patient who developed hypersensitivity pneumonitis after E-cigarette use was investigated in terms of the production of antibodies specific to constituents of the E-liquids.

Abstract

Despite an inhibitory effect on the expression of certain cell-surface markers of DC activation, ECVE treatment of DCs significantly upregulated expression of certain individual signalling molecules expression and induced an overall (or 'global') upregulation of pro-inflammatory signalling molecules in DCs. Moreover, ECVE stimulated the secretion of pro-inflammatory cytokine from DCs. ECVE treatment of DCs also promoted their ability to activate T cells. These results varied between types of stimulation and concentration of ECVE. A clinical case study reported the development of hypersensitivity pneumonitis after E-cigarette use.

In conclusion, this study shows that exposure to ECVE affects the phenotype and functionality of DCs. ECVE generally promoted stimulatory effects on DCs which might lead to excessive immune responses. Therefore, this study raises concerns about the potential negative influence of the long-term use of E-cigarettes.

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Abbreviations

APCs	Antigen presenting cells
BALF	Bronchoalveolar lavage fluid
CCR	Chemokine receptor
cDC	Conventional dendritic cell
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSE	Cigarette smoke extract
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
dsDNA	Double stranded DNA
E-cigarette	Electronic cigarette
eCO	Exhaled carbon oxide
ECVE	Electronic cigarette vapour extract
ELISA	Enzyme linked immunosorbent assay

eNO	Exhaled nitric oxide
ER	Endoplasmic reticulum
ERK	extracellular signal regulated kinase
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FeNO	Fractional exhaled nitric oxide
FITC	Fluorescein isothyocyanate
GM-CSF	Granulocyte macrophage colony stimulating factor
HFOV	High frequency oscillatory ventilation
HLA	Human leukocyte antigen
HP	Hypersensitivity pnumonitis
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ii	Invariant chain
IRAK	Interleukin receptor associated kinase

IRF	Interferon regulatory factor
JNK	c-Jun N terminal kinase
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LSP-1	Lymphocyte specific protein-1
MAPK	Mitogen activated protein kinase
mDCs	Myeloid dendritic cells
MHC	Major Histocompatibility Complex
MFI	Median fluorescence intensity
MLR	Mixed lymphocyte reaction
MMPs	Matrix metalloproteinase
MoDCs	Monocyte-derived dendritic cells
MRSA	Methicillin-resistant Staphylococcus aureus
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
PAMPS	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

pDCs	Plasmacytoid dendritic cells
PE	Phycoerythrin
PG	Propylene glycol
PIP5K1C	Phosphatidylinositol -4- phosphate-5-kinase
Poly I:C	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
RPPA	Reverse phase protein array
SPT	Skin prick test
Th	T helper cells
TLR	Toll-like receptor
TMB	Tetramethyl benzidine
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cells
TSNAs	Tobacco-specific nitrosamines
VG	Vegetable glycerol
VV ECMO	Veno-Venous Extracorporeal Membrane Oxygenation
WHO	World health organization

Chapter 1 General Introduction

1.1 Dendritic cells

The initiation of immune responses depends largely on dendritic cells (DCs) which are professional antigen presenting cells (APC). Due to their ability to recognize and respond to antigens, DCs not only act in first line defence of the body but also play a critical role in bridging innate and adaptive immunity.

DCs are leukocytes that are derived from myeloid and lymphoid progenitor cells in the bone marrow [1]. They circulate in peripheral blood and are abundant at sites that are in contact with the external environment or high antigenic exposure, such as skin and mucosal surfaces. DCs are activated by antigens, process and present them to naïve T cells within the context of major histocompatibility complex (MHC) and co-stimulatory molecules [2]. In addition to T cell activation, DCs promote immune tolerance by distinguishing, and preventing reactivity against, self-proteins [3].

1.1.1 Human DC subsets

The subsets of DCs are categorized by their surface markers and function. There are two major subsets of DCs: myeloid DCs (mDCs) which are also known as conventional or classical DCs, and plasmacytoid DCs (pDCs). mDCs express CD11c and there are two lineages of myeloid DCs distinguished by expression of CD1c or CD141. mDCs expressing CD141 (BDCA-1) are defined as mDC1, and

have a high intrinsic capacity to cross-present antigens via MHC class I, thereby activating CD8+ T cells; they also promote T helper type 1 (Th1) activity through producing high amounts of IL-12 [4,5]. mDC1 also express toll-like receptors (TLR) TLR3, TLR9 and TLR10 that recognize virus nucleic acids and secrete type I and type III interferons (IFN-α/β/λ) which are beneficial for viral clearance [5]. mDC2, which express CD1c+ (BDCA-3), are the major population of myeloid DCs in human blood, tissues and lymphoid organs [5]. mDC2 are equipped with a wide range of lectins, TLRs and other pattern recognition receptors for recognizing glycolipid antigens. mDC2 can stimulate both naïve CD4 T cells and cross-present to CD8 T cells [6]; they secrete IL-12, tumour necrosis factor-α (TNF-α), IL-8 and IL-10, but consistently low quantities of type I and type III interferons [7].

pDCs, which circulate in blood and constitute less than 0.5% of peripheral blood mononuclear cells (PBMCs), are distinguished by surface markers CD304 (BDCA-2), CD303 (BDCA-4) and α-chain of the IL-3 receptor (CD123) [5]. pDCs exert potent antiviral activity via their intracellular TLR7 and TLR9 which recognize single-stranded RNA (ssRNA) and unmethylated CpG DNA, and produce large amounts of type-1 interferon (mainly IFN-α and IFN-β) that trigger CD8+ T cell responses [8,9].

CD16+ monocytes express high levels of MHC II and costimulatory molecules, hence a study categorized CD16+ monocytes as a type of blood DCs [10]. CD16+ monocytes expressing antigen 6-Sulpho LacNAc (SLAN) are called Slan+ DCs and part of the dermal mDC system. Slan+ DCs are anti-inflammatory in steady state,

but become highly inflammatory and able to produce large amounts of TNF-α, IL-1β and IL-12 in response to stimuli [11].

Other subsets of human DCs, like Langerhans cells (LCs) which are resident in the basal epidermis of the skin, are found in other stratified squamous epithelia including bronchus and oral mucous membrane [5]. Human LCs express high levels of the C-type lectin Langerin and invariant MHC-I molecule CD1a [5]. Human LC function not only maintains epidermal health and tolerance to commensals but also induces response against intracellular pathogens and viruses under inflammatory conditions [12,13].

1.1.2 Function of DCs

1.1.2.1 Antigen processing and presentation

Immature DCs include epidermal Langerhans' cells, splenic marginal zone DCs and interstitial DCs. Immature DCs are efficient at antigen uptake mainly through three mechanisms (a) macropinocytosis, (b) phagocytosis, and (c) receptor-mediated endocytosis. Macropinocytosis facilitates the uptake of antigens and allows continuous internalization of large volumes of fluid. Phagocytosis via CD36, αvβ3 and αvβ5 integrins also enables the uptake of apoptotic and necrotic cells by forming phagosomes. Phagosomes then fuse with lysosomes, which contain degrading enzymes, leading to the digestion of phagocytosed material. Receptor-mediated endocytosis involves several receptors, including mannose-like receptors, FcγRI, FcγRII and TLRs. Some of these receptors are PRRs which can identify pathogen-associated molecular patterns (PAMPs) and damage-associated

molecular patterns (DAMPs). PAMPs are related to microbial pathogens and DAMPS are cell components released during cell damage or death. The recognition of PAMPs or DAMPs by PRRs results in activation of downstream signalling pathways, thereby inducing maturation and cytokine production by DCs. Table 1.1 shows the TLRs present on human DCs.

Table 1.1 TLRs present on human DCs.

TLRs	Location	PAMPs	Subset	Main Response
TLR1	Surface membrane	Zymosan, lipoproteins, LPS, lipoteichoic acid, peptidoglycans	mDCs	Produce low IL-12 but high IL-10. Induce Th2 and Treg response
TLR2	Surface membrane	Zymosan, lipoproteins, LPS, lipoteichoic acid, peptidoglycans	mDCs	Produce low IL-12 but high IL-10. Induce Th2 and Treg response
TLR3	Endosome/phagosome membrane	Double strand RNA (dsDNA)	mDCs	Produce IL-12 and IFN-α Induce CD8+ T cell and Th1 response.
TLR4	Surface membrane	LPS	mDCs	Produce IL-12 and IFN-α Induce CD8+ T cell and Th1 response.
TLR5	Surface membrane	Flagellin	mDCs	Produce IL-12 Induce Th1 response

TLR6	Surface membrane	Zymosan, lipoproteins, LPS, lipoteichoic acid, peptidoglycans	mDCs	Produce low IL-12 but high IL-10. Induce Th2 and Treg response
TLR7	Endosome/phagosome membrane	ssRNA	mDCs, pDCs	Produce IFN-α Induce CD8+ T cell and Th1 response
TLR8	Endosome/phagosome membrane	ssRNA	mDCs	Produce IL-12 and IFN-α Induce CD8+ T cell and Th1 response
TLR9	Endosome/phagosome membrane	Unmethylated CpG DNA	pDCs	Produce IFN-α Induce CD8+ T cell and Th1 response
TLR10	Surface membrane	Unknown	mDCs, pDCs	Inhibit pro-inflammatory cytokines

Immature DCs circulate in peripheral blood and are abundant at sites readily exposed to antigens, including skin and mucosal surface. Immature DCs are recruited from the circulation to where they capture antigens or detect danger signals. Immature DCs then process and degrade antigens to fragments and migrate to lymph nodes using homing receptors such as the chemokine receptor CCR7. During migration, DCs are activated to mature forms whilst their antigen capture capacity decreases.

In lymph nodes, DCs present antigen fragments to T cell receptors (TCRs) on antigen-specific T cells via major MHC molecule, leading to T cell activation and differentiation. There are three signals required for naive T cell activation, differentiation and proliferation. MHC molecules presenting antigen peptides represent the first signal for interaction between DCs and T cells. Simultaneously, the antigen-specific expansion of naïve T cells requires a second signal conducted by co-stimulatory molecules, such as CD80 (B7.1), CD86 (B7.2). Both CD80 and CD86 bind to CD28 on T cells, resulting in IL-2 secretion which is needed for T cell proliferation. The expression of co-stimulatory and MHC molecules is upregulated during maturation of DCs. Innate activation of DCs induces cytokine production that is considered as third signal to drive Th cell polarisation towards effector subsets.

1.1.2.2 T cell priming

The antigen presentation by DCs leads to naïve T lymphocyte activation and polarization. The orchestrating processes for T cell priming depend on the types of MHC molecules on DCs and their cytokine signatures interacting with T cells. Upon viral infection, mDCs presenting MHC I-peptide complexes to T cells contribute to

cytotoxic CD8+ T cell activation. However, mDCs expressing MHC II-peptide complexes lead to CD4+ T cell activation. Depending on cytokine produced by DCs during T cell activation, CD4+ T cells differentiate to distinct effector phenotypes. IL-12 secretion by DCs stimulates Th1 responses which are related to pro-inflammatory cytokine production. However, the interaction of OX40 ligands on DCs with OX40 on T cells stimulates Th2 responses which contribute to stimulation of anti-inflammatory cytokines, such as IL-4, IL-5 and IL-13 which promote B cell activation. Humoral responses also can be induced by activated Th2 cells that migrate to B cell zones in lymph nodes to activate naïve, antigenic-specific B cells, promoting their clonal expansion and antibody class switching [14]. In addition to Th1 and Th2 cells, Figure 1 shows other T cells polarized by DCs [15].

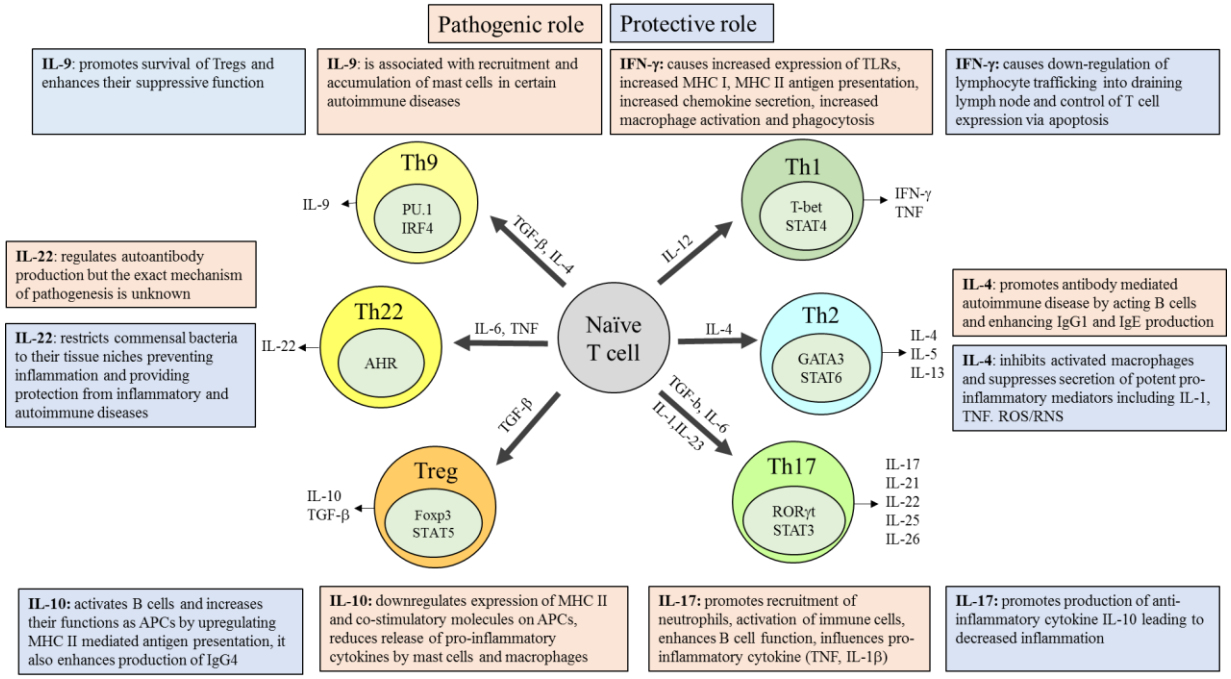


Figure 1.1. T cell polarization by DCs. After interaction with DCs, naïve T cells are polarized into different T cells subsets that exert protective and pathogenic responses via the cytokines they secrete. The figure is adapted from [15].

As well as endogenous antigen presentation, there is another mechanism for DCs to capture exogenous antigens into MHCI complexes called cross-presentation. Antigens involved in cross-presentation are soluble proteins, immune complexes, intracellular bacteria, parasites and cellular antigens. Exogenous antigen is firstly engulfed into endosomes. The exogenous antigens are then transported out of endosomes and degraded to peptides by proteasomes. Peptides are then transported back to endoplasmic reticulum (ER) through TAP complex and bind to MHCI molecules which subsequently travel to cell surface, leading to the cytotoxic T lymphocyte (CTL) responses.

1.1.2.3 Induction of tolerance

DCs are crucial not only for initiation of immune responses but also for induction of immunological tolerance which is essential for preventing pathologic reaction to self-antigens. There are central and peripheral mechanisms of tolerance that are both regulated by DCs. Central tolerance occurs in the thymus when DCs present self-antigens to immature T lymphocytes. Those T lymphocytes with certain levels of autoreactivity then will be programmed to die by apoptosis, called clonal deletion. However, central tolerance is not able to eliminate all the autoreactive T cells: as a result, peripheral tolerance is needed. Generally, there are two mechanisms involved in peripheral tolerance. Those immature DCs expressing low levels of co-stimulatory molecules fail to stimulate T cell activation but provide signal 1, leading a process called anergy (functionally inactive) [16,17]. Moreover, DCs can promote regulatory

T cell (Treg) differentiation or function, which is able to maintain immunological tolerance to self-antigens [18].

1.1.3 Lung DCs

Lung DCs underlie the epithelial layers throughout the lungs, including large and small bronchi and bronchioles and alveoli [19]. Both myeloid and plasmacytoid DCs can be found in the lungs and are present in an immature state in which they exhibit the ability to recognize and capture inhaled antigens [19,20]. After antigen uptake, lung DCs undergo a maturation process and migrate to lymph nodes where they activate naïve T cells. Activated DCs can produce not only pro-inflammatory cytokines that affect other innate cells in lungs but also regulatory cytokines that induce the generation of regulatory T cells. As a result, DCs play a critical role in maintaining tolerance and regulating inflammation in the lungs. Lung DCs can promote the formation of lung lymphoid follicles where DCs also can take up antigen and interact with other cells, driving Th1, Th17 and NK cell responses [21].
Lungs are continuously exposed to foreign particles from the environment that can trigger excessive immune responses, leading to the development of respiratory disorders. Evidence shows that CS and COPD alters lung DC numbers and maturation status [21] which could further induced the proliferation of CD8+ T cells predominantly [22]. CD8+ T cells accumulate in the conducting airways and lungs and produce mediators that leading to the destruction of lung parenchyma. DCs may retain antigen for presentation to T cells over long periods which can cause chronic inflammation in lungs [22].

DCs are also involved in type 2 immunity [20,23]. Allergen exposure stimulates the production of mediators by airway epithelial cells, which in turn trigger lung DCs to secrete chemokines to recruit effector Th2 cells to the lungs, promoting a type 2 allergic inflammatory environment. In addition, those mediators also promote DC migration to lymph nodes and induce polarization of Th2 cells in an OX40L-dependent fashion.

1.2 Electronic cigarettes

1.2.1 Introduction

Electronic cigarettes (E-cigarettes) are handheld electronic devices that generate vapours or aerosols from E-liquid without combustion. The main components of E-liquids are humectants (i.e. propylene glycol, vegetable glycerol), nicotine and flavourings. The main compartments of an E-cigarette comprise a mouthpiece, a cartridge, a heating element/atomizer and a battery (Figure 1.2). E-liquid is stored in the cartridge and then supplied to the atomizer which contains a small coil of electrically resistant wire that heats up when the battery is switched on.

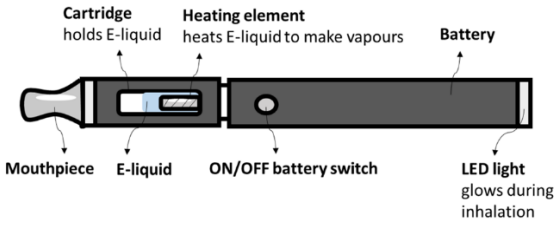


Figure 1.2. Components of E-cigarette (shown here in the form of a 'second generation' E-cigarette).

E-cigarettes have gained rapidly in popularity around the world in recent years; sales of E-cigarette may even surpass conventional cigarettes by 2023 [24]. Since E-cigarettes were invented in 2003, overall usage increased from 3.3% to 8.5% between 2010 and 2013 among adults in the USA [25] and the experience of E-cigarette usage rose from 4.6% to 8.2% in 2014 amongst young people aged 11-18 in the UK [26]. In addition, a rapid rise in E-cigarette use has occurred not only amongst current smokers, but also non-smokers who may therefore develop a smoking habit and/or nicotine addiction [27].

Public Health England promoted the use of E-cigarettes as smoking cessation aids which could be contributing to at least 20,000 successful new quits per year and many E-cigarette smokers believe this to be a benefit of E-cigarettes [28]. Long term E-cigarette use can assist those tobacco smokers who attempt to quit smoking to reduce CS consumption, without severe withdrawal symptoms [29]. The increased prevalence of E-cigarette usage is positively correlated to success in quitting cigarette smoking [30]. Evidence also shows that E-cigarette use helps smokers to commit to smoking cessation, thus leading to fewer smoking-related diseases [31,32].

In addition to promoting abstinence from smoking, E-cigarettes may help those who cannot totally quit smoking to reduce their exposure to tobacco-specific toxicants. Manufacturers also promote E-cigarettes as a safe alternative to conventional cigarettes due to lower levels of carcinogens and toxicants in vapours [33]. These recommendations were supported by The Royal College of Physicians in the UK. The most common reasons given for the use of e-cigarettes among pregnant women were the perception of less harm than CS and help with smoking cessation. An online survey

demonstrated that the majority of the respondents believed that E-cigarettes are less harmful than tobacco smoking and that they have few side effects, with less coughing and better breathing [34]. Furthermore, E-cigarette users had positive attitudes towards E-cigarettes as they regarded the ingredients to be less harmful than CS, although this judgement was based on incomplete information and knowledge [35].

The World Health Organization (WHO) does not recommend the use of E-cigarettes for never smokers, children and pregnant women [36] and strongly advises consumers not to use E-cigarettes until a reputable regulatory body has found them to be safe and effective [37]. The sale of E-cigarettes is already banned in 25 of 68 countries that regulate them [38] and the US Food and Drug Administration (FDA) has proposed a range of regulatory tools to prohibit flavoured E-cigarettes recently [39]. Moreover, despite the positive claims of manufacturers and the perceptions of the public, the use of E-cigarettes remains controversial and a growing literature provides evidence for deleterious effects of E-cigarettes. Discussed here, are the immunological, and other, effects of E-cigarettes on a variety of cell types in humans (Figure 1.3) and in murine models (Figure 1.4), discussing both complete e-liquids and their main constituents. This review might help the public to consider whether to use E-cigarettes based on scientific evidence.

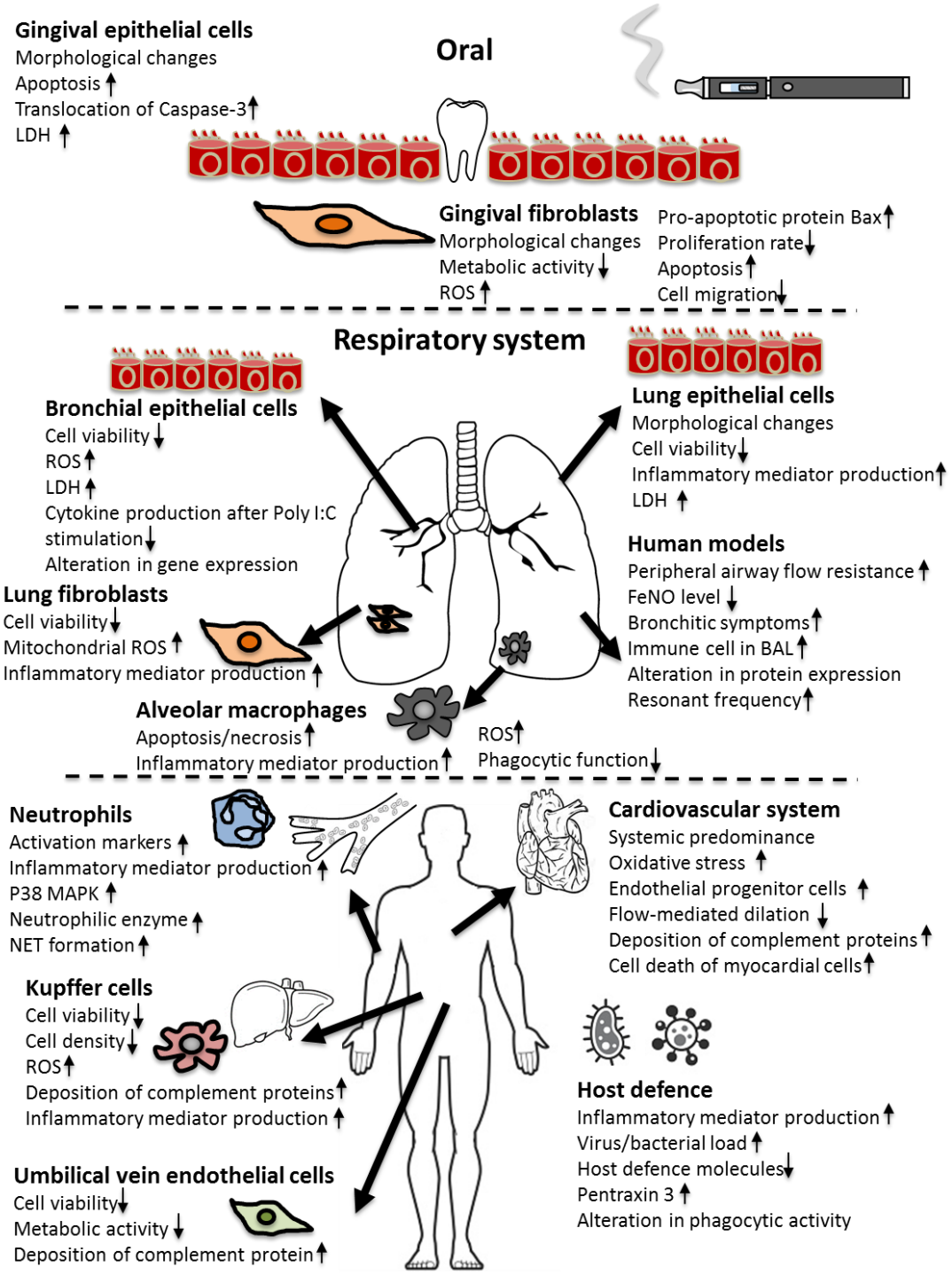


Figure 1.3. Effects of E-cigarettes on human cells and systems. The figure was adapted from the reference [40].

1.2.2 Effects of E-cigarettes in humans

1.2.2.1 Benefits of E-cigarette use

Users of conventional cigarettes who switched to E-cigarettes reported reduced nicotine withdrawal symptoms with less cigarette craving and improved chest tightness and other health effects over the course of 2 weeks [41]. Substitution of cigarettes by nicotine-containing E-cigarettes also reduced the exposure to carcinogens and toxicants commonly found in CS, assessed by urine biomarkers for tobacco-specific nitrosamine, volatile organic compounds and polycyclic aromatic hydrocarbons. A one-year randomized controlled trial of CS smokers who completely stopped smoking or switched to E-cigarette use, showed progressive improvements in respiratory symptoms, particularly coughing and shortness of breath [42]. A one-year prospective randomized control study found restoration of 'fractional nitric oxide concentration in exhaled breath' (FeNO) and reduction in 'exhaled carbon dioxide' (eCO) in CS users who completely stopped cigarette smoking after switching to E-cigarettes, along with improvements in symptom scores [43]. Nitric oxide is involved in antimicrobial activity against lung pathogens and has anti-tumour effects, whereas eCO is a biomarker of smoking that reflects airway inflammation; thus, normalization of FeNO and eCO might support the use of E-cigarettes to reverse the harmful effects of CS in the lungs. A prospective 3.5-year observational study of nine E-cigarette users who never smoked conventional cigarettes found E-cigarette use did not alter healthy outcomes including blood pressure, heart rate, body weight, lung function, respiratory symptoms, exhaled breath nitric oxide (eNO), exhaled carbon monoxide (eCO), or high-resolution computed tomography of the lungs [44].

In addition to helping to reduce harmful effects in smokers with normal lung function, E-cigarette use may also benefit patients with respiratory and systemic diseases. COPD patients who switched to use of E-cigarettes reduced their CS consumption and more than half stopped cigarette smoking completely during a 12 month follow up [45]. They also had significant reduction in annual COPD exacerbation, rate of FEV1 decline and improved symptoms and ability to perform daily physical activities. Similarly, a marked attenuation in CS consumption was found in asthma patients who switched to E-cigarettes, with better asthma control, lung function and lower airway hyper-responsiveness over a two year period [46]. Regular E-cigarette use could help to reduce CS consumption in arterial hypertensive patients, further showing good blood pressure control and disease severity after 12 months follow up [47].

1.2.2.2 Effects of E-cigarettes on the oral cavity (see Table 1.2)

Nicotine has been demonstrated to alter the cytoskeleton [48] and to induce extracellular matrix remodelling [49] in gingival fibroblasts – a cell type that is directly exposed to CS constituents during smoking. CS inhibited metabolic ability of human gingival fibroblasts time dependently, as assessed by MTT assay [50]. Exposure of human gingival fibroblasts to E-liquid, with and without nicotine, reduced their metabolic activity in a dose- and time-dependent manner [51]. E-liquid with nicotine concentrations of greater than 1 mg/ml showed pronounced toxicity that was further exacerbated by vaporising the fluid. Consistent with the study treating human gingival fibroblasts, both CSE and ECVE altered morphology, proliferation rate, apoptosis, migration and wound closure [52]. Of note, CSE and ECVE with nicotine exert a

greater magnitude of damage than those without nicotine. It has been reported that CS triggered the production of reactive oxygen species (ROS) from human gingival fibroblasts [53]. Similarly, ROS production was increased significantly after 24 h exposure of gingival fibroblasts to either nicotine-containing or nicotine-free fluids and vapours, indicating that nicotine was not the only inducer of ROS production [51]. Levels of the pro-apoptotic protein Bax, which is involved in triggering ROS production, is upregulated by CSE [54]. Bax expression was also elevated after exposure to nicotine-containing and nicotine-free E-liquids and vapours for 24 h, followed by cell apoptosis after 48 h [51].

The epithelium forms the first line of defence against toxic substances and periodontal pathogens. CS induced human gingival epithelial cell apoptosis, which was confirmed by upregulated expression of Bax and caspase-3 activity. CS also inhibited gingival epithelial cell migration, which reduced the percentage of healing in a scratch assay. In addition, CS reduced the numbers of epithelial cell layers, resulting in epithelial disorganization [55]. Exposure of primary human gingival epithelial cells to ECV raised levels of apoptosis associated with increased caspase-3 activity accompanied with morphological changes and increased lactate dehydrogenase (LDH) activity [56].

Table 1.2. The effects of CS and E-cigarette on oral cavity

Effects	CS	E-cigarette
Gingival fibroblast		
Metabolic activity	Decrease [50]	Decrease [51]
Alteration in morphology	Yes [52]	Yes [52]
Proliferation rate	Decrease [52]	Decrease[52]
Apoptosis	Increase [52,54]	Increase [51,52]
Cell migration	Decrease [52]	Decrease [52]
Wound closure rate	Decrease [52]	Decrease [52]
ROS production	Increase [53]	Increase [51]
Pro-apoptotic protein Bax expression	Increase [54]	Increase [51]
Gingival epithelial cell		
Apoptosis	Increase [55]	Increase [56]
Caspase-3 activity	Increase [55]	Increase [56]
Alteration in morphology	Yes [55] (disorganization of epithelium)	Yes [56]
Lactate dehydrogenase activity	N/A	Increase [56]

1.2.2.3 Effects of E-cigarettes on the respiratory system (see Table 1.3)

After the oral cavity, airway epithelium is the next site of contact with inhaled substances and serves as an active contributor in defence of the respiratory system. Damage to, or alteration of, airway epithelial cells by inhaled particles can increase the permeability of the epithelium, leading to inflammatory cell influx into the airways. These infiltrating cells contribute to persistent inflammation and the secretion of mediators that cause lung tissue destruction, including defensins, elastase and matrix metallo-proteinases (MMPs). Epithelial cells themselves, through production of these mediators, can also participate in the destructive processes

Several studies have compared the effects of the constituents of E-cigarettes and tobacco on cells of the human respiratory tract, using either primary cells or cell lines. In one study using primary human bronchial epithelial cells (HBECs) obtained at surgery, exposure of the cultured cells to either ECV or CS at an air-liquid interface caused reduced cell viability and increased oxidative stress [57]. These effects were seen using ECV with or without nicotine; indeed, similar deleterious effects were induced by the humectants alone (i.e. propylene glycol and glycerol) that are used in the E-liquids. However, the increase in cell death and oxidative stress of the HBECs was much more pronounced when exposed to CS than to ECV. In contrast, another study observed CSE induced apoptosis and necrosis in human bronchial epithelial cells assessed by increasing caspase 3/7 activity and reducing viable cell protease activities, and disrupted cellular redox hemeostasis characterized by reduction in glutathione ratio and increase in intracellular ROS. However, nicotine-containing ECVE did not elicit those effects [58].

Another study also investigated the exposure of primary HBECs to ECV and CS at an air-liquid interface, using gene-expression profiling as the read-out [59]. Genes upregulated by both CS and ECV were related to apoptosis, xenobiotic stress and oxidative stress. In addition, CS and ECV down-regulated the expression of genes associated with the assembly and movement of cilia, which might cause a reduction of ciliated cells in airway epithelium and compromise mucociliary clearance function – this could contribute to chronic inflammation in the airways. ECV containing nicotine had more pronounced effects than those without nicotine. This study showed that ECV could induce similar effects to CS; again, however, the effects of CS were more pronounced.

Human cell lines derived from lung epithelium (A549) and skin keratinocytes (HaCaT) have also been used to study the effects of CS, ECV containing nicotine and/or balsamic flavouring, and ECV humectants alone [60]. CS and, to a lesser extent, ECV containing balsamic flavouring or nicotine triggered morphological changes, elevated LDH release and reduced viability in both cell types. The influence of flavouring was greater than nicotine and the ECV humectants alone did not have these effects. However, exposure to the humectants did induce release of multiple cytokines by the lung epithelial cells. This paper concluded that ECV was less cytotoxic than CS and that the deleterious effects of E-cigarettes were more related to the flavourings than to nicotine. A study examining the effects of CS and ECV on the HBEC cell line BEAS-2B also concluded that the deleterious effects of E-cigarettes were less severe than those of CS [61].

ECVE also significantly increased LDH release from bronchial epithelial cells (BECs) in both healthy and COPD patients and induced production of cytokines, including IL-6 and IL-8. Moreover, both CS and ECVE exposure to BECs from COPD patients showed much lower IL-6, CXCL10 and CCL5 production than those from healthy donors after Poly I:C stimulation. Due to the role in antiviral responses by recruiting and activating lymphocytes, suppression of CXCL10 and CCL5 could reduce antiviral activity in bronchial epithelium, leading to elevated susceptibility to infection [62].

Surfactant helps to reduce surface tension of alveolar fluid thereby less energy is required to inflate the lungs and prevent alveolar collapse. High surface tension is a known characteristic of asthma, pneumonia and COPD. Both CS and ECVs affected surfactant microstructure analysed by Infasurf®, a calf lung surfactant extract used as a lung surfactant model. However, surface tension was increased by CS but not ECVs. Interestingly, tar was the only component in CS able to increase surface tension, suggesting E-cigarette did not alter surface tension due to lack of tar [63].

Experiments with the human lung fibroblast line, HFL-1, showed that these cells, like lung epithelial cells, are sensitive to ECV. Again, however, the cells were more sensitive to CS than to ECV in the sense that CS, but not ECV, reduced the fibroblast viability in relatively large culture volumes whereas ECV did reduce the viability only of smaller cultures [64]. Certain flavours of ECV, as well as CS, also induced IL-6 and IL-8 production [64,65]. Acute increase of mitochondrial ROS was also observed in the human lung fibroblast cell line treated with nicotine-containing ECVs [65], consistent with findings using CS [66].

E-cigarette vapour condensate (ECVC) caused more reduced viability of alveolar macrophages than E-liquid [67]. ECVC also induced apoptosis, necrosis, ROS production and pro-inflammatory cytokines (i.e. IL-6, TNF-α, IL-8), chemokine MCP-1 and proteolytic enzyme MMP-9 production but decreased phagocytic function of alveolar macrophages, and these impacts were ameliorated by anti-oxidant, suggesting the role of oxidative stress in the effects of E-cigarettes. Similar to ECV, CS also has been reported to trigger apoptosis [68], and the production of inflammatory mediators [69-71] and ROS [72], but to attenuate phagocytosis by alveolar macrophages.

CS is known to have deleterious effects on the respiratory system in humans [73,74] and studies in human subjects also raised concerns over the safety of E-cigarette use. Healthy smokers, who used E-cigarettes for 5 minutes, had increased levels of peripheral airway flow resistance and oxidative stress illustrated by lower fractional exhaled nitric oxide (FeNO), demonstrating the rapid side effects of E-cigarettes [75]. Adolescents using e-cigarettes for 12 months showed a two-fold increase in rates of chronic bronchitic symptoms, such as chronic cough, phlegm or bronchitis [76]. Acute E-cigarette use (regardless of nicotine content) by never-smokers altered transcriptomes in small airway epithelium including downstream targets of p53 signalling related to apoptosis, cell cycle arrest and DNA damage [77]. Moreover, E-cigarette use changed transcriptomes in alveolar macrophages, thereby inhibiting their migration and phagocytic ability but increased susceptibility of E-cigarette users to infection [77]. Both chronic CS and E-cigarette users had more erythematous and irritable airway mucosa and there were approximately 200 proteins altered (78 proteins

altered in both groups and 113 specifically altered in vapers) in bronchial epithelium, including mucin MUC5AC that facilitates mucus secretion. Of note, some proteins upregulated in vapers like MUC5AC, CYP1B (generates covalent adducts to damage DNA) and STIM1 (controls Ca^{2+} homeostasis) were also elevated by aerosolized PG/VG in human bronchial epithelial cells in vitro [78]. Aside from direct use, a 30-minute passive exposure to E-cigarette vapour in healthy non-smokers caused small airway irritation and inflammation detected by increased resonant frequency and reduced FeNO, raising the possibility of potential risk to humans exposed to ECV in public places and environments [79]

Overall, the studies considered here indicate that ECV is less deleterious to the respiratory system than CS, but still has significant toxic and inflammatory effects whose long-term consequences are currently unknown.

Table 1.3. The effects of CS and E-cigarette on respiratory system

Effects	CS	E-cigarette
Bronchial epithelial cells		
Cell viability	Decrease [57]	Decrease [57]
Oxidative stress	Increase [57,58]	Increase [57]

		No [58]
Apoptosis	Increase [58]	No [58]
Necrosis	Increase [58]	No [58]
Caspase 3/7 activity	Increase	No [58]
Glutathione ratio	Increase [58]	No [58]
Alteration in gene expression	Yes [59]	Yes [59]
LDH release	No [62]	Increase [62]
Cytokine production (i.e. IL-6, CXCL10 and CCL5) after Poly I:C stimulation	Decrease [62]	Decrease [62]
Lung Epithelial cells		
Morphological changes	Yes [60]	Yes [60]
LDH release	Increase [60]	Increase [60]
Cell viability	Decrease [60,61]	Decrease [60] No [61]
Oxidative stress	Increase [61]	No [61]
Inflammatory mediator production (i.e. IL-8,MCP-1 and GRO-α)	Decrease [61]	No [61]

IL-6 production	Increase [61]	Increase [61]
Surfactant		
Alteration in microstructure	Yes [63]	Yes [63]
Surface tension	Yes [63]	Yes [63]
Lung fibroblasts		
Cell viability	Decrease [64]	Decrease [64]
Inflammatory mediator production (i.e. IL-6 and IL-8)	Increase [64]	Increase [64,65]
Mitochondrial ROS production	Increase [66]	Increase [65]
Alveolar macrophage		
Apoptosis/necrosis	Increase [68]	Increase [67]
Inflammatory mediator production	Increase [69-71]	Increase [67]
ROS production	Increase [72]	Increase [67]
Phagocytic function	Decrease [70]	Decrease [67]
Human models		
Peripheral airway flow resistance	Increase [73]	Increase [75]
FeNO level	Decrease [73]	Decrease [75,79]

Bronchitic symptoms	Increase [74]	Increase [76]
Alteration in transcriptomes/protein expression	Yes [78]	Yes [77,78]
Resonant frequency	N/A	Increase [79]

1.2.2.4 Effects of E-cigarettes on other cells and tissues (See Table 1.4)

Inhaled substances can be absorbed into the bloodstream and distributed systemically. It is therefore important to consider possible effects of ECV constituents on other cells and tissues in addition to effects on cells of the respiratory tract.

Neutrophils are an important component of innate immunity that act rapidly against invading pathogens by phagocytosis, degranulation and release of neutrophil extracellular traps. They are abundant in the circulation and migrate to sites of infection in response to chemokines and other chemo-attractants. Neutrophils also release cytokines which can recruit and activate other cells during inflammation. Neutrophils treated with ECV extract for up to 6h showed increased expression of the neutrophil activation markers CD11b and CD66b [80]; this is in contrast with a report that CD11b was upregulated in circulating neutrophils from tobacco smokers only with COPD, not from tobacco smokers with normal lung function [81]. Also, the levels of neutrophil elastase, MMP-9 and IL-8 showed similar changes, accompanied by p38 MAPK activation without a reduction in neutrophil viability. CD66b cross-linkage between neutrophils was increased by ECV extract, which might cause the secretion of IL-8. Neutrophil granulocytes possess two mechanisms for antimicrobial activity:

degranulation of stored mediators and release of NETs. The levels of neutrophilic enzymes associated with chronic lung diseases, such as neutrophil elastase, myeloperoxidase and matrix metalloproteinase-9 and NET formation, were elevated in sputum derived from both CS and E-cigarette users, with increasing neutrophil count by CS only [82]. In addition, E-cigarettes suppressed antimicrobial activity and viscoelastic properties of the mucus barrier, as demonstrated by decreasing innate defence proteins secreted by the airway epithelium. Interestingly, these proteins were elevated in tobacco smokers. Increased total mucin concentration, an important parameter for failed mucus transport in muco-obstructive disease and COPD, was shown in both CS and E-cigarette users. These results suggested ECV might affect neutrophilic response and defence proteins in lungs in a similar way to that seen in CS users.

Habitual E-cigarette users had a shift in cardiac autonomic balance toward sympathetic predominance and higher systemic oxidative stress levels, which are known risks for cardiovascular disease (CVD) [83]. The levels of endothelial progenitor cells, the biomarker for endothelial function and risk marker for CVD based on their function in maintenance, differentiation and regeneration of endothelial cells following vascular injury or necrosis, was elevated in participants exposed to ECV acutely, similar to exposure to CS [84]. Flow-mediated dilation, the other marker for endothelial function and CVD, was decreased after CS and E-cigarette use [85]. Markers of oxidative stress were elevated but anti-oxidant vitamin E was suppressed by both acute CS and E-cigarette exposure [85]. Viability and metabolic activity of human umbilical vein endothelial cells (HUVECs) was inhibited by CSE and nicotine-containing ECVE but

not pure nicotine [86]. Furthermore, the deposition of C1q and C5b-9, C3b and the expression of the receptors for C1q onto endothelial cells was enhanced by CSE and nicotine-containing ECVE while C4d was increased by CSE only. Since complement deposition onto endothelial cells is known to promote CVD progression, both CS and E-cigarette may increase the risk for CVD. Inhibition of endothelial cell migration is a mechanistic link between CS and CVD. CS suppressed HUVEC migration dose-dependently but ECVE did not exert this effect [87]. The abundance of oxidants and free radicals in CS can lead to oxidative damage, apoptosis and necrosis in endothelium, this paper, in contrast, proposed that E-cigarette use may reduce the risk of CVD by reducing exposure to oxidant species in users [87]. Myocardial cells are another example of cells in which oxidative stress is induced by CS. A rat myocardial cell line exposed to CS extract suffered large scale cell death and morphological changes; by contrast, ECV extract produced from only a minority of brands of E-cigarette caused significant cell death, especially those with tobacco flavouring, raising the possibility that several tobacco impurities may be present in these E-liquids [88]. Indeed, nicotine and humectants alone did not induce cytotoxicity, suggesting that flavourings and other components in E-cigarettes play a significant role in causing harm (see below).

Kupffer cells are liver resident macrophages that locate to the liver sinusoids. They clear foreign substances by adhering to platelets, which then return to the systemic circulation. Some studies indicate that liver inflammation might cause alterations in the circulation, leading to the development of systemic diseases. Exposure of immortalized rat Kupffer cells to CS, ECV extract, or nicotine significantly decreased

cell viability and density [89]. The deposition of complement proteins C1q, C4d, C3b and C5b-9 on the Kupffer cells was augmented by ECVE, CS and nicotine. As C5b-9 is the main lytic moiety of the complement cascade, the authors hypothesized that increased C5b-9 deposition on Kupffer cells might be one reason for the reduction of Kupffer cell viability. Elevated expression of C1q receptors (C1qR) on Kupffer cells was also observed. No significant differences were observed between the effects of CS and ECV extract or various concentration of nicotine in ECV extract. In addition, although nicotine alone was detrimental to these cells, E-cigarettes further enhanced the effects, suggesting that other components in E-cigarettes initiate inflammatory responses. ECVE also induced ROS and pro-inflammatory cytokine secretion by the Kupffer cells.

Table 1.4. The effects of CS and E-cigarette on other cells and tissues

Effect	CS	E-cigarette
Neutrophils		
Activation marker (i.e. CD11b and CD66b)	No [81]	Increase [80]
Inflammatory mediator production	Increase [80]	Increase [80]
p38 MAPK activation	N/A	Increase [80]
Neutrophilic enzyme	Increase [82]	Increase [82]

NET formation	Increase [82]	Increase [82]
Cell number	Increase [82]	No [82]
Cardiovascular system		
Sympathetic predominance	N/A	Yes [83]
Oxidative stress	Increase [85]	Increase [83,85]
Endothelial progenitor cells	Increase [84]	Increase [84]
Flow-mediated dilation	Decrease [85]	Decrease [85]
Anti-oxidant Vitamin E	Decrease [85]	Decrease [85]
Umbilical vein endothelial cells		
Cell viability	Decrease [86]	Decrease [86]
Metabolic activity	Decrease [86]	Decrease [86]
Complement deposition	Increase [86]	Increase [86]
Migration	Decrease [87]	No [87]
Myocardial cells		
Cell death	Increase [88]	Increase [88]
Morphological changes	Yes [88]	No [88]
Kupffer cells		

Cell viability	Decrease [89]	Decrease [89]
Cell density	Decrease [89]	Decrease [89]
Complement deposition	Increase [89]	Increase [89]
Oxidative stress	Increase [89]	Increase [89]
Inflammatory mediator production	Increase [89]	Increase [89]

1.2.3 Inflammatory effects of E-cigarettes in murine models in vivo (See Figure 1.4 and Table 1.5)

Increased airborne levels of nicotine and cotinine in homes of E-cigarette users are associated with passive exposure to ECVs [17]. Children may absorb higher amounts of ECV constituents than adults because of their higher respiratory rates and increased dermal absorption; in addition, they have diminished nicotine clearance [18]. Murine experiments have therefore been performed to better understand the potential effects of ECV constituents in the young. Newborn mice exposed to ECV containing nicotine for 10 days after birth had significantly lower body weight, and there was a positive correlation between reduction of body weight and plasma cotinine levels [90]. Nicotine-containing ECV also attenuated alveolar growth in neonatal mice, assessed by a significant reduction in expression of the cell proliferation marker Ki67 [90]. Exposure of mice to CS has also been reported to lead to reduced body weight [91] and lung development [92].

Acute lung injury and greater IL-6 secretion was observed in adult mice after 3 days of CS exposure and, to a lesser extent, after ECV exposure [93]. On the other hand, only CS (and not ECV) induced oxidative stress and secretion of IL-1β and TNF-α, indicating less toxicity of E-cigarettes [93]. In other studies, mice treated with tobacco-flavoured ECVs for 3 days were observed to have significantly elevated levels of IL-6, MCP-1, IL-1α and IL-13 in BALF [64], and pentraxin-3 was elevated in mice exposed to ECV over a period of 4 weeks [94]. These data demonstrate that acute exposure to e-cigarette aerosol in mouse lung induces inflammatory responses. Chronic exposure of mice to nicotine-containing ECV over a 4-month period induced respiratory resistance and alveolar airspace enlargement reminiscent of features of COPD [95], as also found in CS-exposed mice [96-98]. Moreover, the nicotine-containing ECV induced increased levels of IL-1β and MCP-1 in the lungs, as well as protease, collagenase and cathepsin expression, all of which are involved in the pathogenesis of COPD. These changes were not induced by ECVs lacking nicotine, thus supporting the direct harmful effects of nicotine. All these inflammatory mediators and enzymes were similarly found to be stimulated in other experiments treating mice with CS [96-100].

In addition to inducing COPD-like symptoms, another murine study demonstrated that both CS and E-cigarette fluid can exacerbate the signs and symptoms of experimental asthma induced by sensitization of mice to ovalbumin. Airway hyper-responsiveness was significantly increased by CS in OVA-sensitized mice [101,102] and also by intra-tracheal instillation of E-liquid twice a week for 10 weeks [103]. Furthermore, the total number of leukocytes in BALF was higher in OVA-sensitized mice also exposed to

CS [101] or E-liquid [103], as was the percentage/number of eosinophils [101-104]. CS elevated OVA-specific IgE in serum [101,102] and increased OVA-induced Th2 cytokines IL-4 and IL-5 in BALF [102] whereas the production of IL-13 varied [101,102]. Levels of OVA-specific IgE in serum and IL-4, IL-5 and IL-13 in BALF, were all higher in OVA-sensitized mice with E-liquid than those without, whereas IFN-γ was lower which was contradictory to one study using CS [104]. Th2 cytokines are the crucial players in the pathogenesis of atopic asthma and IFN-γ counteracts the Th2 immune responses; thus, a reduction in IFN-γ levels caused by E-liquid exposure might exacerbate Th2-dominated inflammatory responses. In conclusion, these results indicate that E-liquid exposure aggravated allergen-induced asthmatic inflammation and use of E-cigarettes in asthmatic people might exacerbate their symptoms.

In addition to respiratory effects, mice exposed to CS [91] or injected with nicotine and nicotine containing E-liquid for 4 weeks [105] had significantly less food and energy intake. Furthermore, nicotine or E-liquid (with or without nicotine) elevated blood glucose levels but inhibited hepatic glycogen rate. Also, nicotine or E-liquid (with or without nicotine) might help lipid profile by decreasing total cholesterol and low-density lipoprotein (LDL) but increase hepatotoxicity. Therefore, E-liquid might induce metabolic disorder by disturbing both glucose and cholesterol homeostasis, independent of nicotine. It should be borne in mind, however, that direct application of E-liquids themselves (as in the above studies) may not be equivalent to ECV in the effects exerted. The relevance of these findings to ECV inhalation may therefore be questioned.

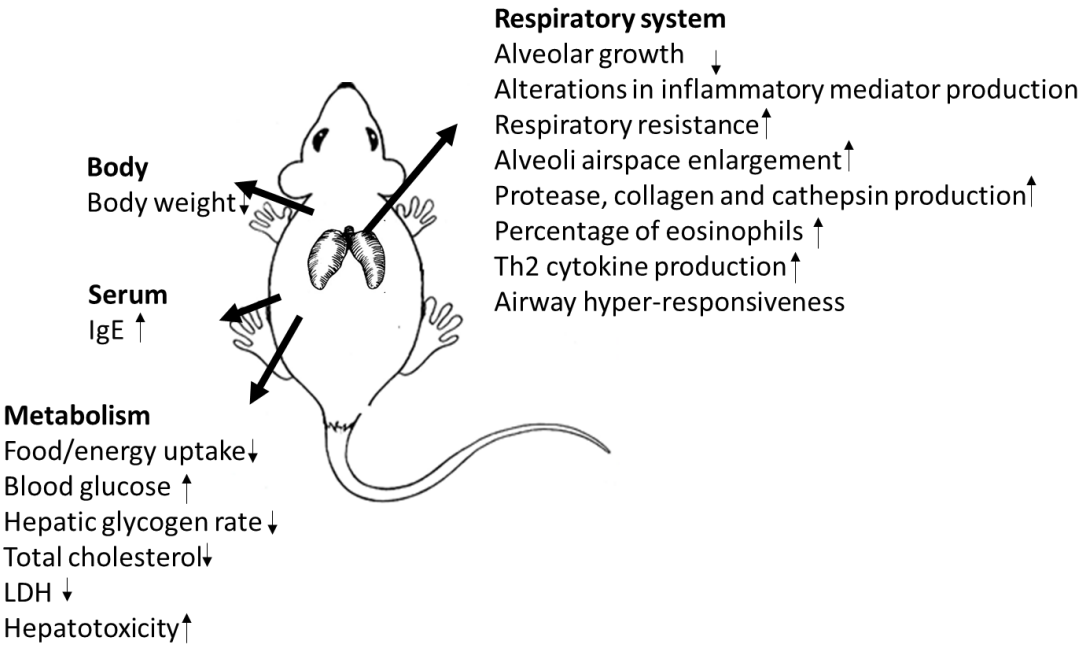


Figure 1.4. The effects of E-cigarette on murine models in vivo. The figure was adapted from the reference [40].

Table 1.5. The effects of CS and E-cigarette on murine models in vivo

Effect	CS	E-cigarette
Body weight	Lower [91]	Lower [90]
Alveolar/lung growth	Decrease [92]	Decrease [90]
IL-6 production in BALF	Increase [93,96]	Increase [93]
IL-1β production in BALF	Increase [93,100]	No [93]
TNF-α production in BALF	Increase [93,97,98]	No [93]
Oxidative stress in lung	Increase [93]	No [93]

Other inflammatory mediators in BALF/Lung	Increase [97,98]	Increase [64,94,95]
Respiratory resistance	Increase [96]	Increase [95]
Alveolar space enlargement	Increase [97,98]	Increase [95]
Enzyme production (i.e. protease, collagen and cathepsin)	Increase [97,98]	Increase [95]
Airway hyper-responsiveness	Increase[101,102]	Increase [103]
Total leucocytes in BALF	Increase [101] No [102]	Increase [103]
Percentage/number of eosinophils in BALF	Increase [101,102]	Increase [103]
IgE in serum	Increase [101,102]	Increase [103]
Th2 cytokine production (i.e. IL-4, IL-5) in BALF	Increase [102]	Increase [103]
IL-13 production in BALF	No [101] Increase [102]	Increase [103]
IFN-γ production in BALF	Increase [104]	Decrease [103]
Food/energy uptake	Decrease [91]	Decrease [105]

Blood glucose	N/A	Increase [105]
Hepatic glycogen rate	N/A	Decrease [105]
Total cholesterol	N/A	Decrease [105]
LDL release	N/A	Decrease [105]
Hepatotoxicity	N/A	Increase [105]

1.2.4 Effects of E-cigarettes on resistance to infection (See Table 1.6)

Platelet-activating factor receptor (PAFR) is used by pneumococci to adhere to host cells and CS increases pneumococcal adhesion by up-regulating PAFR expression in human lower airway epithelial cells [106]. Increased levels of nasal PAFR were seen in adults inhaling E-cigarette vapour for 5 minutes [107]. ECVE (with or without nicotine) also increased pneumococcal adhesion to a human epithelial cell line, and this was related to ROS production. Since pneumococcal infection is the commonest cause of bacterial pneumonia, E-cigarette use has the potential to facilitate pneumococcal diseases.

ECV may impair defence against bacterial and viral infections, resulting in increasing pathogen burden and pro-inflammatory cytokine production whilst suppressing phagocytic and defensive abilities of cells. For example, treatment of normal human tracheobronchial epithelial cells with tobacco-flavoured e-liquid, with or without nicotine, increased the virus load after human rhinovirus (HRV) infection and augmented HRV-induced IL-6 production. Moreover, SPLUNC1, a host defence

molecule against HRV infection, was suppressed by e-liquids with and without nicotine [108].

CS caused higher pulmonary bacterial burden of methicillin-resistant Staphylococcus aureus (MRSA) in mice, which was due to augmented evasion of phagocytosis and neutrophil extracellular trap–mediated killing [109]. With regard to immunity to bacteria, exposure to ECV prior to bacterial infection led to greater numbers of MRSA in epithelial cells, alveolar macrophages and human polymorphonuclear leukocytes, which are responsible for clearance of inhaled pathogens and host defence of the lung against bacteria. This reduction in anti-bacterial activity was seen upon treatment with nicotine or humectants, as well as complete ECV. Increased virulence of the bacteria themselves was also induced by exposing them to ECV extract [94].

Bacterial burden was significantly increased in the lungs of both CS- [110] and ECV-exposed [111] mice after Streptococcus pneumoniae infection, and this was associated with reduced bacterial clearance by alveolar macrophages. ECV exposure also caused greater severity of influenza infection in mice, with higher viral titres and increased mortality, but reduced levels of IL-17A which is associated with protective immunity against influenza infection [111].

Table 1.6. The effects of CS and E-cigarette on resistance to infection

Effect	CS	E-cigarette
PAFR level	Increase in lower airway epithelial cells [106]	Increase in nasal epithelial cells [107]
Pathogen adhesion	Increase to lower airway epithelial cells [106]	Increase to nasal epithelial cells [107]
Pathogen burden	Increase [109,110]	Increase [94,108,111]
IL-6 production by normal human tracheobronchial epithelial cells	N/A	Increase [108]
Host defence molecule SPLUNC1 of normal human tracheobronchial epithelial cells	N/A	Decrease [108]
IL-17A production in BALF	N/A	Decrease [111]

1.2.5 The effects of E-liquid constituents other than nicotine (See Table 1.7)

The studies described above show the potentially deleterious effects of E-cigarettes. These effects are not entirely attributable to nicotine, indicating that other components

in E-liquids may also be damaging. In addition to nicotine, humectants (i.e. propylene glycol and vegetable glycerol) and flavourings are the major ingredients of E-liquids. Humectants are the bases, or carriers, for nicotine and flavourings and most E-liquids contain both propylene glycol (PG) and vegetable glycerol (VG) at a ratio of 50/50 or 70/30. PG is widely used in food and inhaled therapeutic products and can deliver a strong throat hit which may cause a slight irritation after use. VG, which has a slightly sweet taste, can soften the feeling in the throat and generate thicker and more visible vapours. The levels of oxidants and/or ROS detected in aerosols of PG and VG were significantly higher than in air alone [64]. Both PG and VG also induced death of normal human bronchial epithelial cells and the effects of glycerol were more potent than complete E-liquid [57]. Moreover, cells exposed to PG or VG showed higher levels of oxidative stress than when exposed to air [57]. PG/VG mixture also had negative effects on cell viability of embryonic kidney 293 cells, human adenocarcinoma alveolar basal epithelial cells and human airway smooth muscle cells, in a dose-dependent manner [112].

More than seven thousand flavourings for E-cigarettes are now available, with over 70% of e-liquids containing greater than 1% flavoured chemical levels by weight [113]. Although most of the flavourings are defined as safe to be used in food products, the potential toxicity associated with degradation products generated by vaping at high temperatures is unknown. Significantly elevated ROS production was discovered in classic tobacco-flavoured (16 mg of nicotine) and menthol-flavoured (0 mg of nicotine) ECVs [64]. However, classic tobacco-flavoured ECVs gave relatively reduced ROS production when nicotine was also present. Similarly, e-liquids with

either high or low concentration of nicotine had significantly less ROS than those without nicotine, suggesting nicotine is not a crucial inducer of ROS production. Moreover, E-liquids with non-tobacco flavouring had higher ROS than tobacco flavoured E-liquid, indicating that the occurrence of ROS in e-liquids is associated with the type of flavourings. The levels of ROS present in fruit-flavoured ECVs can be up to 3-fold higher than in tobacco-flavoured ECV [114].

Flavouring also has an impact on inflammatory responses: for example, nicotine-free cinnamon roll-flavoured ECV induced high IL-8 secretion from human lung fibroblasts [64]. 2,5-dimethylpyrazine, an odorant compound in dark chocolate, reduced the capacity of airway epithelial cells to respond to cAMP and exogenous ATP which are crucial factors for normal airway epithelial cell functions, like mucociliary clearance [115]. 2,5-dimethylpyrazine also increased ion conductance by evoking protein kinase A-dependent (PKA) activation of the cystic fibrosis transmembrane conductance regulator ion channel. Menthol, coffee and strawberry-flavoured ECVs reduced both cell viability and metabolic activity while coffee and strawberry-flavoured ECVS also induced cytokine production in human bronchial cells [116]. CALU3 airway epithelial cell line proliferation was inhibited by all 13 flavoured E-liquids tested in a dose dependent manner [117]. Peanut Butter cookies, Banana Pudding, Kola, Hot Cinnamon Candies and Menthol Tobacco caused significantly reduced cell numbers and viability compared with the PG/VG mixture. Moreover, Kola, Hot Cinnamon Candies and Menthol Tobacco increased production of the cytotoxic marker LDH. Aerosolized E-liquids had similar toxicity profiles to neat E-liquids. Sini-cide and Kola flavoured E-liquid altered phagocytic ability and

cytokine production in alveolar macrophages [118]. Moreover, Hot cinnamon candies, Banana pudding, Menthol tobacco, Sini-cide affected phagocytosis by human airway neutrophils in comparison to PG/VG mixture. Kola and Sini-cide also altered formation of neutrophil extracellular traps (NETs) which contain chromatin filaments and granule protein to expel extracellular pathogens and killing reduced the efficiency of NK cells. These three flavourings all contained cinnamaldehyde which, on its own, showed similar effects, suggesting the potential of cinnamaldehyde for impairing respiratory immune cell function. Another study also pointed out the deleterious effects of cinnamaldehyde: both flavouring chemicals and flavoured e-liquid without nicotine reduced cell viability and IL-8 secretion of monocytic cell lines dose-dependently, with cinnamaldehyde showing the highest cytotoxicity [119]. Flavouring chemicals and 7 types of aerosolized E-liquids including American tobacco, café latte and cinnamon roll induced cell-free ROS production [119]. Interestingly, mixing a variety of flavours induced higher cytotoxicity and cell-free ROS levels compared to individual flavours. This might give a warning of greater toxic effects to those users who mix E-liquids or those who are exposed to multiple flavourings in public areas.

A comprehensive study examined the effects of 35 flavours of E-liquid on human embryonic stem cells (hESC), mouse neural stem cells (mNSC), and human pulmonary fibroblasts (hPF) [120]. Cinnamon Ceylon flavour was the most cytotoxic to all three cell types, whilst butterscotch was defined as having relatively low cytotoxicity. However, the possibility of harm being caused by the latter cannot be excluded, since some workers at microwave popcorn packaging plants developed the irreversible obstructive lung disease, bronchiolitis obliterans, due to exposure to the

butter flavouring component diacetyl [121]. Three E-liquids all named butterscotch flavour induced different levels of cytotoxicity, although they were from the same company. hESC and mNSC were more sensitive to E-liquids than hPF (except for caramel and Menthol Arctic, which had stronger effects on hPF), suggesting that cells from embryos and newborns are generally more sensitive to E-cigarette constituents. There was no correlation between cytotoxicity and nicotine levels, as the cells did not survive better in E-liquids without nicotine.

In addition to humectants and flavourings, other chemicals and metals (e.g. copper, nickel and titanium) are detected in E-liquids. Copper metal nanoparticles could stimulate the production of mitochondrial ROS and reduction of mitochondrial membrane potential in human lung fibroblasts [64]. A quarter of e-liquids contain aldehyde (e.g. benzaldehyde and vanillin) [113] and significant amounts of formaldehyde, acetaldehyde and propionaldehyde were found in E-liquid heated at 150°C [122]. Aldehydes are recognized as primary irritants of the mucosa of the respiratory tract and acetaldehyde exposure impairs mucus clearance, leading to decreased host defence. Benzaldehyde, which can cause irritation of the respiratory airways and eyes, is a major ingredient in fruit-flavourings. Cherry-flavoured E-liquid was found to contain the greatest concentration of benzaldehyde and the benzaldehyde doses inhaled with 30 puffs from e-cigarettes were often higher than doses inhaled from conventional cigarettes [123]. Tobacco-specific nitrosamines (TSNAs) are known carcinogenic compounds in conventional cigarettes, especially 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN), which exert the strongest carcinogenicity. TSNAs were also detected in both liquid

[124] and vapour [33] of E-cigarettes. However, conventional cigarettes contain up to 1800 times higher amounts of NNK and NNN than E-cigarettes [125].

Table 1.7. The effects of E-liquid constituents other than nicotine

Effect		Other constituents and flavourings
Increase oxidative stress	Normal human bronchial epithelial cells	Humectants [57]
	in vapours	Humectants [64], classic tobacco [64], Menthol [64]
Alteration in cytokine production	Human lung fibroblast	Cinnamon roll [64]
	Human bronchial cells	Coffee[116], Strawberry[116],
	Alveolar macrophages	Sini-cide [118], Kola [118]
	Monocytic cell lines	cinnamaldehyde [119]
Reduce cAMP response in normal airway epithelial cells		2,5-dimethylpyrazine [115]
Reduce exogenous ATP response in normal airway epithelial cells		2,5-dimethylpyrazine [115]

Increase PKA activation by normal airway epithelial cells		2,5-dimethylpyrazine [115]
Reduce cell viability	Human bronchial epithelial cells	Humectants [57]
	Human adenocarcinoma alveolar epithelial cells	Humectants [112]
	Human airway smooth muscle cells	Humectants [112]
	Human bronchial cells	Menthol[116], Strawberry [116], Coffee [116]
	CALU3 airway epithelial cells	Peanut Butter Cookie [117], Banana Pudding [117], Kola [117], Hot Cinnamon Candies [117]
Reduce metabolic activity of human bronchial cells		Menthol[116], Strawberry [116], Coffee [116]
Reduced cell number of CALU3 airway epithelial cells		Peanut Butter Cookie [117], Banana Pudding [117], Kola [117], Hot Cinnamon Candies [117],

Reduce cell proliferation of CALU3 airway epithelial cells	Captain Black Cigar [117], Peanut Butter Cookie [117], T-bone [117], Popcorn, Black Licorice [117], Energon [117], Vanilla Tobacco [117], Banana Pudding [117], Kola [117], Hot Cinnamon Candies [117], Menthol Tobacco [117], Solid Menthol [117]
Increase LDH release from of CALU3 airway epithelial cells	Kola [117], Hot Cinnamon Candies [117], Menthol Tobacco [117]
Decrease phagocytosis of alveolar macrophages	Sini-cide [118], Kola [118]
Decrease phagocytosis of airway neutrophils	Hot Cinnamon Candies [118], Banana pudding [118], Menthol tobacco [118], Banana , Sini-cide[118]
Alteration in NET formation	Sini-cide [118], Kola [118]

1.2.6 Case reports and discussion

E-cigarettes are battery-operated devices that generate vapours by heating liquid mainly containing humectants, nicotine and flavourings. E-cigarettes have seen a rapid rise in use since the introduction to the market because of the perception that they are safer than conventional cigarettes, and their appealing design; however, there are many unanswered questions regarding their health impacts on human. There is accumulating evidence demonstrating the effects of E-cigarettes on both human and murine models, and comparison between E-cigarette and conventional cigarettes have also been made. With respect to the latter, numerous studies have indicated that ECV can induce similar effects to CS, but to a lesser extent. This may mean that smoking E-cigarettes is unlikely to be as deleterious to health as smoking conventional cigarettes; however, the relatively recent widespread introduction of E-cigarettes means that it is too early to know the potential consequences, for an individual, of using E-cigarettes for several decades. A very important aspect of this is the carcinogenic potential of E-cigarettes compared to conventional cigarettes. Once again, however, it is too early to draw conclusions about the actual carcinogenicity of ECVs.

Gingival epithelia cells, the most abundant structural cells in gingiva, are in direct contact with vapours from smoking devices. As described in previous sections, both CS and ECV induced death of gingival epithelial cells, associated with the elevation of caspase-3 activity, which is a frequently activated protease in cell apoptosis. Moreover, ECV altered the morphology of the cells, resulting in large faint nuclei, enlarged cytoplasm and compromised integrity of the plasma membrane. Gingival fibroblasts are important in wound healing through their adhesion, proliferation and

migration which were affected negatively by both CS and ECV. As CS is a well-established risk factor for periodontitis, these findings indicate that ECV might also provide an unhealthy oral environment and promote periodontal disease. Indeed, two clinical cases were reported of patients with more than a 10-year history of E-cigarette use, but no other identifiable risk factors, who developed basaloid squamous cell oral carcinoma, suggesting a potential role for ECV in oral cancer development [126].

A clinical case report described a previously healthy subject who started coughing, with whitish secretions, and who developed progressive dyspnoea after e-cigarette use for 48h; wheezing was revealed by auscultation after 4 weeks. However, these symptoms improved gradually, and were abolished without any treatment, a week after cessation of e-cigarette use [127]. Another case report described a presentation of hypersensitivity pneumonitis associated with E-cigarette use [128]. Also, a 46-year-old healthy man developed respiratory distress, night sweats, fever, and weight loss after using E-cigarettes for a month. He was found to have BALF that contained abundant lipid-laden macrophages, eosinophils, and neutrophils. The symptoms were fully abolished after E-cigarette abstinence [129]. These clinical cases illustrate how E-cigarettes can have a negative impact on respiratory health. Respiratory epithelium is a ciliated epithelium forming the integral barrier against inhaled agents to prevent infection and injury by trapping and expelling particles (mucociliary clearance), phagocytosing and killing pathogens and recruiting immune cells. It is known that CS alters respiratory epithelium by compromising its integrity and impairing mucociliary clearance. According to the findings described in a previous section, ECV also induced morphological changes, cell death, LDH release and inflammatory mediator

production by bronchial epithelial cells. Furthermore, ECV might compromise mucociliary clearance by down-regulating the expression of genes related to assembly and movement of cilia. Although some studies observed a lower magnitude of effects than those caused by CS, ECV is also able to damage respiratory epithelium and evoke an inflammatory environment. Using human models, both direct and passive ECV exposure was found to facilitate adverse respiratory effects including alterations in transcriptome/protein expression related to various biological functions, increased bronchitic symptoms, airway flow resistance and oxidative stress measured by FeNO.

Oxidative stress, which reflects an imbalance between oxidants and antioxidants, is critical in injurious and inflammatory responses in respiratory diseases [130]. Oxidative stress causes damage to airway epithelium by increasing epithelial cell lysis and detachment after CS exposure, and triggers the release of inflammatory mediators such as IL-1 and IL-8 which are involved in activation of the NF-κB pathway. Tobacco smokers have higher levels of oxidative stress in the lung, which arises not only from oxidants in cigarettes, but also ROS from macrophages and neutrophils via the NADPH system. Excessive ROS damage cells and tissue, leading to induction of cell death [131]. These mechanisms might also explain oxidative stress-induced deleterious effects of E-cigarettes since ECV causes high levels of oxidative stress in a variety of cell types, as discussed in previous sections.

Alveolar macrophages are crucial in innate immune responses in lungs. As mentioned in previous sections, both CS and ECV induced the production of pro-inflammatory mediators and proteolytic enzymes which damage connective tissues and parenchymal cells of lungs, thereby providing cellular mechanisms that connect smoking/vaping

and inflammation and tissue damage. Moreover, increase ROS production can help the killing of intracellular pathogens; however, phagocytosis of alveolar macrophages was compromised by both CS and ECV, suggesting their roles in impairing defence ability. Excessive inflammatory mediators and functional impairment of alveolar macrophages could contribute to lung pathogenesis including chronic bronchitis and emphysema. In previous sections, both CS and ECV reduced the production of inflammatory mediators associated with an anti-viral response by bronchial epithelial cells, showing that both CS and ECV might increase susceptibility to infection, which is the main factor in the acute exacerbation of COPD. It was reported in animal models that chronic exposure to CS suppressed bacterial clearance from the lungs and induced COPD-like damage in the lungs. ECV also impaired resistance to infection in mice, leading to higher burden and adhesion of pathogens, which might be due to inhibition of defence mechanism (i.e. lower levels of SPLUNC1, IL-17 and clearance activity by leukocytes).

CS has been documented to have a negative impact on many aspects of human health and to be a risk factor for various diseases. In particular, tobacco smoking is a risk factor for a range of cancers, respiratory diseases and infections, cardiovascular diseases and deleterious effects on the immune system [132,133]. ECV also exerts systemic effects on various cells and tissues. Neutrophils are recruited to the lung and stimulate enzymes that break down collagen, elastin and extracellular matrix and mucus production after CS exposure, leading to the development of emphysema and chronic bronchitis, major characteristics of COPD. In addition, smokers who developed COPD have higher numbers of circulating neutrophils [134]. We have

described in previous sections that sputum of E-cigarette users has a raised neutrophil count, neutrophilic enzyme production and NET formation, which promote antimicrobial ability. Moreover, ECV could activate neutrophils and inflammatory mediator production in vitro. ECV might be harmful to the cardiovascular system as it was found to reduce endothelial functions, decrease cell viability and induce oxidative stress and complement deposition: all these factors could promote the development of cardiovascular disease. Habitual E-cigarette users showed sympathetic predominance and higher oxidative stress levels. Viability and density of Kupffer cells was decreased by ECV while complement deposition, oxidative stress and inflammatory mediators were induced, suggesting that ECV might trigger inflammation in the liver. In murine models, ECV affected not only respiratory system, but also body growth, energy intake and metabolism.

A clinical case report described an E-cigarette user who developed respiratory symptoms that coincided with her onset of e-cigarette use and was given a diagnosis of lipid pneumonia [135]. Lipid pneumonia results from accumulation of either exogenous or endogenous lipids in the alveoli. Glycerine is obtained from plants by heating palm or coconut oils or soap via a fatty-acid splitting operation; therefore, glycerine-based oil in E-cigarettes was suspected to be the exogenous source of lipids that induced lipid pneumonia in this individual. Flavourings have increased the appeal of E-cigarettes to adolescents and previous intermittent smokers; the FDA planned to limit sale of flavoured E-cigarette to crackdown on the ‘’epidemic’’ use of E-cigarettes among the young [39]. Indeed, as discussed in previous sections, many flavoured E-cigarettes had adverse effects in vitro and there are many more E-cigarette flavourings

on the market waiting to be investigated. Although humectants, nicotine and flavourings are labelled as the ingredients in most commercial E-liquids, metals and other chemicals such as carbonyls and tobacco-specific nitrosamines, which are toxic and carcinogenic, have been detected in E-liquids.

There are limitations studying the effects of E-cigarette, such as types of E-cigarette devices and E-liquid used and patterns of E-cigarette exposure (i.e duration and frequency). Quantifying E-cigarette exposure is more challenging than conventional cigarette exposure, which can be calculate by the number of cigarettes smoked per day. In those human studies, it is also difficult to certain that the participants did not use conventional cigarette surreptitiously or have been exposed to a CS-containing environment.

1.3 Aims of the study

E-cigarettes are gaining in popularity around the world and manufacturers claim that they are safe alternatives to conventional cigarettes. Considering the alterations of several cell types and tissues caused by E-cigarettes, it was hypothesized that E-cigarettes may change the phenotype and function of human DCs. The aim of this study was to investigate the effects of E-cigarettes on DCs by creating an in vitro cell culture model using human monocyte-derived DCs (MoDCs), which might help to understand the potential roles of E-cigarettes in immunological and inflammatory processes.

Objectives:

1. To investigate the effects of ECVE on surface marker expression on DCs using flow cytometry.
2. To evaluate the effects of ECVE on cell death of DCs using flow cytometry.
3. To elucidate the effects of ECVE on the modulation of intracellular proteins of DCs using reverse-phase protein array.
4. To investigate the effects of ECVE on the cytokine production of DCs using ELISA.
5. To investigate the effects of ECVE on the communication of DCs with allogenic T cells by detecting T cell proliferation using flow cytometry.

Chapter 2 Materials and Methods

2.1 Peripheral blood mononuclear cell (PBMC) isolation

Anonymised buffy coats were obtained from the Regional Blood Transfusion Service (Sheffield, UK) and were diluted 1:3 with RPMI-1640 medium (Sigma-Aldrich, Gillingham, UK). Fresh venous blood was taken into heparinised Vacu-tainer tubes from healthy volunteers with ethical approval (FMHS REC ref: 121-1706) and informed consent; the fresh venous blood was diluted 2:1 with RPMI-1640 medium (Sigma-Aldrich, Gillingham, UK). Diluted blood was layered onto Histopaque (Histopaque-1077, Sigma-Aldrich, Gillingham, UK) and centrifuged at 800 g for 22 minutes. PBMC were collected from the fluid interface into a Falcon tube. Cells were washed twice by topping up with 50 ml RPMI-1640 medium and centrifuging at 300 g for 10 minutes. Thereafter, supernatant was discarded and cell pellets were resuspended in RPMI-1640 medium supplemented with 100 μg/ml Penicillin-Streptomycin, 2mM L-Glutamine, 10mM HEPES and 10 % FBS (called RPMI-1640 complete medium) and cell counting was performed.

2.2 Cell counting

A haemocytometer and coverslip were cleaned with 70% ethanol. Equal volumes of cell suspension and Trypan Blue were mixed in an Eppendorf tube and then pipetted into the haemocytometer chamber. A microscope was used to count the number of

cells in three 4 x 4 grids of haemocytometer. The cell concentration was calculated using the following formula: X cells/ml = cell number count x dilution factor x 10^4.

2.3 Magnetic isolation of CD14+ monocytes

A MACS LS column (Miltenyi Biotec, Bisley, UK), magnet and MACS buffer were placed in the fridge before use. A cell pellet of isolated PBMCs was resuspended with MACS buffer (phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA) and 2 mM ethylenediamine tetra-acetic acid (EDTA)) at a concentration of 80 µl MACS buffer per 10^7 cells and 20 µl CD14 microbeads (Miltenyi Biotec, Bisley, UK). Subsequently, the samples were incubated at 4 ℃ for 15 minutes. After incubation, 5-10 ml MACS buffer per 10^7 cells was added and centrifuged at 300 g for 10 minutes. The cell pellet was resuspended with 500 µl MACS buffer after centrifugation. Next, an LS column was placed in the magnetic field and the column was rinsed with 3 ml MACS buffer. Cell solution was applied to the column and the solution passed through the column was collected as CD14- cells. The column was then washed with 3 ml MACS buffer three times. After washing, the column was removed from the magnet and washed through with 5 ml MACS buffer into a sterile tube. This solution, containing CD14+ cells was centrifuged at 300 g for 5 minutes. Cell pellets were resuspended with RPMI-1640 complete medium then a cell count was performed according to the protocol described previously. Finally, samples were centrifuged at 300 g for 10 minutes and the cells were resuspended with RPMI-1640 complete medium at $5x10^5$ cells/ml.

2.4 Generation of monocyte-derived DCs

1 ml of CD14+ cell suspension ($5x10^5$ cells) was added to each well in a 24-well plate (Costar, High Wycombe, UK) in the presence of 50 ng/ml GM-CSF (Peprotech, London, UK) and 400 IU IL-4 (Peprotech, London, UK) and incubated at 37 ℃, 5% CO_2 for five days. Fresh medium and cytokines were added on day three. On day five, the immature DCs that had been generated were stimulated with 100 ng/ml LPS (Sigma-Aldrich, Gillingham, UK) or 30 μg/ml polyinosinic polycytidylic (Poly I:C) (Sigma-Aldrich, Gillingham, UK) and incubated at 37 ℃, 5% CO_2 for 24 h.

2.4.1 DC generation by adherence method

The adherence method was performed as described previously [136]. 10-15 x 10^6 PBMC per flask were seeded into T25 cell culture flasks and incubated at 37℃, 5% CO_2 for 2 hours in 5 ml of RPMI-1640 complete medium. After incubation, the non-adherent cell suspension was removed from the flasks and fresh RPMI-1640 complete medium was added to each flask. DC differentiation was carried out over five days in the presence of 50 ng/ml GM-CSF and 400 IU IL-4. Fresh medium and cytokines were added on day three. On day five, the immature DCs that had been generated were stimulated with 100 ng/ml LPS and incubated at 37 ℃, 5% CO_2 for 24 h.

2.5 Flow cytometric analysis

Samples were placed in fluorescence-activated cell sorter (FACS) tubes and then 1 ml of PBA buffer (PBS, 1% BSA and 0.1% sodium azide) was added to each tube. The tubes were centrifuged at 300 g for 5 minutes. The supernatant was discarded and the cell pellets were resuspended by gently tapping the tubes. Antibodies were then added and the samples were incubated in the dark at room temperature for 30 minutes. All antibodies used are summarized in table 2.1. After incubation, 2 ml of PBA was added to remove excessive and unbound antibodies, followed by centrifugation at 300 g for 5 minutes. Finally, the cells were fixed with 0.5% formaldehyde and the samples were incubated at 4℃ until flow cytometric analysis.

Single staining was used for compensation of the flow cytometry and isotype-matched antibodies were used as controls. Flow cytometry was carried out on the FC500 Flow Cytometer (Beckman Coulter, High Wycombe, UK) and data was analysed using Weasel Software v3.0.2. Fluorescence minus one was performed for quadrant setting using the Weasel software.

Table 2.1. Antibodies used for flow cytometric analysis. All antibodies used in this project and their conjugation and manufactures are shown.

Antibody	**Clone**	**Isotype**	**Fluorochrome**	**Company**
CD3	IM2635U	IgG1	ECD	Beckman Coulter
CD14	IM0645U	IgG1	FITC	Beckman Coulter
CD19	IM1285U	IgG1	PE	Beckman Coulter

CD56	IM2474U	IgG1	APC	Beckman Coulter
CD80	IM1853U	IgG1	FITC	Beckman Coulter
DC-SIGN	A07407	IgG1	PE	Beckman Coulter
CD86	REA968	IgG1	FITC	Miltenyi Biotec
CD83	REA714	IgG1	PE	Miltenyi Biotec
HLA-DR	REA805	IgG1	APC	Miltenyi Biotec
CD40	REA733	IgG1	PE-Vio 770	Miltenyi Biotec
CD36	REA760	IgG1	FITC	Miltenyi Biotec
CD1a	REA736	IgG1	PE	Miltenyi Biotec
CXCR-4	REA649	IgG1	APC	Miltenyi Biotec
CD74	REA1103	IgG1	PE-Vio 770	Miltenyi Biotec
PD-L1	MIH1	IgG1	APC	BD Biosciences
CD8	REA734	IgG1	PE	Miltenyi Biotec
CD4	REA623	IgG1	PE-Vio 770	Miltenyi Biotec

2.6 Electronic cigarette vapour extract (ECVE) preparation

E-cigarette devices and a DIY E-liquid Mixing kit were purchased from VAPEMATE. The E-liquid contained 50/50 PG/ VG and 12 mg/ml nicotine. The smoking apparatus consisted of a three-way stopcock attached to a 50 ml syringe, the E-cigarette and a bottle containing 20 ml RPMI complete medium. The vapour was drawn into the syringe, resulting in 200 mg of E-liquid being consumed and then bubbled into the 20 ml RPMI complete medium at a flow rate of 50 mL/min. It took approximately 25 cycles to consume 200 mg of E-liquid. The medium was then filter-sterilized with a 0.45 μm pore-size filter. The EC cartridge was weighed before and during the process

to confirm that 200 mg of liquid was consumed. The resulting extract was considered as 100% ECVE. Further dilution was needed to generate 1% and 3% ECVE, which were used to treat DCs. The choice of final ECVE concentrations was based on nicotine content as follows: The E-liquid contained 12mg of nicotine per gram of liquid. Therefore, 200mg of E-liquid contained 2.4mg nicotine. This resulted in 1% ECVE containing approximately 1.2μg/ml nicotine and 3% ECVE containing 3.6μg/ml nicotine. This is similar to the nicotine concentrations in 0.5% to 3% CS extract used in previous studies [137] .

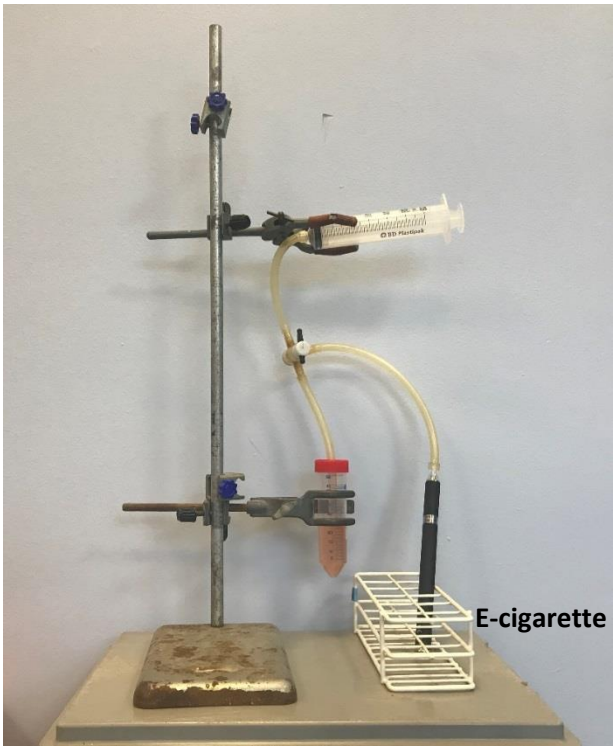


Figure 2.1. The apparatus for ECVE preparation

2.7 Reverse phase protein array (RPPA)

2.7.1 Cell lysate preparation

$5x10^5$ DCs were harvested and centrifuged for 5 min at 300 g. The pellets were re-suspended in RIPA buffer (Thermo Scientific, Loughborough, UK), supplemented with phosphate/protease inhibitor (Thermo Scientific, Loughborough, UK) and Benzonase nuclease ultrapure (Sigma-Aldrich, Gillingham, UK). Samples were then incubated on ice for 30 minutes with frequent shaking followed by centrifugation at 10,000 g at 4 ℃ for 10 minutes. The supernatants were collected and stored at -80℃ until used.

2.7.2 RPPA preparation and processing

Before conducting RPPA, a BCA protein assay was performed using a Micro BCA™ Protein Assay Kit (Thermo Scientific, Loughborough, UK) to confirm the protein concentration of lysates. Lysates then were diluted to 500 μg/ml with 4x SDS printing buffer containing 0.4M DTT and heated at 95 ℃ for 5 minutes. Subsequently, lysates were transferred to a 396-well plate and were robotically spotted onto nitrocellulose-coated glass slides by microarrayer (MicroGrid II, Digilab, Marlborough, USA) and dried in the air. Printed slides were stored at -20 ℃ until they were processed.

Slides were incubated in appropriate blocking buffer for 1 h. After washing with 0.5% Tween-20 in PBS 3 times for 5 minutes each time, slides were incubated with primary antibodies (Table 2) diluted in blocking buffer and incubated for 2 h at room temperature. Mouse anti-β-actin (Sigma Aldrich, Gillingham, UK), diluted 1:1000 in blocking buffer, was used to monitor this ‘housekeeping’ protein to control for

differences in protein loading. After washing 3 times with 0.5 % Tween-20 in PBS, the slides were incubated with biotinylated secondary antibodies diluted 1:20,000 in blocking buffer and incubated for 2 h at room temperature. Next, the slides were incubated with streptavidin conjugated to infrared dyes, IRDYE-800CW (1:10,000 in blocking buffer, LI-COR Biotechnology, Cambridge, UK) for 30 minutes at room temperature. Lastly, the slides were rinsed with distilled water, centrifuged dry and scanned with a Licor Odyssey scanner (LI-COR Biotechnology, Cambridge, UK) at 800 nm.

The resultant TIFF images were processed with GenePix Pro-6 Microarray Image Analysis software (Molecular Devices, Sunnyvale, USA). Protein signals were finally determined by using background subtraction and normalization to the internal housekeeping targets.

Table 2.2. Primary antibodies used in RPPA against the proteins of interest in DC lysates. All antibodies were purchased from Cell Signalling Technology (Beverly, USA).

Antibody Specificity	**Species**	**Dilution**	**Antibody specificity**	**Species**	**Dilution**
c-Rel (12707)	Rabbit	1:200	pSTAT3 (9145)	Rabbit	1:50
pMST1 (3681)	Rabbit	1:200	pHSP27 (2401)	Rabbit	1:50
HMGB1 (6893)	Rabbit	1:200	p44/42 MAPK (4370)	Rabbit	1:250
LSP1 (3812)	Rabbit	1:100	pAKT Threonine (9275)	Rabbit	1:50

nNOS (4231)	Rabbit	1:100	pAKT Serine (9271)	Rabbit	1:50
PIPK1C (3296)	Rabbit	1:100	pC-Jun (9261)	Rabbit	1:250
CD74 (77274)	Rabbit	1:400	pNF-κB P65 (3031)	Rabbit	1:50
IRAKM (4369)	Rabbit	1:50	pSTAT1 (9177)	Rabbit	1:250
TAB2 (3744)	Rabbit	1:250	pROS (3078)	Rabbit	1:250
IRF3 (4302)	Rabbit	1:2000	pc-Fos (5348)	Rabbit	1:500
pIRF3 (4947)	Rabbit	1:2000	Bcl-xL (2764)	Rabbit	1:250
pTAB2 (8155)	Rabbit	1:500	IRAK1 (4362)	Rabbit	1:250
NALP1 (4990)	Rabbit	1:500	IRAK2 (4367)	Rabbit	1:50
pIRF7 (5184)	Rabbit	1:500	pP38 (4511)	Rabbit	1:2000
SOD1 (4266)	Mouse	1:400			

2.8 Mixed lymphocyte reaction (MLR)

2.8.1 CD3+ T cell isolation

A cell pellet of isolated PBMCs was resuspended with MACS buffer at a concentration of 80 µl MACS buffer per 10^7 cells and 20 µl CD3 microbeads (Miltenyi Biotec, Bisley, UK). Subsequently, the samples were incubated at 4 ℃ for 15 minutes. After incubation, 5-10 ml MACS buffer per 10^7 cells was added and centrifuged at 300 g for 10 minutes. The cell pellet was resuspended with 500 µl MACS buffer after centrifugation. Next, an LS column was placed in the magnetic field and the column was rinsed with 3 ml MACS buffer. Cell solution was applied to the column which was then washed with 3 ml MACS buffer three times. After washing, the column was

removed from the magnet and washed through with 5 ml MACS buffer into a sterile tube. Cell solution was centrifuged at 300 g for 5 minutes and then cell pellets were resuspended with RPMI-1640 complete medium.

2.8.2 CFSE staining

T cell proliferation was determined by CFSE staining. CD3+ T cells were centrifuged at 300 g for 5 minutes and resuspended with PBS to 10^6 cells/ml. 1 ul of CFSE (Invitrogen, Loughborough, UK) was added per 10^6 cells which were then incubated at 37 ℃ in the dark for 20 minutes. RPMI-1640 complete medium was added to 4 times the volume of the cell suspension, followed by incubation at 37 ℃ in the dark for 20 minutes. Finally, cells were centrifuged at 300 g for 5 minutes and resuspended in RPMI-1640 complete medium.

2.8.3 DC-T cell co-culture

LPS-matured DCs treated with or without ECVE were co-cultured in 96-well round-bottom plates (Corning Life Sciences) with 1 x 10^5 CFSE-stained CD3+ allogenic T cells at different ratios for 5 days. Cells were then harvested and stained with fluorochrome-labelled antibodies to detect CD3, CD4 and CD8 by flow cytometry.

2.9 Enzyme-linked immunosorbent assay (ELISA)

2.9.1 Cytokine detection

Cell-free culture supernatants were collected and stored at -20°C before analysis. The levels of cytokines IL-6, IL-8, IL-10, IL-12p70, and TNF-α were measured by sandwich ELISA using Duo Set ELISA kits (R&D, Abingdon, UK) according to the manufacturer's instructions.

ELISA plates (Thermo Scientific, Loughborough, UK) were coated with capture antibodies diluted with PBS (IL-6: 2 ug/ml, IL-8, IL-10, IL-12p70, and TNF-α: 4 ug/ml) overnight. Plates were washed with 5% Tween-20 in PBS (PBST) 3 times between steps. Plates were then blocked with 1X reagent diluent (R&D, Abingdon, UK) for 1 h at room temperature. Supernatants were applied in duplicates and incubated for 2 h at room temperature, followed by 2 h-incubation with detection antibodies diluted with 1X reagent diluent (IL-6: 50 ng/ml, IL-8: 20 ng/ml, IL-10: 75 ng/ml, IL-12: 100 ng/ml, TNF-α: 50 ng/ml). (Standard curves which were performed by applying cytokine standards, instead of culture supernatants, as serial 2-fold dilutions across 7 concentrations (IL-6: 600-9.38 pg/ml, IL-8, 10, 12 and TNF-α: 2000-31.2 pg/ml) in 1X reagent diluent. The diluted standards were applied in duplicates, incubated for 2 h at room temperature.) Subsequently, plates were incubated with Streptavidin-HRP (R&D, Abingdon, UK) 1:40 in PBS for 20 minutes at room temperature. Substrate reagent, tetramethyl benzidine (TMB, Sigma-Aldrich, Gillingham, UK), was then added and incubated for 20 minutes at room temperature. Finally, the stop solution, 1.8 M sulphuric acid, was added and the absorbance was measured at 450 nm using a spectrophotometer (Glomax, Promega, UK).

2.9.2 Immunoglobulin (Ig) detection

ELISA plates (Thermo Scientific, Loughborough, UK) were coated with 1 mg/ml goat anti-human IgG capture antibody or 4 mg/ml goat anti-human IgM capture antibody (Sigma-Aldrich, Gillingham, UK), E-liquid 1, E-liquid 2, or 80/20 vegetable glycerol (VG)/propylene glycol (PG) (VAPEMATE,UK) overnight at room temperature. Plates were then washed 3 times with 0.5% PBST and blocked using PBS-3% BSA. Human IgG standard curves were performed by serial 2-fold dilutions across 15 concentrations (200 ng/ml – 0.006 ng/ml, Bethyl Laboratories, Montgomery, USA) in PBST-1% BSA. Human IgM standard curves were performed by serial 2-fold dilutions across 12 concentrations (100 ng/ml – 0.05 ng/ml, Bethyl Laboratories, Montgomery, USA) in PBST-1% BSA. The diluted standards were applied in duplicates to anti-Ig coated wells and incubated for 2 h at room temperature.

To test for E-liquid specific antibodies, human sera were diluted with PBST-1% BSA (1:1500 to measure specific IgG; 1:10 to measure specific IgM). The diluted sera were clarified by centrifugation for 10 minutes at 10,000g, applied to E-liquid or VG/PG coated wells and incubated for 2 h at room temperature. Plates were then washed 3 times with PBST. Biotinylated anti-human IgG (Biosource, Camarillo, USA) was diluted 1:15,000 with PBS, added to plates and incubated for 2 h at room temperature. Biotinylated anti-human IgM (Biosource, Camarillo, USA) was diluted 1:15,000 with PBS, added to plates and incubated for 2 h at room temperature. Plates were then washed 3 times with PBST and incubated with Streptavidin-HRP (R&D, Abingdon, UK) 1:40 in PBS for 20 minutes at room temperature. After the incubation, the plates were washed 3 times with PBST; then the substrate reagent, TMB (Sigma-Aldrich,

Gillingham, UK), was added and incubated for 20 minutes at room temperature. Finally, the stop solution, 1.8 M sulphuric acid, was added and the absorbance was measured at 450 nm using a spectrophotometer (Glomax, Promega, UK). Absolute values for IgG or IgM binding to E-liquid or VG/PG coated wells were interpolated from the IgG or IgM standard curves, respectively. The concentrations of IgG or IgM specific for GS or PB were then determined by subtracting the values for IgG or IgM binding, respectively, to VG/PG from the values for binding to the E-liquids.

To measure total IgG levels in serum, human sera were diluted to 1:16,000,000 with PBST-1% BSA. The diluted sera were clarified by centrifugation for 10 minutes at 10,000g and applied to anti-human IgG coated wells. The further steps were the same as described previously.

2.10 Statistical analysis

Data were analysed using GraphPad Prism v7.02 for Windows (GraphPad Software, CA) and values from independent experiments were expressed as dot scatter plots with the horizontal bars showing medians, unless otherwise stated. Normal distribution test was performed and either paired t test or paired one-way ANOVA was used if the data were normally distributed (i.e. parametric); Wilcoxon test or Friedman test were used if the data were not normally distributed (i.e. non-parametric). A p value <0.05 was considered statistically significant.

Chapter 3 ECVE did not alter the expression of CD80 and DC-SIGN, or cell death of DCs

3.1 Introduction

The impact of E-cigarette use remains controversial, so the effects of ECVE on DCs were investigated (which play an important role in linking both innate and adaptive immune responses) by generating an in vitro cell culture model using MoDCs. Circulating DCs in peripheral blood are approximately only 1-2 % of PBMCs, which makes it difficult to isolate them for in vitro studies; thus MoDCs are widely used for studying DCs instead. MoDCs are generated from monocytes in response to cytokines – particularly GM-CSF and IL-4. However, there were some difference and limitations in MoDCs compared to DCs isolated from blood. For example, MoDCs have different capacities of endocytosis [138] and T cell stimulation [139]. Moreover, cytokine concentrations used for generating MoDCs are higher than would be expected to occur in vivo and GM-CSF, which is not an essential cytokine for normal DC differentiation in vivo, could hinder the development of physiologically normal pathways [140]. MoDCs survive for only short periods of time in culture, and there is no differentiation of DCs from progenitors to replace dying cells [140]. Despite these problems, MoDCs are effective APCs and share some characteristics with myeloid DCs [140]. Thus, MoDCs are a useful tool to study DCs in vitro.

In our study, DCs were generated from CD14+ monocytes by magnetic isolation and adherence methods. Cell surface molecule DC-SIGN (CD209) and co-stimulatory

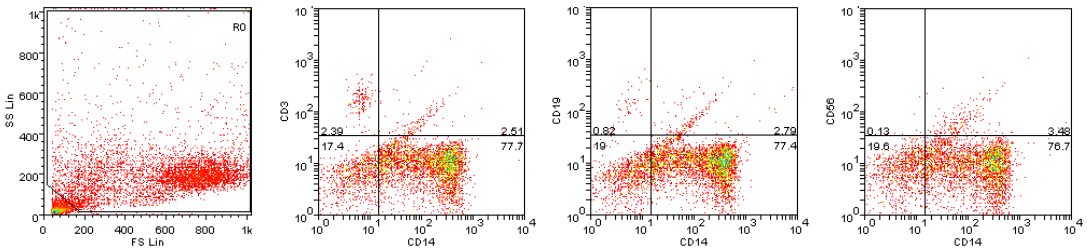
molecule CD80 were analysed by flow cytometry after cell-surface staining to confirm the generation of DCs. In the immature state, DCs express high levels of DC-SIGN which is involved in antigen binding. Upon antigen uptake, immature DCs become mature and express high levels of CD80 which is required for activation of naïve T cells in the lymphoid tissues. DCs were then further stimulated and treated with different concentrations of ECVE to examine their changes under different maturation stages and conditions. Furthermore, cell death of DCs altered by ECVE was also measured by propidium iodide (PI) staining using flow cytometry.

3.2 Results

3.2.1 Purity of CD14+ monocytes

CD14+ monocytes were separated from PBMC using positive magnetic isolation. The cell suspension of CD14+ monocytes was stained with anti-CD3-ECD, anti-CD14-FITC, anti-CD19-PE and anti-CD56-APC. Flow cytometry was used to check purity and Weasel v3.0.2 software was used to analyse the data. The results showed the purity of CD14+ monocytes ranged from 71-81%, which was far below expected levels. An example of the staining is shown in Figure 3.1.

A.



B.

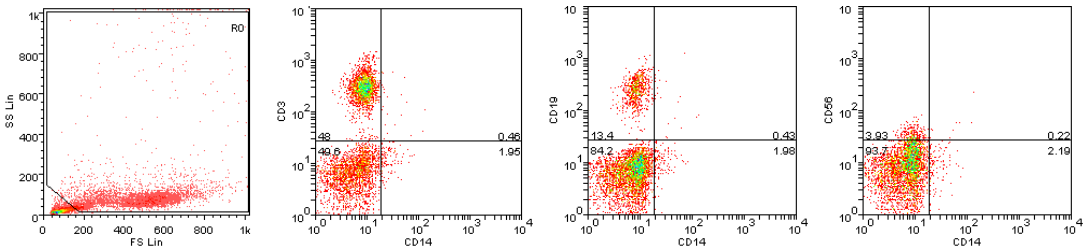
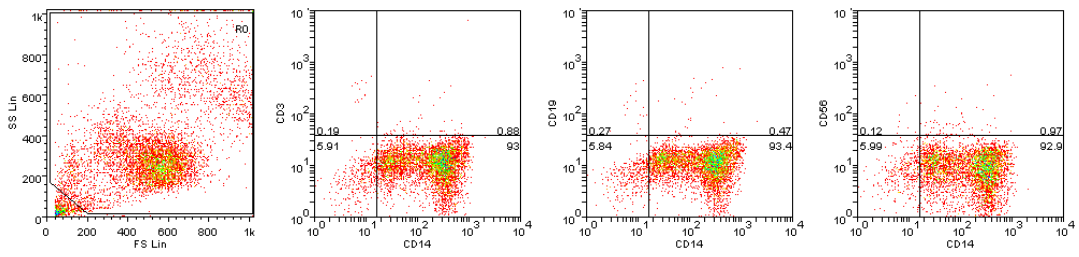


Figure 3.1. Assessment of the purity of CD14+ monocytes. CD14+ monocytes generated from magnetic isolation were stained with anti-CD3-ECD (T cells), anti-CD14-FITC (monocytes), anti-CD19-PE (B cells) and anti-CD56-APC (NK cells) to access the purity of cells using an FC500 flow cytometer. All cell populations were gated and debris was excluded (named gate R0). Fluorescence Minus One was applied to determine the quadrants. (A) CD14+ fraction indicated 71-81% of pure population of monocytes from buffy coat (B) CD14- fraction indicated cell populations collected as the waste during magnetic isolation. This figure is representative for three different individual samples.

3.2.2 Improvement of the purity of CD14+ monocytes

Due to the purity of CD14+ monocytes obtained previously not being high enough, optimization of the CD14+ magnetic isolation procedure was needed. Cell clumps were observed after CD14+ magnetic isolation, which might cause reduced purity of CD14+ cells. 5% EDTA was added to the complete medium and the cell suspension went through two-rounds of the protocol for magnetic isolation as mentioned previously. Then, the cells were centrifuged for 5 minutes instead of 10 minutes after magnetic isolation. The other steps remained the same. Both buffy coat and fresh blood were tested by this optimized protocol. The purity of CD14+ monocytes increased to 93% in buffy coat (Fig. 3.2A).and 95% in fresh blood (Fig. 3.2B).

A.



B.

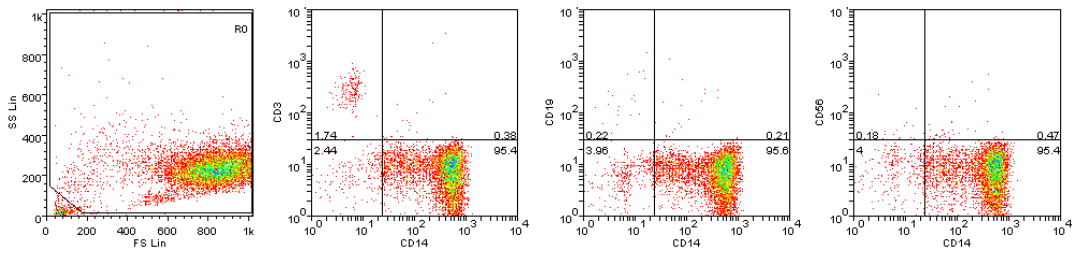


Figure 3.2. Assessment of the purity CD14+ monocytes from fresh blood by optimized protocols. Following the optimized protocols for magnetic isolation of CD14+ monocytes, cells were stained with anti-CD3-ECD (T cells), anti-CD14-FITC (monocytes), anti-CD19-PE (B cells) and anti-CD56-APC (NK cells) to access the purity of cells using an FC500 flow cytometer. All cell populations were gated and debris was excluded (gate R0). Fluorescence Minus One was applied to determine the quadrants. (A) CD14+ fraction indicated 93% of pure population of monocytes from buffy coat. (B) CD14+ fraction indicated 95% of pure population of monocytes from fresh blood. This figure is representative for two different individual samples.

3.2.3 Generation of immature and mature DCs

To confirm the generation of immature DCs on day five from CD14+ cells incubated with GM-CSF and IL-4, the expression of the DC specific marker DC-SIGN and the co-stimulatory molecule CD80 were analysed using an FC500 flow cytometer after extracellular staining. The results showed high-level expression of DC-SIGN but low-level of CD80 (Fig. 3.3A, 3.3B), confirming the generation of immature DCs.

To generate mature DCs, immature DCs on day five were stimulated with 100 ng/ml LPS or 30 ug/ml Poly I:C for 24 h. That is, mature DCs were harvested on day six followed by extracellular staining for cell surface markers. There was a trend towards a decrease of DC-SIGN in response to both LPS and Poly I:C (Fig. 3.3A, 3.3B). In contrast to immature DCs, mature DCs expressed significantly upregulated levels of CD80 (Fig. 3.3A, 3.3B), demonstrating generation of mature DCs. No difference of

CD80 expression between LPS-matured and Poly I:C matured DCs was noted (Fig. 3.3B).

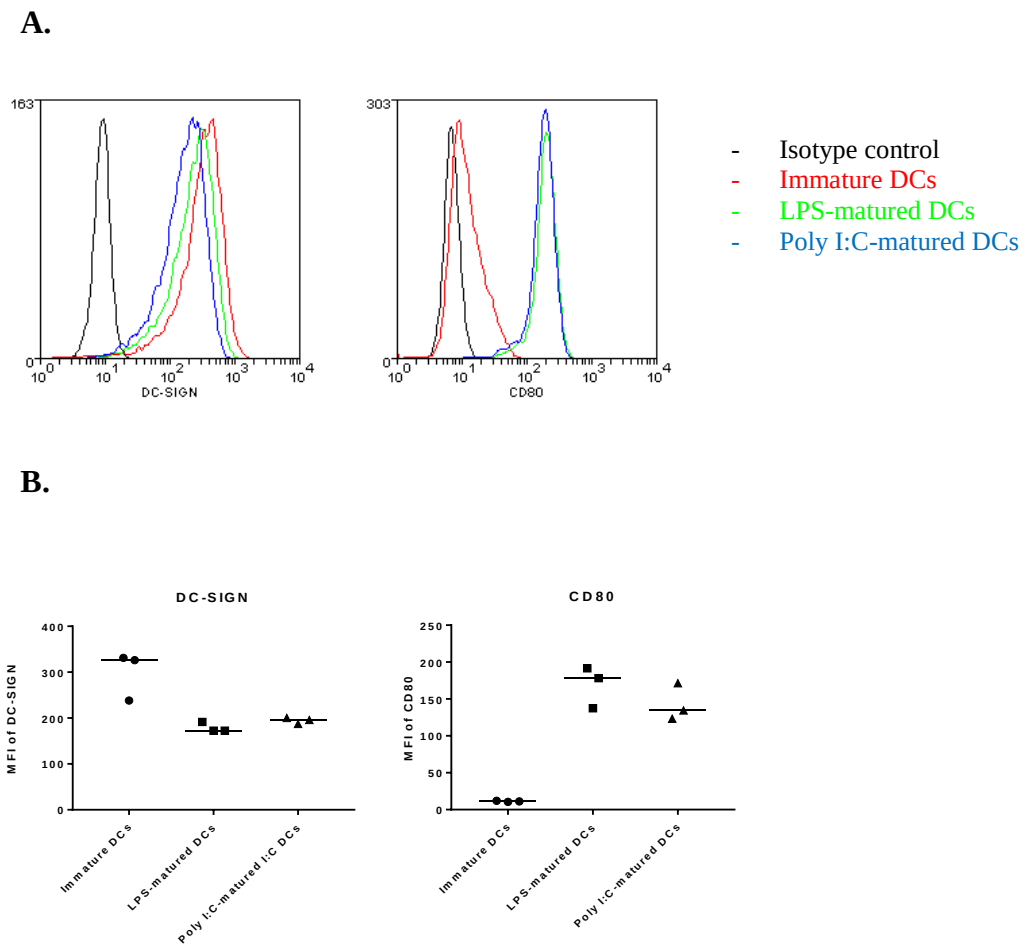


Figure 3.3. Generation of immature and mature DCs. (A) Histograms showing the distribution of DC-SIGN and CD80 expression by immature and mature DCs. High levels of DC-SIGN and low levels of CD80 expression were seen on immature DCs. There was an increase in CD80 expression by LPS/Poly I:C matured DCs. (B) The

comparison between median fluorescence intensity (MFI) of immature and LPS/Poly I:C matured DCs showed a trend toward a decrease of DC-SIGN and clear upregulation of CD80 in response to both LPS and Poly I:C. The horizontal bars represent the medians of three independent experiments.

3.2.4 Generation of DCs by adherence method

The adherence method is another common technique used to isolate monocytes from blood. PBMCs were seeded into T25 cell culture flasks and adherent cells isolated as described in Materials and Methods. The adherent cells were then incubated with GM-CSF and IL-4 for five days. Immature DCs were generated. Subsequently, cells were stimulated with 100 ng/ml LPS for 24 hours to induce maturation. Cells were stained for expression of DC-SIGN and CD80 and analysed using an FC500 flow cytometer. Immature DCs expressed high levels of DC-SIGN but low levels of CD80 (Fig. 3.4). In contrast, there was an increase of CD80 expression in mature DCs. These results confirmed the generation of immature and mature DCs by the adherence method. Several disadvantages of adherence method have been reported in section 3.4 [141]: therefore, magnetic isolation as described in section 3.2.2 was used in the rest of the study.

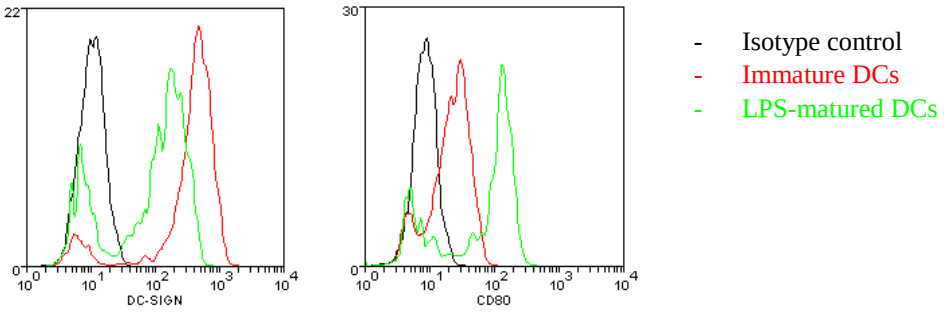


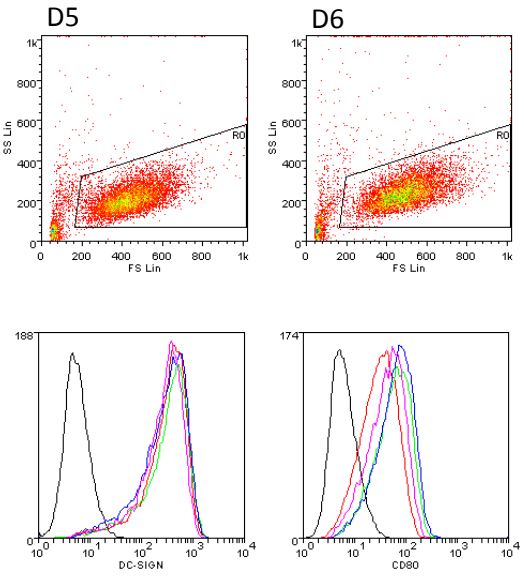
Figure 3.4. The expression of cell surface molecules on immature and mature DCs generated by the adherence method. A comparison of histograms of immature and mature DCs for expression of DC-SIGN and CD80 are shown. High levels of DC-SIGN but low levels of CD80 were seen, confirming immature DC generation. There was a shift to increased CD80 expression in LPS-matured DCs. This figure is representative of three experiments.

3.2.5 24 h-ECVE treatment did not alter DC-SIGN and CD80 expression on DCs

To examine the effects of ECVE on DCs, immature and LPS/Poly I:C-matured DCs were exposed to ECVE for 24 h (i.e. Day 6). Immature DCs on day five were stained for the DC specific marker DC-SIGN and for the co-stimulatory molecule CD80. The same staining was performed following incubation without or with 1% or 3% ECVE for 24 h. FC500 flow cytometry was used to detect the expression of cell surface molecules. Compared to immature DCs without ECVE, no significant changes of DC-SIGN and CD80 (Fig. 3.5A, 3.6A) expression was shown following 1% and 3% ECVE exposure for 24 h.

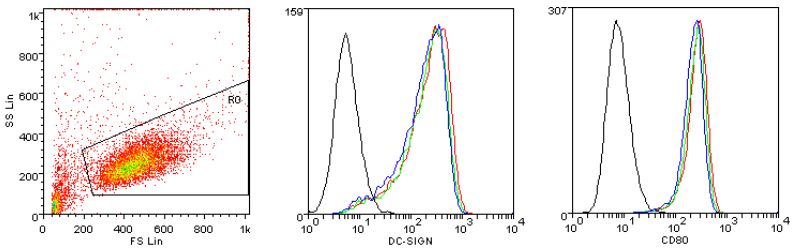
Immature DCs were stimulated with LPS or Poly I:C in the absence or presence of 1% and 3% ECVE for 24 h. Significantly elevated levels of CD80 were induced, confirming mature DC generation. Again, neither DC-SIGN nor CD80 (Fig. 3.5B, 3.5C, 3.6B, 3.6C) expression on LPS/Poly I:C-matured DCs was affected by 24 h-ECVE treatment in comparison to mature DCs without ECVE. These results indicated that 24 h-exposure to ECVE at different concentrations did not influence the cell surface molecule expression and maturation status of immature and mature DCs.

A. Immature DCs



- Isotype control
- D5 + 0% ECVE
- D6 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

B. LPS-matured DCs



- Isotype control
- + 0% ECVE
- + 1% ECVE
- + 3% ECVE

C. Poly I:C-matured DCs

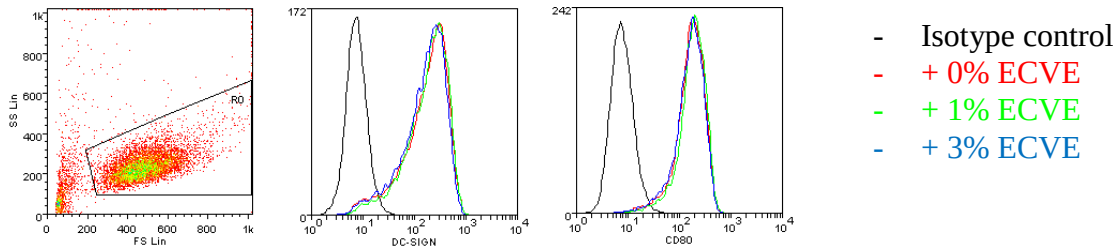


Figure 3.5. The expression of DC-SIGN and CD80 on immature and mature DCs treated with ECVE for 24 h. (A) The dot plot and comparison of histograms of DC-SIGN and CD80 expression on immature DCs treated without or with ECVE. (B) The comparison of histograms of DC-SIGN and CD80 expression on LPS-matured DCs treated without or with ECVE. (C) The comparison of histograms of DC-SIGN and CD80 expression on Poly I:C-matured DCs treated without or with ECVE. The DC population was gated (named gate R0) and this was applied during the analysis. This figure is representative of six different individual experiments.

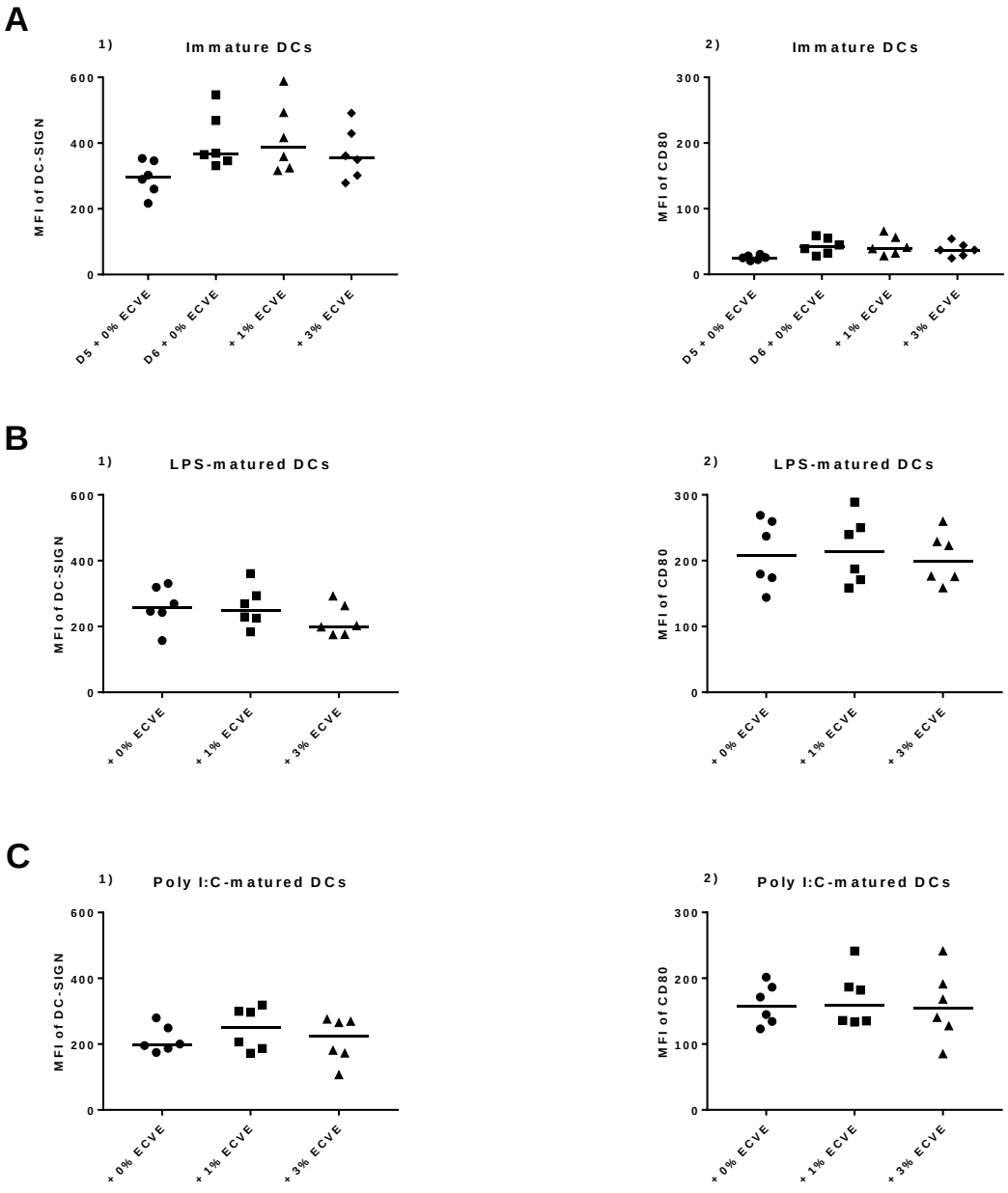


Figure 3.6. 24 h-ECVE treatment did not alter DC-SIGN and CD80 expression on either immature or mature DCs. (A) The MFI of DC-SIGN and CD80 expression comparing immature DCs without ECVE and immature DCs treated with 1% and 3% ECVE. (B) The MFI of DC-SIGN and CD80 comparing LPS-matured DCs without ECVE and LPS-matured DCs treated with 1% and 3% ECVE. (C) The MFI of DC-

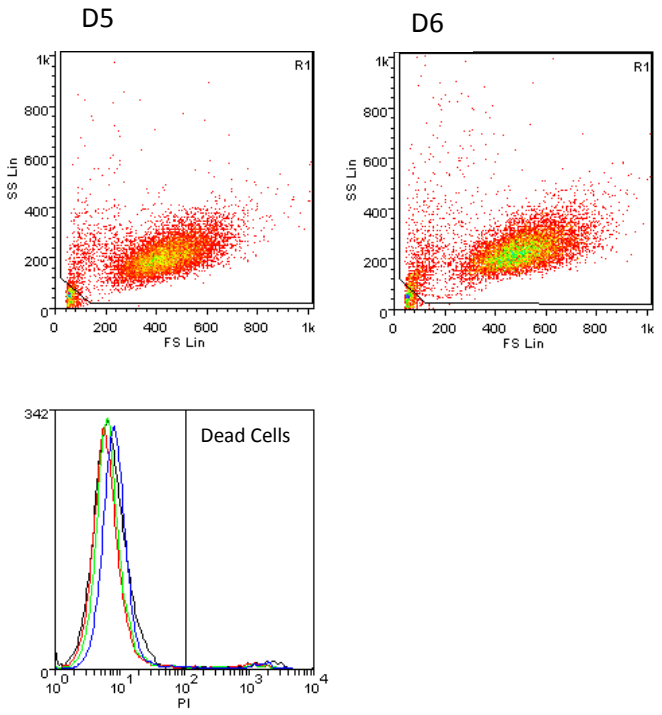
SIGN and CD80 expression comparing Poly I:C-matured DCs without ECVE and Poly I:C-matured DCs treated with 1% and 3% ECVE. 24 h-ECVE exposure had no effects on expression of DC-SIGN and CD80 in either immature or mature DCs. The horizontal bars represent the medians of six independent experiments. All data was normally distributed so paired one-way ANOVA was used.

3.2.6 24 h-ECVE treatment did not alter cell viability of DCs

Both immature and LPS/Poly I:C-matured DCs were stained with propidium iodide (PI) to analyse their viability after ECVE exposure. The experiment was repeated six times and flow cytometry was used to analyse PI expression. Compared to immature DCs without ECVE on day five, no increase of cell death was noted in immature DCs without ECVE on day six (Fig. 3.7A, 3.7B). Moreover, no significant cell death was induced in either immature or mature DCs in the presence of 1% or 3% ECVE exposure for 24 h in comparison to immature or mature DCs without ECVE, suggesting that ECVE is not cytotoxic to either immature or mature DCs (Fig. 3.7A, 3.7B).

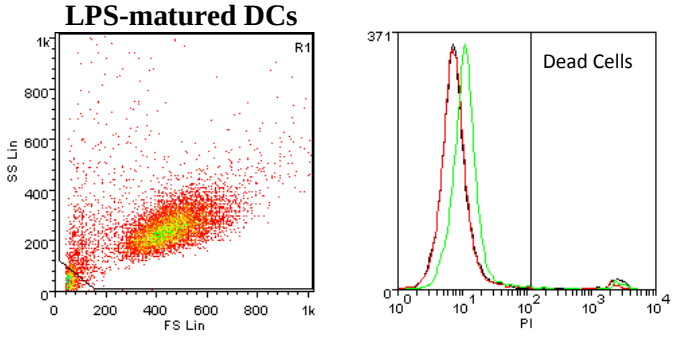
A.

Immature DCs



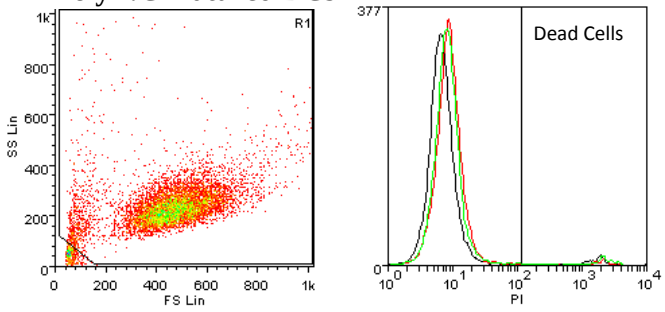
- D5 + 0% ECVE
- D6 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

LPS-matured DCs



- + 0% ECVE
- + 1% ECVE
- + 3% ECVE

Poly I:C matured DCs



- + 0% ECVE
- + 1% ECVE
- + 3% ECVE

B.

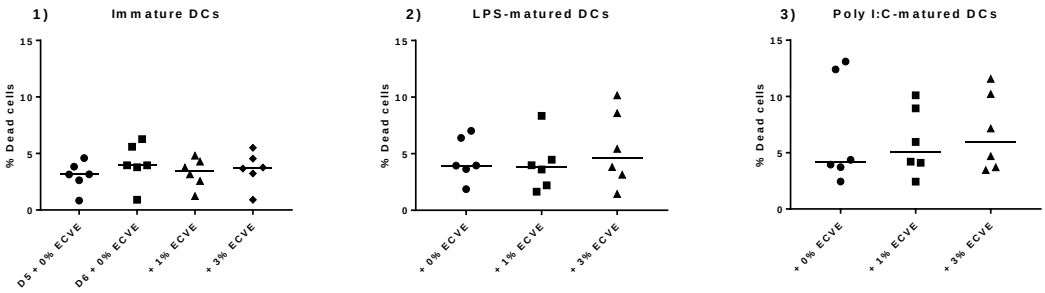


Figure 3.7. 24 h-ECVE treatment did not alter cell death of either immature or mature DCs. Immature and LPS/Poly I:C-matured DCs were treated without or with 1% or 3% ECVE for 24 h. DCs were then stained with PI to detect the percentage of dead cells. (A) Dot plots and comparison of histograms of PI-stained cells for immature DCs and LPS/Poly I:C-matured DCs treated without or with ECVE. All populations were gated but the debris was excluded (named gate R1), and this was applied during analysis. (B) Comparison of percentage of dead cells (PI-stained) for immature DCs and LPS/Poly I:C-matured DCs treated without or with ECVE. No significant cell death was induced in DCs by exposure to 1% or 3% ECVE for 24 h, suggesting that ECVE is not be cytotoxic to either immature or mature DCs. The horizontal bars represent the medians of six independent experiments. If data was normally distributed, paired one-way ANOVA was used (Fig. 3.7B1, 3.7B2), otherwise Friedman test was used (Fig. 3.7B3).

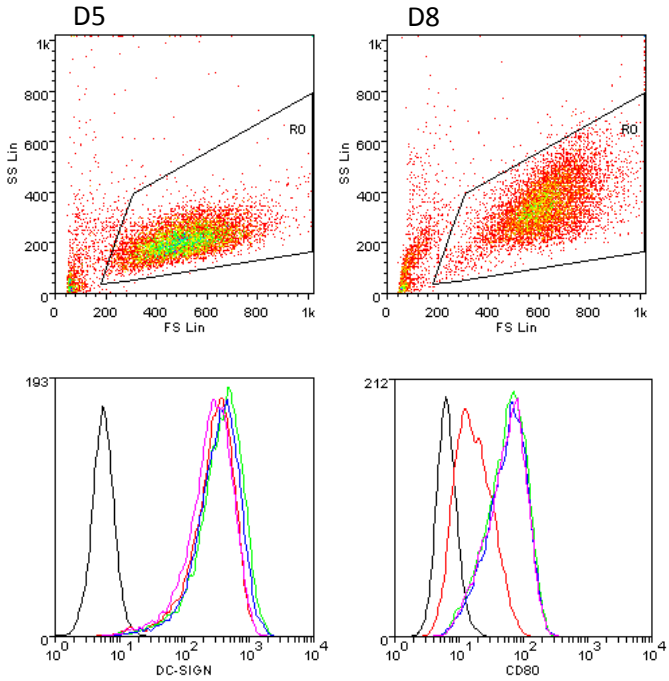
3.2.7 72 h-ECVE treatment did not alter DC-SIGN or CD80 expression on DCs

To investigate the effects of prolonged ECVE exposure, both immature and mature DCs were treated with ECVE for 72 h. On day five, immature DCs were treated with 1% or 3% ECVE for 72 h; that is, cells were harvested on day eight. Changes of surface molecule DC-SIGN and CD80 were assessed by flow cytometry after cell-surface staining. Firstly, on day 5, high levels of DC-SIGN and low levels of CD80 expression were observed, confirming the generation of immature DCs (Fig. 3.8A, 3.9A). Immature DCs without ECVE on day eight had significantly higher CD80 expression than immature DCs without ECVE on day five (Fig. 3.8A, 3.9A, p=0.0076). Nevertheless, there was no difference in CD80 expression between immature DCs without ECVE on day eight and immature DCs treated with 1% and 3% ECVE for 72 h (Fig. 3.8A, 3.9A). These results demonstrated the elevation of CD80 expression could be attributed to prolonged incubation of DCs rather than being due to ECVE exposure. No alterations in DC-SIGN expression were observed (Fig. 3.8A, 3.9A).

In order to examine the 72 h effects of ECVE on mature DCs, immature DCs were stimulated on day five with LPS or Poly I:C and incubated without or with 1% or 3% ECVE for 72 h; i.e. cells were harvested on day eight. DC-SIGN expression was detected and the levels of CD80 increased dramatically (Fig. 3.8C, 3.9C), supporting the generation of mature DCs. Both LPS- (Fig. 3.8B, 3.9B, p=0.0304) and Poly I:C- (Fig. 3.8C, 3.9C, p=0.057) matured DCs without ECVE on day eight had significantly higher CD80 expression than mature DCs on day six not treated with ECVE. However, there was no difference of CD80 expression between LPS/Poly I:C-matured DCs without ECVE on day eight and LPS/Poly I:C-matured DCs treated with ECVE for 72

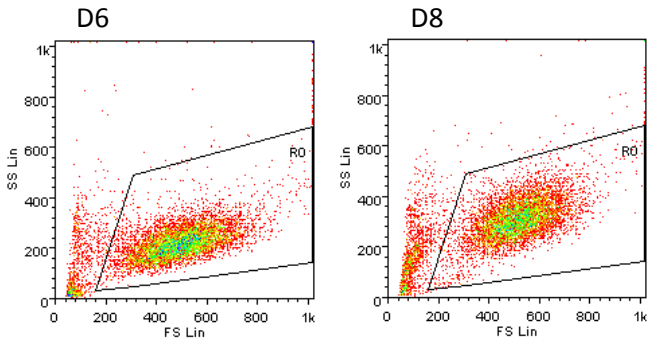
h. These results were similar to treatment in immature DCs and also supported the elevation of CD80 expression was attributed to prolonged incubation of DCs. No alterations in DC-SIGN expression were seen (Fig. 3.8B, 3.8C, 3.9B, 3.9C).

A. Immature DCs



- Isotype control
- D5 + 0% ECVE
- D8 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

B. LPS-matured DCs



- Isotype control
- D6 + 0% ECVE
- D8 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

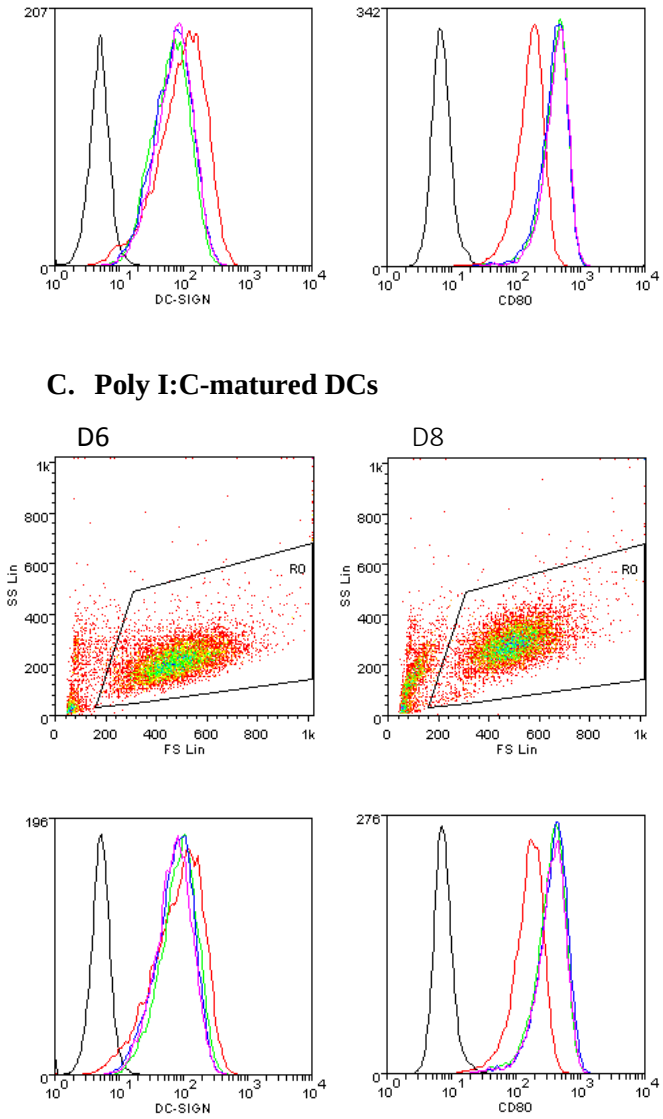


Figure 3.8. The expression of DC-SIGN and CD80 on immature and mature DC treated with ECVE for 72 h. (A) Comparison of histograms of DC-SIGN and CD80 expression on immature DCs treated without or with ECVE. (B) Comparison of histograms of DC-SIGN and CD80 expression on LPS-matured DCs treated without or with ECVE. (C) The comparison of histograms of DC-SIGN and CD80 expression on Poly I:C-matured DCs treated without or with ECVE. The DC population was gated (named gate R0) and this was applied during analysis. This figure is representative of six different individual experiments.

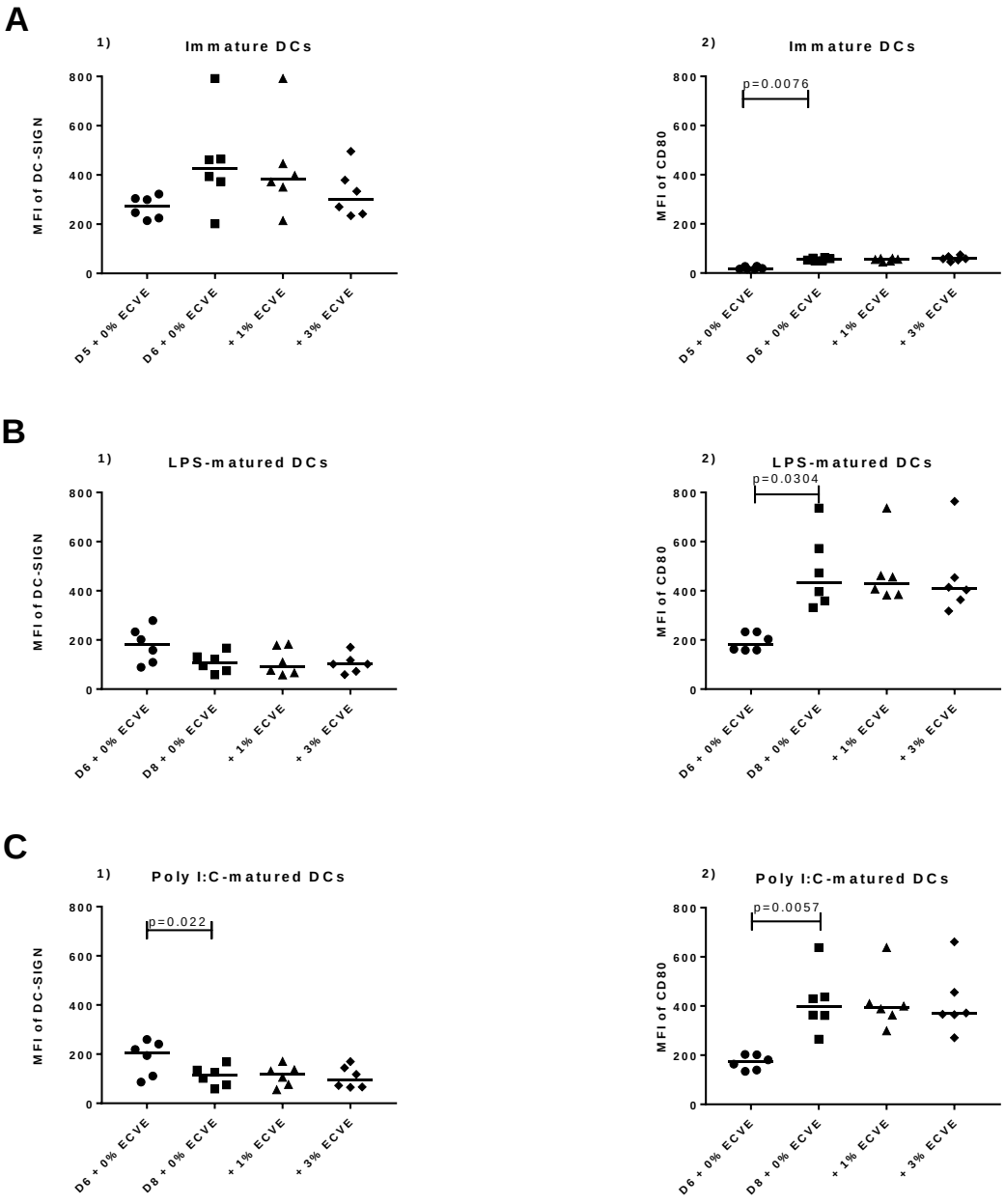


Figure 3.9. 72 h-ECVE treatment did not alter DC-SIGN or CD80 expression on either immature or mature DCs. (A) The MFI of DC-SIGN and CD80 expression comparing immature DCs without ECVE and immature DCs treated with 1% and 3% ECVE. (B) The MFI of DC-SIGN and CD80 expression comparing LPS-matured DCs

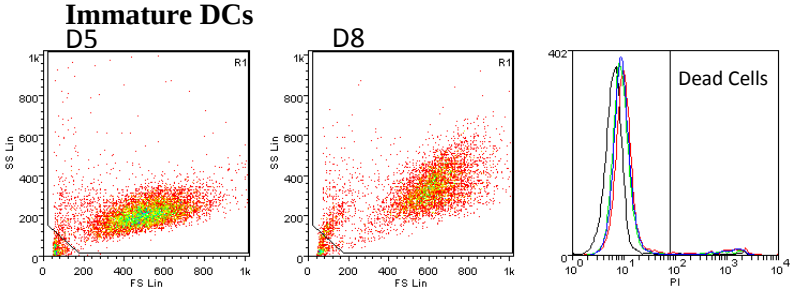
without ECVE and LPS-matured DCs treated with 1% and 3% ECVE. (C) The MFI of DC-SIGN and CD80 expression comparing Poly I:C-matured DCs without ECVE and Poly I:C-matured DCs treated with 1% and 3% ECVE. 72 h-ECVE exposure had no effects on expression of DC-SIGN and CD80 in either immature or mature DCs. The horizontal bar represents the median of six independent experiments. If data is normal distributed, paired one-way ANOVA was used (Fig. 3.9A1, 3.9A2, 3.9B1, 3.9C1 and 3.9C2), otherwise paired Friedman test was used (Fig 3.9A2).

3.2.8 72 h-ECVE treatment did not alter the viability of DCs.

Both immature and LPS/Poly I:C-matured DCs were stained with propidium iodide (PI) to analyse their viability after ECVE exposure. The experiment was repeated six times and an FC500 flow cytometer was used to check PI expression after staining. 72 h-ECVE exposure did not have an effect on cell viability of immature DCs and LPS-matured DCs (Fig. 10A, 10B). Compared to Poly I:C-matured DCs without ECVE harvested on day six, percentage of dead cells in Poly I:C-matured DCs without ECVE harvested on day eight increased significantly ($P=0.041$). Nevertheless, there was no difference in cell viability between Poly I:C-matured DCs without ECVE on day eight and Poly I:C-matured DCs treated with different concentrations of ECVE for 72 h (Fig. 10A, 10B). That is, cell death after 72 h treatment was due to the prolonged incubation rather than ECVE.

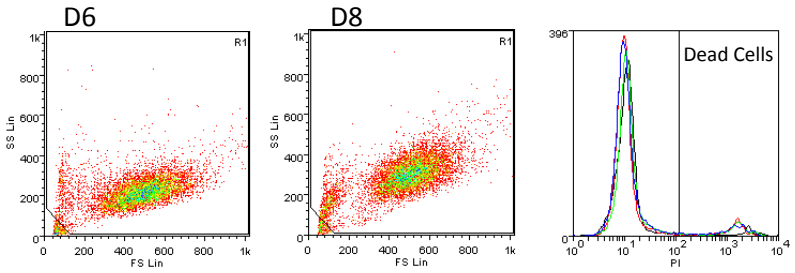
A.

Immature DCs



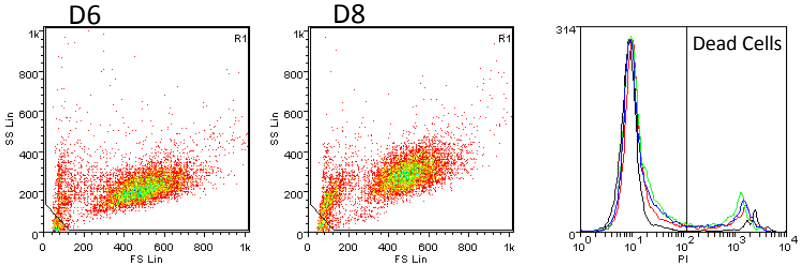
- D5 + 0% ECVE
- D8 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

LPS-matured DCs



- D5 + 0% ECVE
- D8 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

Poly I:C-matured DCs



- D5 + 0% ECVE
- D8 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

B.

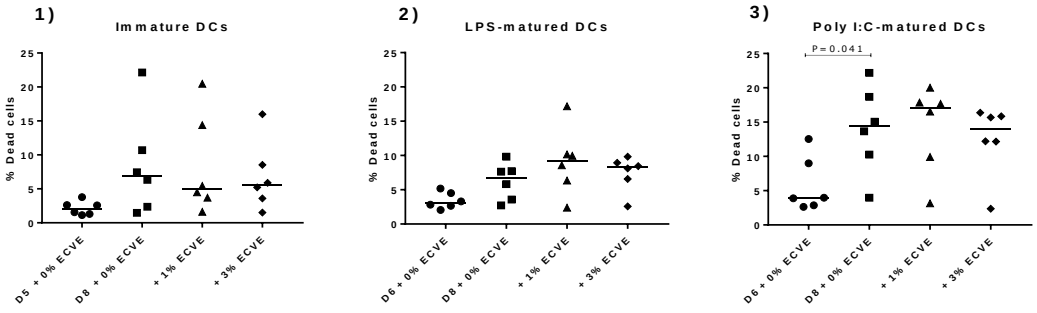


Figure 3.10. 72 h-ECVE treatment did not alter cell death of either immature or mature DCs. Immature and LPS/Poly I:C-matured DCs were treated without or with 1% and 3% ECVE for 72 h. DCs were then stained with PI to detect the percentage of dead cells. (A) Dot plots and comparison of histograms of PI-stained cells for immature DCs and LPS/Poly I:C-matured DCs treated without or with ECVE. All populations were gated and the debris was excluded (named gate R1), and this was applied during analysis. (B) The comparison of percentage of dead cells (PI-stained) for immature DCs and LPS/Poly I:C-matured DCs treated without or with ECVE. No significant cell death was induced in either immature or mature DCs by exposure to 1% or 3% ECVE for 72 h. Nevertheless, prolonged incubation of cells induced cell death. The horizontal bars represent the medians of six independent experiments. If data was normally distributed, paired one-way ANOVA was used (Fig. 3.10B1, 3.10B2), otherwise paired Friedman test was used (Fig. 3.10B3).

3.3 Discussion

Monocyte-derived dendritic cells (MoDCs) are widely used for studying DCs in vitro. In order to generate MoDCs in vitro, PBMCs were firstly separated from buffy coats which were used due to the high numbers of PBMCs and easy accessibility. Subsequently, positive magnetic selection of CD14+ monocytes from PBMCs was performed. The purity of CD14+ monocytes was examined by flow cytometry. Initially, the average purity was approximately 80% and clumps of cells were noticed, which may contribute to the low purity. Therefore, 5% EDTA, which helps to avoid clumping, was added to the RPMI-1640 complete medium. In addition, the magnetic isolation procedure was performed twice. The purity of CD14+ monocytes improved to 95% in both buffy coat and fresh blood. As a consequence, EDTA was added to the complete medium and double magnetic isolation was used in all further experiments.

Adherence of cells to tissue culture plastic is another technique that can be used to isolate CD14+ monocytes from PBMCs. Although the adherence method is simple, cheap and time-saving, the purity can be very low and variable, depending on the type of surface to which cells adhere [142]. In addition, the process of detaching monocytes can cause cell damage [142] and cell activation [142,143], or modify functional activities [144]. Therefore, CD14+ cell isolation by positive magnetic selection is preferable and was used throughout our study. The CD14 antigen belongs to the LPS receptor complex. Binding of antibody to CD14 does not trigger signal transduction since CD14 lacks a cytoplasmic domain.

Monocytes can differentiate into DCs in the presence of GM-CSF and IL-4 when cultured in vitro. CD14+ monocytes were therefore cultured with these two cytokines

for five days and immature DCs were generated. Immature DCs were analysed with DC-SIGN (CD209) and co-stimulatory molecule CD80. DC-SIGN, which is a C-type lectin receptor present exclusively on the surface of DCs, acts both as a pathogen recognition receptor and adhesion molecule. DC-SIGN recognizes carbohydrate structures in numerous pathogens including bacteria, viruses and fungi. Upon pathogen recognition, DC-SIGN triggers intracellular signalling by Raf-1 activation, leading to acetylation of NF-κB subunit p65 [145]. Also, DC-SIGN can bind to ICAM-2 on endothelial cells, thereby inducing the trans-endothelial migration of DCs [146]. DC-SIGN also facilitates adhesion between DCs and naïve T cells via its ligand ICAM-3 [147]. The co-stimulatory molecule CD80 (known as B7.1), present on mature DCs, can bind to CD28 on T cells and induce the second signal which is essential for T cell activation. In our study, immature DCs that were generated expressed high levels of DC-SIGN but low levels of CD80, confirming the generation of immature DCs.

Subsequently, immature DCs were stimulated with 100 ng/ml of LPS or 30 ug/ml of Poly I:C for 24 hours to induce maturation. LPS is a component of Gram-negative bacteria that binds to TLR4 on the surface of immature DCs, leading to the activation of cells. On the other hand, Poly I:C is a double-stranded RNA and activates DCs via TLR3 which localizes in the endosomal membranes. The decrease of DC-SIGN expression seen in our study was consistent with previous findings [148]. In contrast, the levels of CD80 elevated dramatically, confirming the mature status of DCs.

E-cigarettes are devices that vaporize liquid to simulate the feeling of conventional cigarette smoke. They have been claimed to be safe alternatives to conventional

cigarette smoking due to the lack of combustion of tobacco-specific toxicants. Research has shown the adverse effects of conventional cigarette smoke on DCs; however, there is a lack of studies focusing on e-cigarettes. Therefore, the effects of electronic cigarette vapour exposure on DCs were investigated. Both immature and mature MoDCs were treated with different concentrations of ECVE (1% and 3%), which were chosen relying on previous work, for 24 and 72 hours. No changes in expression of the surface markers DC-SIGN and CD80, or in cell death, were observed in either immature or mature DCs, suggesting e-cigarettes did not affect DC maturation. Interestingly, some studies demonstrated alterations of CD80 expression by CSE or nicotine. Treatment with CSE or nicotine for 24 hours did not suppress the expression of several co-stimulatory molecules, including CD80, on immature DCs; however, both CSE and nicotine suppressed their expression in LPS-matured DCs [149]. Mice exposed to cigarette smoke showed dramatically reduced expressions of CD80 in DCs within thoracic lymph nodes [150] and lung tissue [151]. Conversely, upregulation of CD80 in mDCs was detected from bronchoalveolar lavage fluid (BALF) of COPD patients who had a smoking history of at least 10 pack-years [152]. As mature DCs interact with naïve T cells to induce Th1 antigen-specific activation while immature DCs are tolerogenic, elevation of CD80 might be one of the factors that induce robust inflammatory responses thereby causing inflammatory or autoimmune diseases. On the other hand, there is also evidence that CD80 tends to induce suppression of T cell proliferation, whereas CD86 induces activation of T cells [153].

Elevation of CD80 has been reported in several types of diseases. CD80 level on myeloid DC derived from secondary progressive multiple sclerosis (SP-MS) patients was higher than in the relapsing-remitting form or healthy controls [154]. CD80 expression could induce IL-12 and TNF-α production and this is consistent with findings that the higher percentage producing IL-12 and TNF-α in SP-MS [154], thereby demonstrating SP-MS patients tend to have Th1 responses. DCs from synovial fluid in rheumatoid arthritis (RA) expressed higher levels of MHCII, CD40 and CD80 and had potent ability to stimulate T cells [155], suggesting those DCs are phenotypically and functionally more mature than normal and RA peripheral blood DCs. Monocyte-derived DCs and freshly isolated peripheral blood myeloid DCs from systemic lupus erythematosus patients displayed high level of CD80 [156]. Furthermore, CD80 on mDC1, mDC2 and pDCs from lung surgical specimens of COPD patients increased remarkably with worsening disease severity [157].

Significantly elevated CD80 and reduced cell viability were observed in both untreated immature and mature DCs after 72 h incubation, indicating that prolonged incubation of DCs might affect surface molecule expression and cell death. This was not affected by exposure to ECVE. As a results, 24 h was chosen as the assay time point for rest of the study.

3.4 Conclusion

Positive magnetic isolation of PBMCs was successful in yielding high purity of CD14+ monocytes from both buffy coat and fresh blood when using optimized

protocols. Monocyte-derived immature DCs were generated by culturing CD14+ monocytes with GM-CSF and IL-4 for five days. Immature DCs were then stimulated with LPS or Poly I:C for 24 h to become mature forms which were confirmed by investigating the expression of cell surface molecules. ECVE exposure for either 24 h or 72 h did not have effects on the expression of the cell surface molecules DC-SIGN and CD80, or cell death of monocyte-derived DCs. However, altered cell surface molecule expressions and induced cell death were noted following prolonged incubation of cells. Therefore, 24 h ECVE exposure will be used as the time point in the rest of the experiments, which was commonly used in studies investigating the effects of conventional cigarettes on DCs.

Chapter 4 Reverse phase protein microarray

4.1 Introduction

In this part of the study, the aim was to examine the effects of ECVE on the expression of intracellular proteins in DCs using reverse phase protein array (RPPA). The intracellular proteins that were selected for analysis were based on a study concerning the effects of conventional cigarette on DCs conducted in our group previously [137]. The chosen candidates cover major signaling pathways associated with several DC functions, which might provide a more complete picture of biological changes from proteomic aspects.

RPPA, a miniaturized dot plot, allows measurement of proteins in phosphorylated, glycosylated, cleaved or total cellular forms across many samples simultaneously. Compared with other proteomic methods, RPPA is a sensitive and high-throughput technology which yields a large volume of quantitative or semi-quantitative molecular information in an experiment using only nano-litres of samples [158]. Various types of samples can be used in RPPA, including cell lysates, tissues, serum or plasma and biological fluids like synovial fluid and cerebrospinal fluid [158].

The general process of RPPA includes sample preparation, printing, immunostaining, signal detection and data analysis. Samples are spotted on microarray slides, which are most commonly coated with nitrocellulose due to its good binding capacity, retention of molecular structure and compatibility with a variety of detection methods [159]. The printed slides are then probed with specific antibodies against the proteins of interest. Subsequently, incubation with biotinylated secondary antibody followed by

streptavidin conjugated with infrared dye facilitates the detection via infrared scanner. Amplification methods can be applied to enhance signals. Signal intensities of spotted samples detected are used for further analysis after subtracting background signals. The workflow of RPPA is illustrated in figure 4.1.

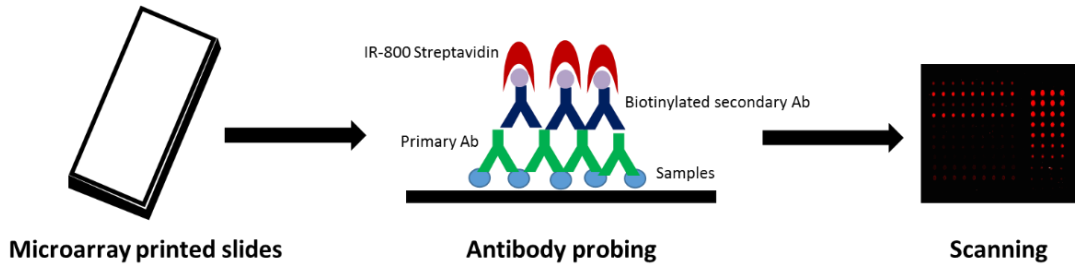


Figure 4.1. General framework of RPPA. Samples are printed on nitrocellulose-coated microarray slides using BioRobotics microGrid II Arrayer. The printed slides are blocked and incubated with specific antibodies against the proteins of interest. This is followed by a biotinylated secondary antibody and streptavidin conjugated with infrared dye for signal detection. Signal intensities of spotted samples detected by LI-COR infrared scanner are used for further analysis after subtracting background signals.

RPPA has been successfully used for the profiling of post-translational modifications and cell signalling pathways in cell and tissue lysates [160]. In addition to applications in basic research, preclinical and clinical importance of signaling pathway using RPPA have flourished due to its rapid turnaround time. RPPA has been used to study signaling pathways related to angiogenesis, DNA damage, apoptosis and other cellular functions in cancer research. RPPA could also be a diagnostic tool to address prospective

biomarkers which are correlated with the state and progression of diseases, thereby providing appropriate therapies to patients based on their individual requirements and also minimizing the adverse effects caused by incorrect treatments. Furthermore, RPPA has been adopted to study mechanisms involving signaling pathways underlying drug-resistance. Evidence demonstrates the utility of RPPA to evaluate therapeutic effects in clinical trials, which supports the feasibility of using RPPA routinely in clinical fields in the future [158,161].

Several factors including printing setup, blocking buffers and detection methods could affect the performance of RPPA. Therefore, in this part of the study, firstly the protocols for RPPA were tested and improved, and then the optimized protocols were used to analyze expression of signaling molecules using DC lysates.

4.2 Results

4.2.1 Serum free medium altered the DC-SIGN and CD80 expression on DCs

When preparing lysates for microarray, cells are sometimes cultured in serum free RPMI-1640 medium for 24 h prior to stimulation. In order to confirm that both immature and mature DCs function properly in serum free medium, DC-SIGN and CD80 expression on DCs cultured in serum-containing or serum-free media were analysed. Due to limited sample size in these pilot experiments, it was not possible to examine statistical significance of these data. On day 5, immature DCs were centrifuged and resuspended in serum-free RPMI-1640 medium and incubated for 24 h. This experiment was repeated four times. Immature DCs incubated in serum-free medium for 24 h had a trend to reduced levels of DC-SIGN compared to those in serum-containing medium for 24 h and immature control on day 5 (Fig. 4.2A).

Furthermore, a trend of CD80 upregulation was only observed in immature DCs cultured with serum-containing medium (Fig. 4.2A).

On day 5, immature DCs were resuspended in serum-free medium for 24 h prior to Poly I:C stimulation for another 24 h; that is, DCs were cultured in serum-free medium for 48h in total and harvested on day 7. CD80 expression of Poly I:C-matured DCs was induced in both serum-containing (Fig. 4.2B) and serum-free media (Fig. 4.2C) compared to immature DCs. In addition, in serum-free medium, Poly I:C-matured DCs had higher levels of DC-SIGN than immature DCs (Fig. 4.2C), which was in contrast to the culture using serum-containing medium (Fig. 4.2B). In addition, there was a trend of decreasing DC-SIGN and CD80 expression of Poly I:C-matured DCs in serum-free media compared to those in serum-containing medium (Fig. 4.2D). Serum free medium increased the average percentage of dead cells according to their forward and side scatter plots (i.e. immature DCs incubated for 24h: increased from 17.61% to 28.72%; immature DCs incubated for 48h: increased from 14.18% to 41.63%; Poly I:C-mature DCs incubated for 48h: increased from 18.01% to 39.4%).

These finding therefore indicate that serum-free medium adversely affects surface molecule expression on both immature and mature DCs. Therefore, serum-containing medium was used in the cultures for the preparation of cell lysates for microarray.

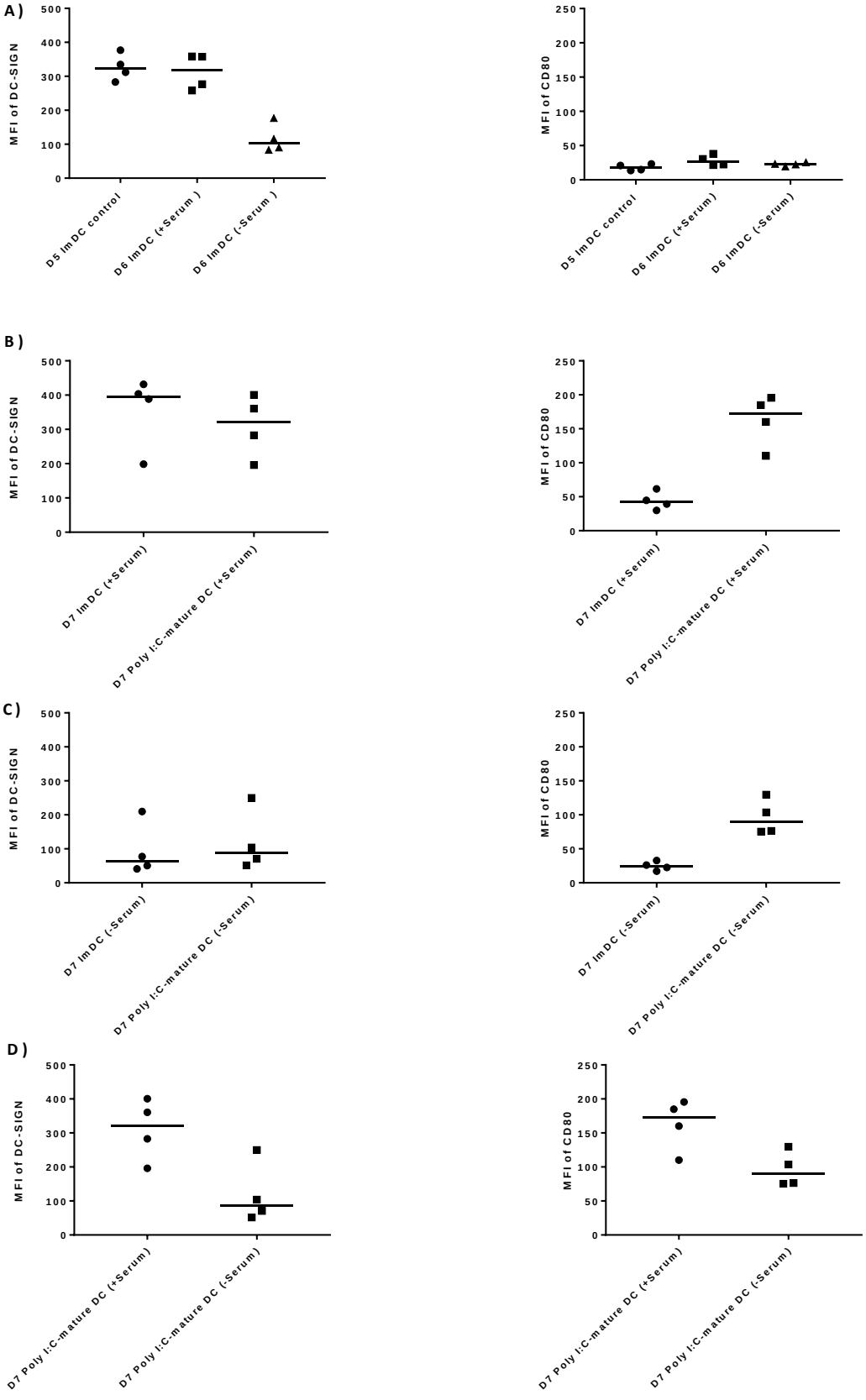

A)
MFI of DC-SIGN
500
400
300
200
100
0
D5 imDC control
D6 imDC (+Serum)
D6 imDC (-Serum)
MFI of CD80
250
200
150
100
50
0
D5 imDC control
D6 imDC (+Serum)
D6 imDC (-Serum)
B)
MFI of DC-SIGN
D7 imDC (+Serum)
D7 Poly I:C-mature DC (+Serum)
MFI of CD80
D7 imDC (+Serum)
D7 Poly I:C-mature DC (+Serum)
C)
MFI of DC-SIGN
D7 imDC (-Serum)
D7 Poly I:C-mature DC (-Serum)
MFI of CD80
D7 imDC (-Serum)
D7 Poly I:C-mature DC (-Serum)
D)
MFI of DC-SIGN
D7 Poly I:C-mature DC (+Serum)
D7 Poly I:C-mature DC (-Serum)
MFI of CD80
D7 Poly I:C-mature DC (+Serum)
D7 Poly I:C-mature DC (-Serum)

Figure 4.2. Serum free media altered the DC-SIGN and CD80 expression on immature and mature DCs. (A) Comparing the MFI of DC-SIGN and of CD80 between untreated immature DCs on day 5 and immature DCs treated with serum-containing or serum-free medium for 24 h. (B) Comparing the MFI of DC-SIGN and CD80 of immature DCs and Poly I:C-matured DCs incubated in serum-containing medium. (C) Comparing the MFI of DC-SIGN and CD80 of immature DCs and Poly I:C-matured DCs incubated in serum-free medium. (D) Comparing the MFI of DC-SIGN and CD80 in Poly I:C-matured DCs incubated with serum-containing or serum-free medium. The horizontal bars represent the medians of four independent experiments

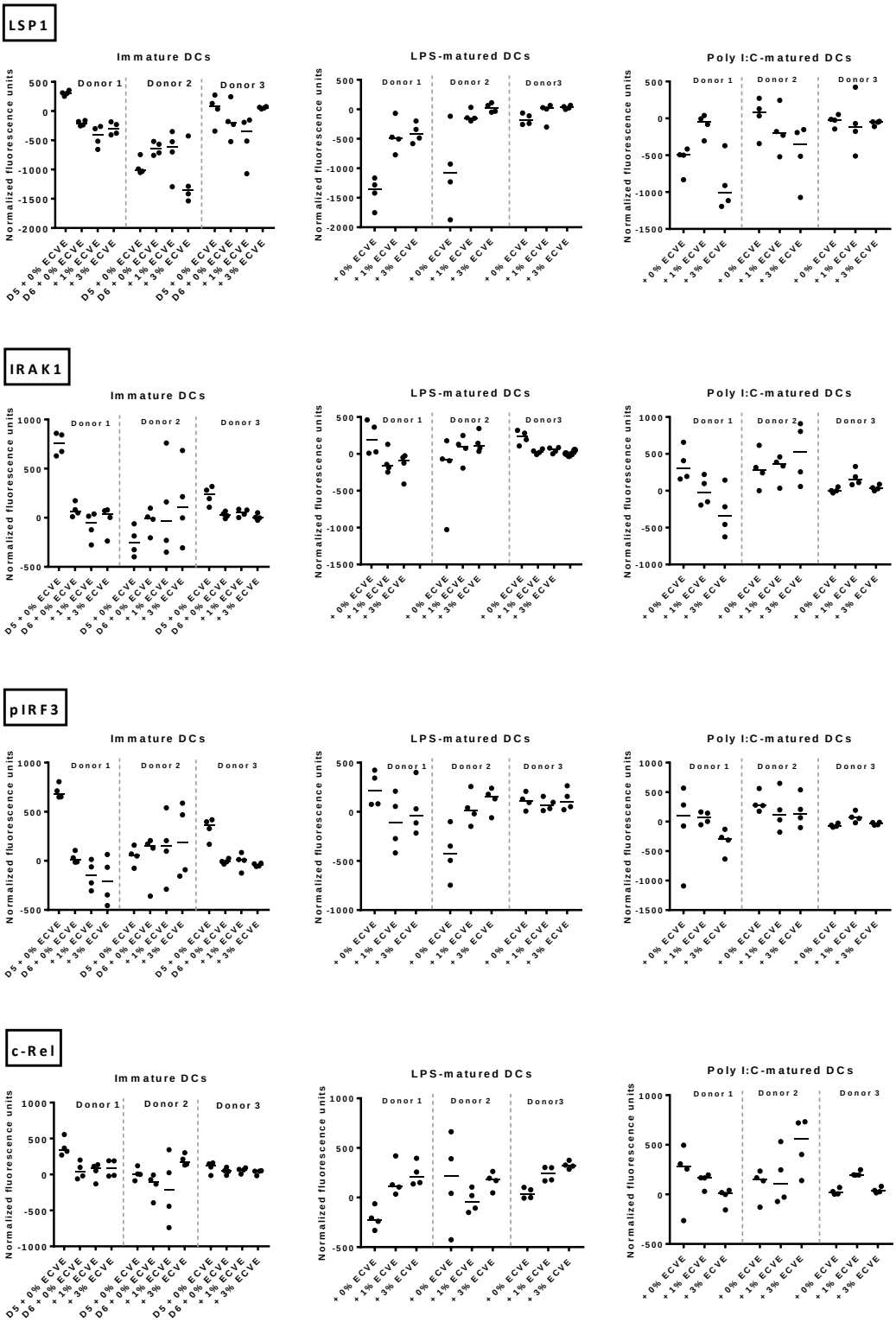
4.2.2 Optimization of the RPPA technique

4.2.2.1 Pre-test of signalling molecule analysis using RPPA

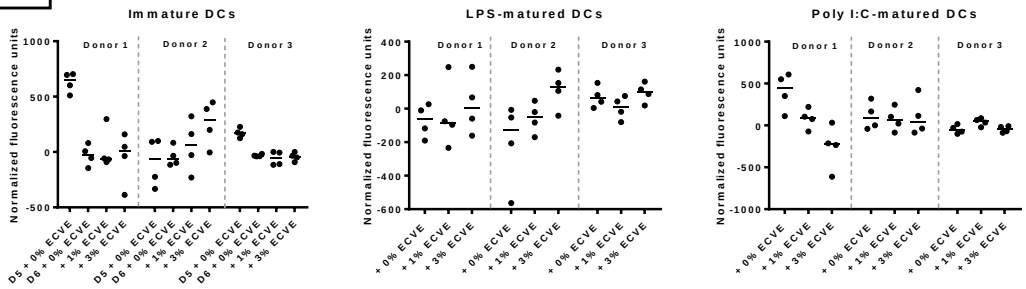
The expression of a number of signalling molecules was investigated under three different maturation conditions: immature, LPS-matured or Poly I:C-matured DCs with different concentrations of ECVE exposure for 24 h. DCs were generated from three buffy coats and were then prepared as cell lysates after ECVE treatment. Cell lysates were printed in quadruplicate onto nitrocellulose slides which were then blocked using Super G blocking buffer (Grace Bio-Labs, Bend, USA), followed by immunostaining, signal detection and data analysis.

After subtracting background signals, the levels of expression of most signalling molecules in both immature and mature DCs were very low, or even negative, with two rounds of amplification using streptavidin for detection (Fig. 4.3). It was therefore

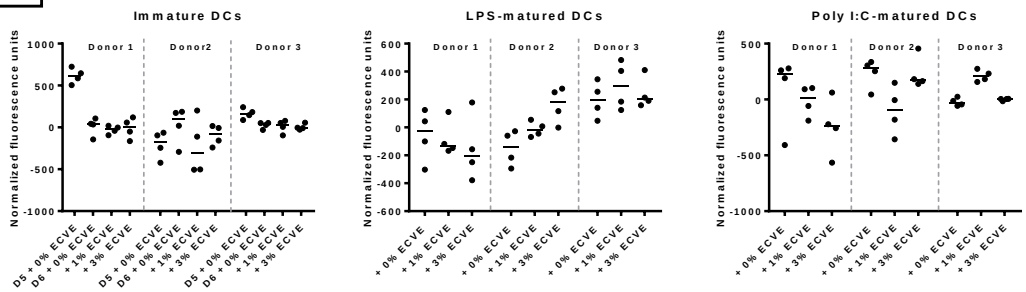
necessary to optimize the protocols to amplify the signals. In addition, the trends of signalling molecule expression were opposite between experiment one and two. A possible reason for this was that one of the donors might have a smoking history. However, it was not possible to access background information of the donors of the buffy coats, including their smoking history. Therefore, in order to minimize such variations, we decided to generate DCs using fresh blood from known healthy donors without any history of smoking, including E-cigarettes and Shisha. In addition, buffy coats had been left at room temperature at least overnight before delivery to the lab, which may affect the quality of blood and the cell viability.


LSP1
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
IRAK1
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
pIRF3
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
c-Rel
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
Normalized fluorescence units
Donor 1
Donor 2
Donor 3
D5 + 0% ECVE
D6 + 0% ECVE
+ 1% ECVE
+ 3% ECVE

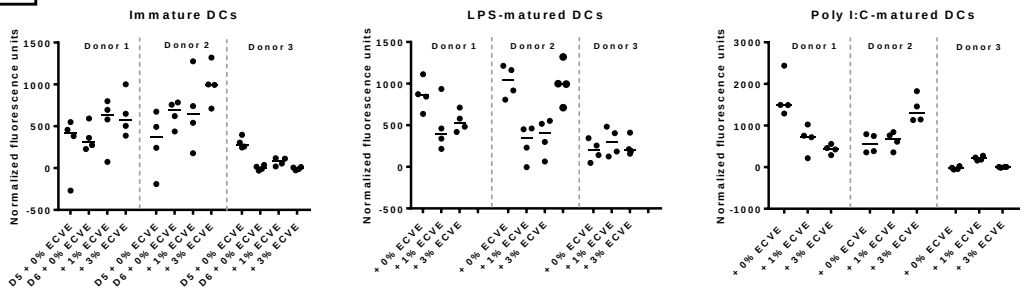
NALP1



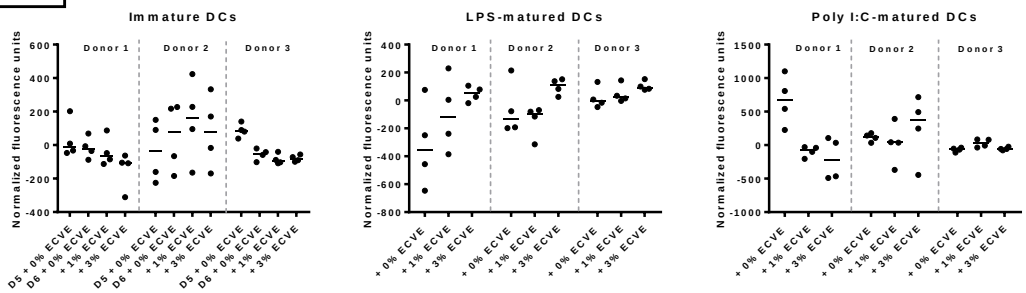
pIRF7



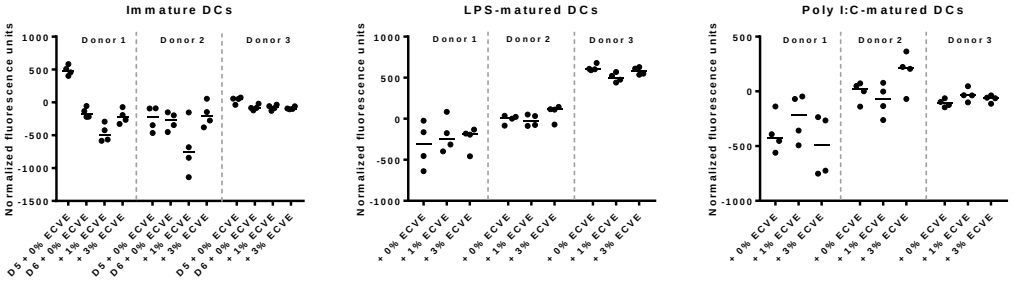
pP38



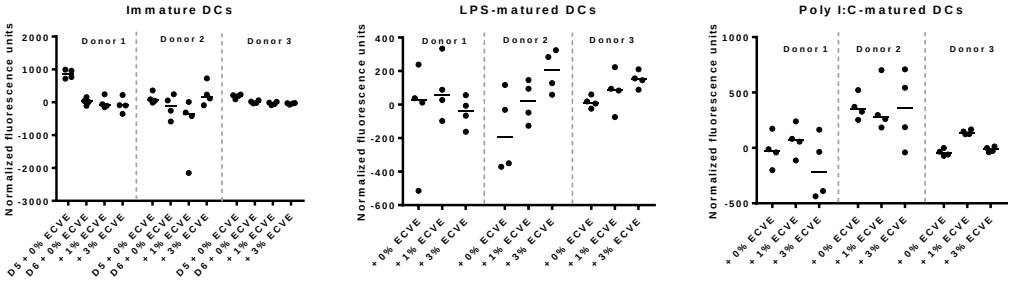
pHSP27



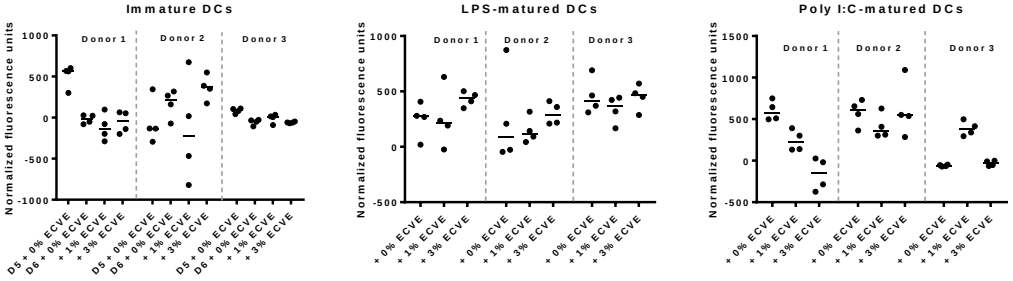
pc-Jun



pP65



pSTAT1



pc-Fos

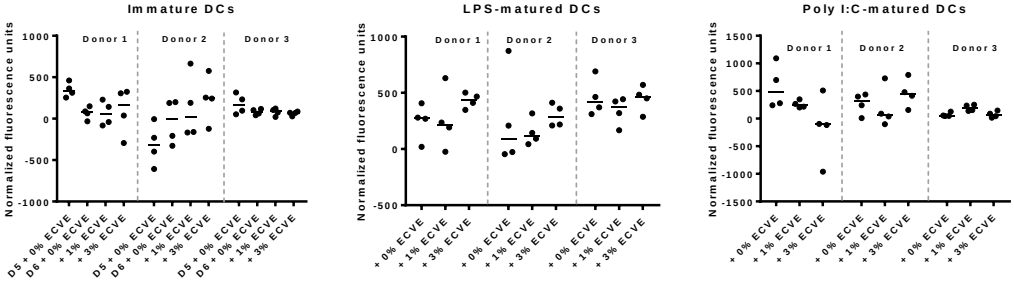


Figure 4.3. Pre-test of RPPA for DCs with 24 h-ECVE exposure. RPPA was used to analyse 12 signalling molecules which are presented as scatter plots showing the median with three individuals (donors). Samples were printed as four replicates and each dot represents a single replicate.

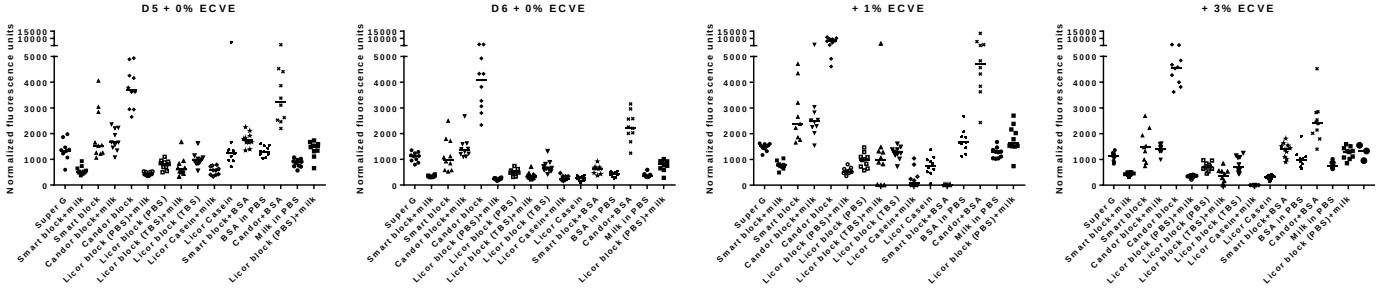
4.2.2.2 Super G, Candor block and Smart block were chosen as more suitable blocking buffers

In order to improve the levels of signalling molecule detection, experiments were undertaken to identify an optimal blocking buffer to reduce background signals. Lysates of immature and LPS/Poly I:C-matured DCs with or without 24 h-ECVE exposure were printed in 10 replicates onto nitrocellulose slides which were then blocked with 16 different blocking buffers, both commercial or produced in-house (Table 4.1), followed by immunostaining. Two rounds of amplification were performed using labelled streptavidin. The experiment were repeated twice using cells from different donors. Among 16 types of blocking buffers, Super G not only gave substantial signals, but also showed small variation between replicates for most signalling molecules (Fig 4.4). Candor block and Smart block gave the highest signals, although the variations of the replicates were slightly larger. Therefore, these three blocking buffers were chosen as candidates for further testing.

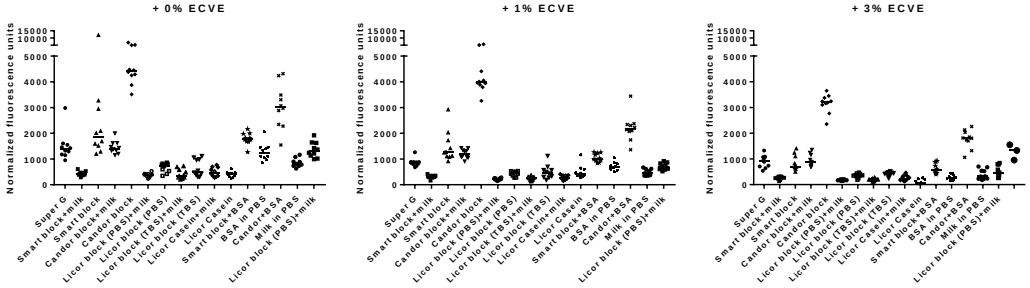
Table 4.1. Sixteen types of blocking buffer used in blocking buffer test

Blocking buffer	Manufacture
Super G	Grace Bio-Labs, Bend, USA
Smart block	Candor Bioscience, Weissenberg, Germany
The Blocking Solution (Candor block)	Candor Bioscience, Weissenberg, Germany
Odyssey® Blocking Buffer (PBS)	LI-COR Bioscience, Lincoln, USA
Odyssey® Blocking Buffer (TBS)	LI-COR Bioscience, Lincoln, USA
Casein Blocking buffer	LI-COR Bioscience, Lincoln, USA
PBS-3% BSA	Made in-house
PBS-5% skimmed milk	Made in-house
Smart block-5% skimmed milk	Made in-house
The Blocking Solution-5% skimmed milk	Made in-house
Odyssey® Blocking Buffer (PBS)-5% skimmed milk	Made in-house
Odyssey® Blocking Buffer (TBS)-5% skimmed milk	Made in-house
Casein Blocking buffer-5% skimmed milk	Made in-house
Smart block-3% BSA	Made in-house
The blocking solution-3% BSA	Made in-house
Odyssey® Blocking Buffer (TBS)-3% BSA	Made in-house

Immature DCs



LPS-matured DCs



Poly I:C-matured DCs

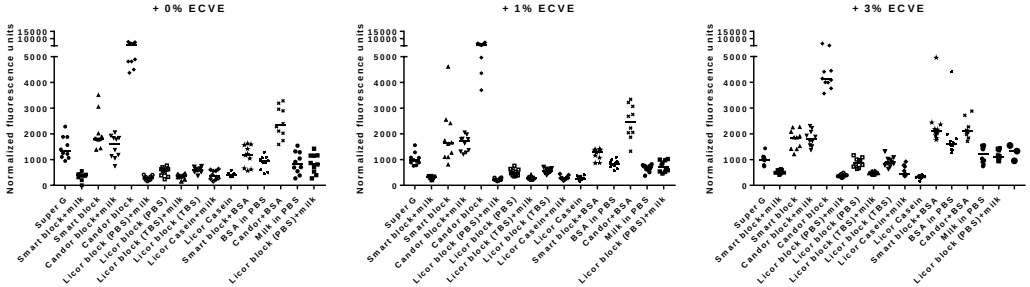


Figure 4.4. **Super G, Candor block and Smart block were chosen as more suitable blocking buffers as candidates for further testing.** Following 24 h-ECVE exposure, lysates of immature and mature DCs were printed on nitrocellulose slides. 16 types of blocking buffer were then used to block the slides. Data is from one experiment and is presented as scatter plots with the median. Samples were printed as 10 replicates and each dot represents a single replicate.

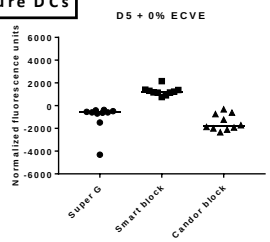
4.2.2.3 Neither streptavidin amplification method nor BIO-RAD amplification module improved signals

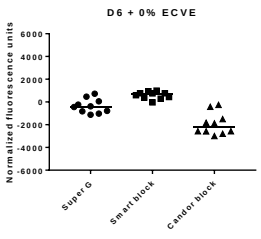
In addition to reducing the background signals, different detection methods may also help to generate stronger signals. Lysates of immature and LPS/Poly I:C-matured DCs, with or without 24 h-ECVE exposure, were printed in 10 replicates onto nitrocellulose slides which were then blocked with blocking buffer: either Super G, Smart block or Candor block, followed by immunostaining for c-Rel, signal detection and data analysis. For signal detection, amplification using 3 rounds of streptavidin or a commercial amplification module (BIO-RAD, Hercules, USA) were used.

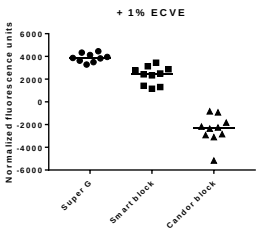
Most signals were very low, or even negative, after subtracting background signals – especially those blocked with Candor block (Fig 4.5). No improvement of signals was found when using either 3-round streptavidin amplification or the commercial amplification kit. Therefore, some of the materials that had been used were suspected to have lost function. Due to the small variations of replicates using Super G, it was decided to use this blocking buffer in the following experiments.

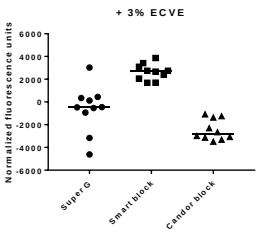
Immature DCs

3-round Streptavidin amplification method

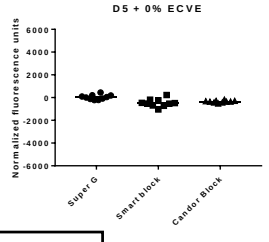


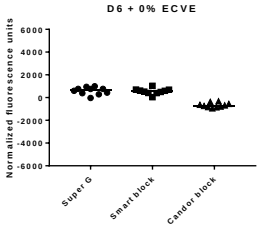


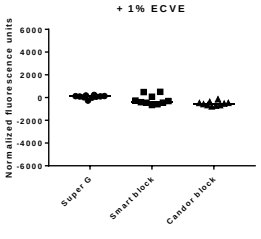


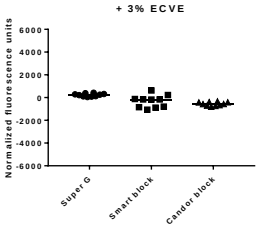


BIO-RAD amplification module



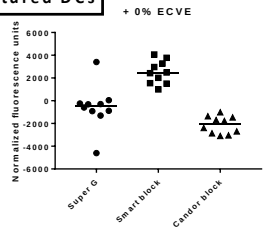


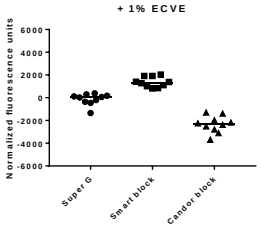


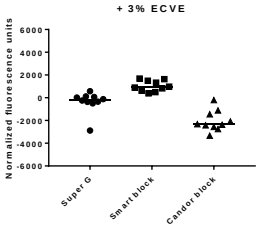


LPS-matured DCs

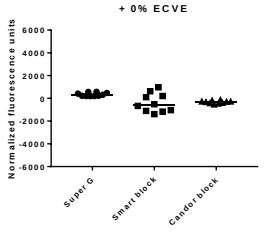
3-round Streptavidin amplification method

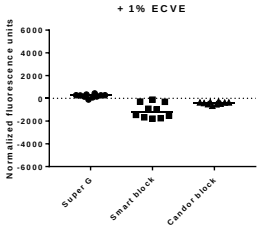


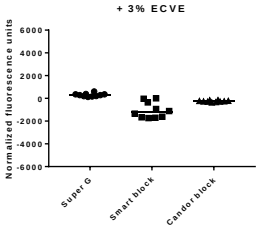




BIO-RAD amplification module







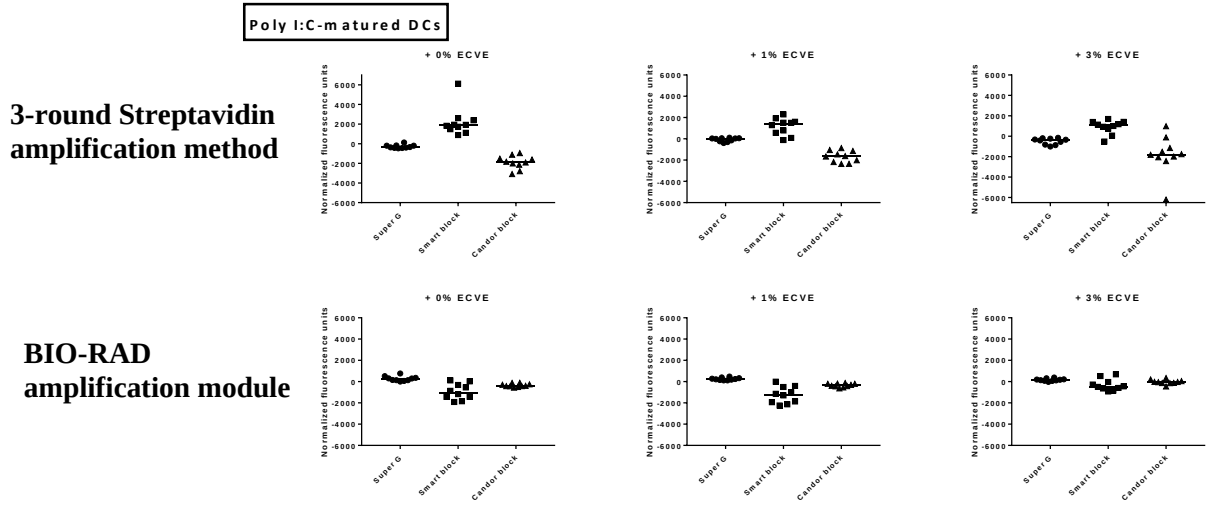


Figure 4.5. Neither streptavidin amplification method nor BIO-RAD amplification module improved signals. After printing lysates of immature and mature DCs (treated with or without 24 h-ECVE exposure) on nitrocellulose slides, Super G, Smart block and Candor block were used to block slides followed by immunostaining and signal detection. For signal detection, 3-rounds of streptavidin amplification or a commercial BIO-RAD amplification module were used to compare their effects on improvement of signals. Data is from one experiment and presented as scatter plots showing the median. Samples were printed as 10 replicates and each dot represents single replicate.

4.2.2.4 Biotinylated goat anti-rabbit secondary antibodies and IRDdye® 800CW streptavidin being used had lost their functionality

In order to compare and confirm the functionality of the reagents, a test assay using solid pins or silicon pins was set up to print a primary antibody to c-Rel (rabbit species, Cell Signalling, UK) with different concentration in 8 replicates onto nitrocellulose slides. Slides then were blocked with Super G, followed by immunostaining, signal detection and data analysis. Various types of secondary antibodies combined with different conjugation of streptavidin were used (Table 4.2)

Variation of replicates printed with solid pins were smaller than those printed with silicon pins (Fig 4.6), supporting the use of solid pins for printing in future experiments. The signals generated using either biotinylated goat anti-rabbit secondary antibody (the one that was used before) or biotinylated swine anti-rabbit secondary antibody were not high enough, especially those followed by signal detection using IRDye® 800CW streptavidin (the one that was used before) compared to IRDye® 680LT streptavidin, suggesting inappropriate function of IRDdye® 800CW streptavidin. Moreover, IRDye 680LT conjugated goat anti-rabbit secondary antibody gave signals twice as high as those given by biotinylated secondary antibodies, suggesting limited functionality of the biotinylated secondary antibodies. Therefore, new secondary antibody and streptavidin dye were required for further experiments.

Table 4.2. Reagents used to test their functions

Reagents	Manufacture
Biotinylated goat anti-rabbit secondary antibody (B8895)	Sigma Aldrich
Biotinylated swine anti-rabbit secondary antibody (E0353)	Agilent Dako
IRDye 680LT conjugated goat anti-rabbit secondary antibody (926-68021)	LI-COR Biotechnology
IRDdye® 680LT Streptavidin (926-68031)	LI-COR Biotechnology
IRDdye® 800CW Streptavidin (925-32230)	LI-COR Biotechnology

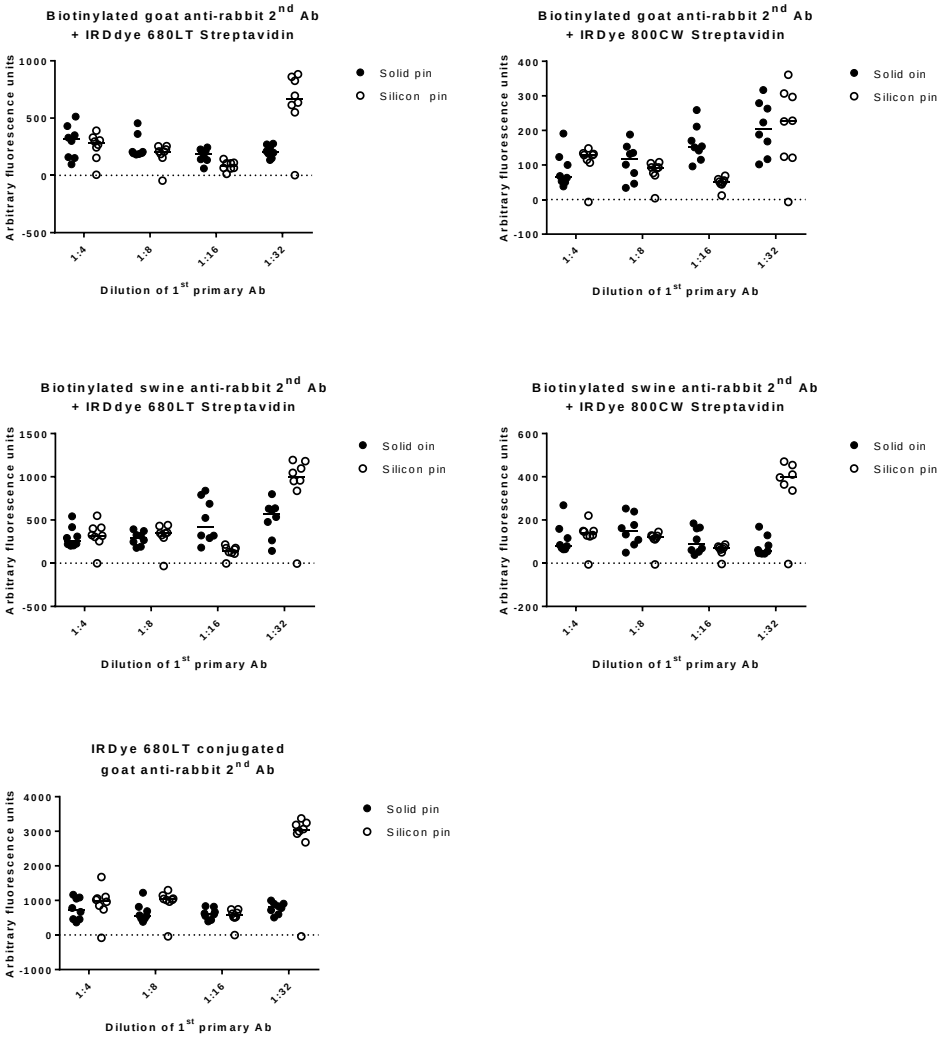


Figure 4.6. Biotinylated goat anti-rabbit secondary antibodies and IRDdye® 800CW streptavidin being used had lost their function

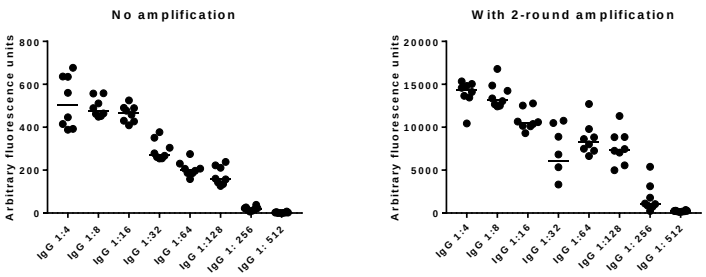
A primary anti-c-Rel antibody at different concentrations was printed using a solid pin or a silicon pin. Slides then were blocked, immunostained and signal detection performed using various types of secondary antibodies combined with different streptavidin conjugates. Data is from one experiment and is presented as scatter plots showing the medians. Samples were printed as 8 replicates and each dot represents a single replicate.

4.2.2.5 New materials improved the RPPA signals

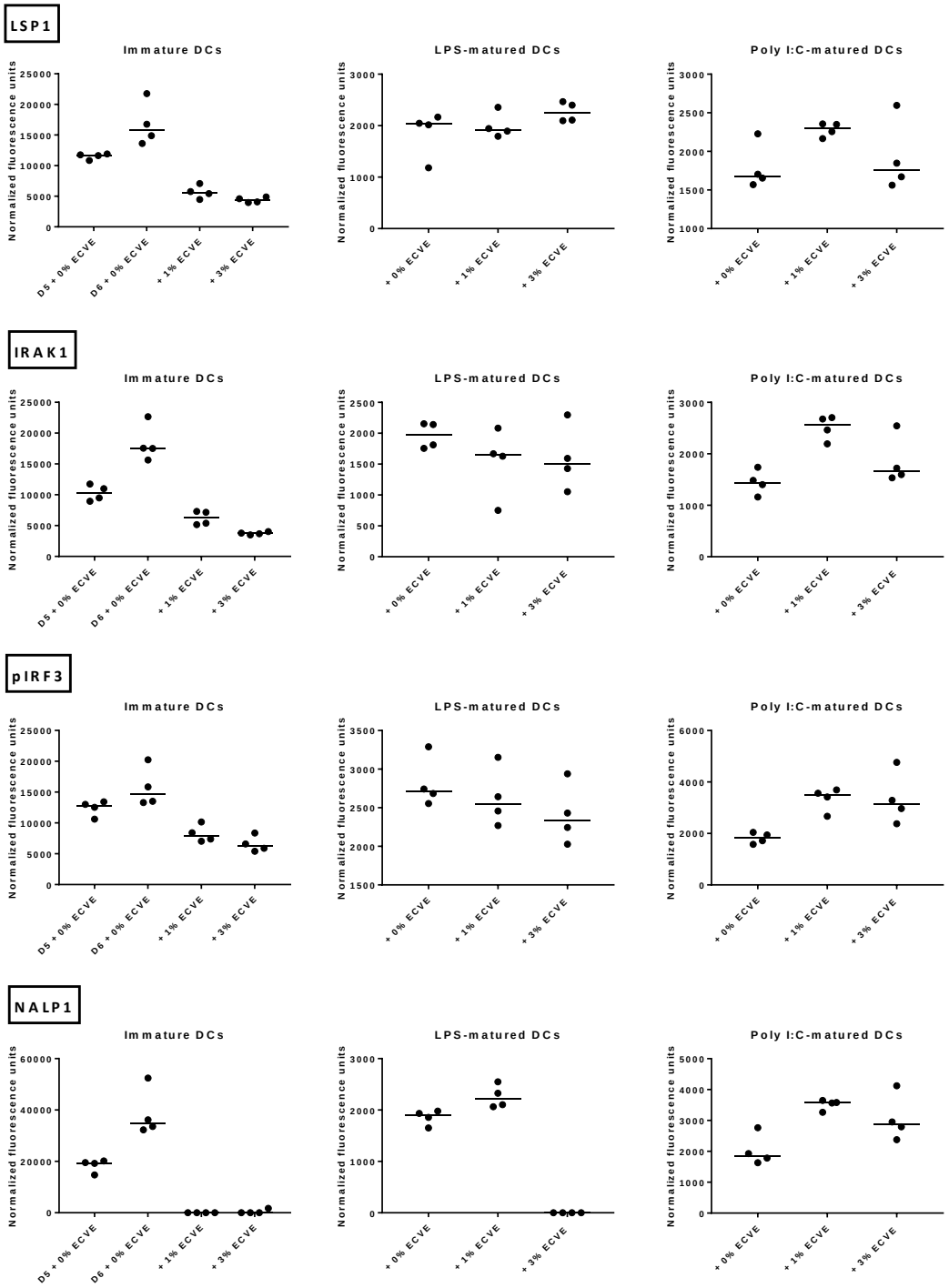
In order to validate the newly purchased biotinylated anti-rabbit secondary antibody and IRDdye® 800CW Streptavidin, eight different dilutions of 1 mg/ml rabbit IgG (Sigma-Aldrich, Gillingham, UK) were printed in 8 replicates onto nitrocellulose slides. Slides then were blocked with Super G, followed by immunostaining with biotinylated anti-rabbit secondary antibody, signal detection and data analysis. For signal detection, IRDdye® 800CW Streptavidin was used with or without amplification.

Signals were detected which decreased with decreasing IgG concentrations, and two rounds of streptavidin amplification dramatically elevated the signals (Fig. 4.7A), confirming the validation of the new materials and detection method. The technique was further applied to DC lysate samples: figure 4.7B shows the expression of signalling molecules in immature and mature DCs after ECVE treatment for 24 h. This shows that the technique now gave acceptable signals in terms of arbitrary fluorescence units, and that differences were apparent in the ECVE treated samples compared to the samples without ECVE treatment.

A.



B.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Poly I:C-matured DCs
Normalized fluorescence units
D5 + 0% ECVE
D6 + 0% ECVE
+ 1% ECVE
+ 3% ECVE
IRAK1
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
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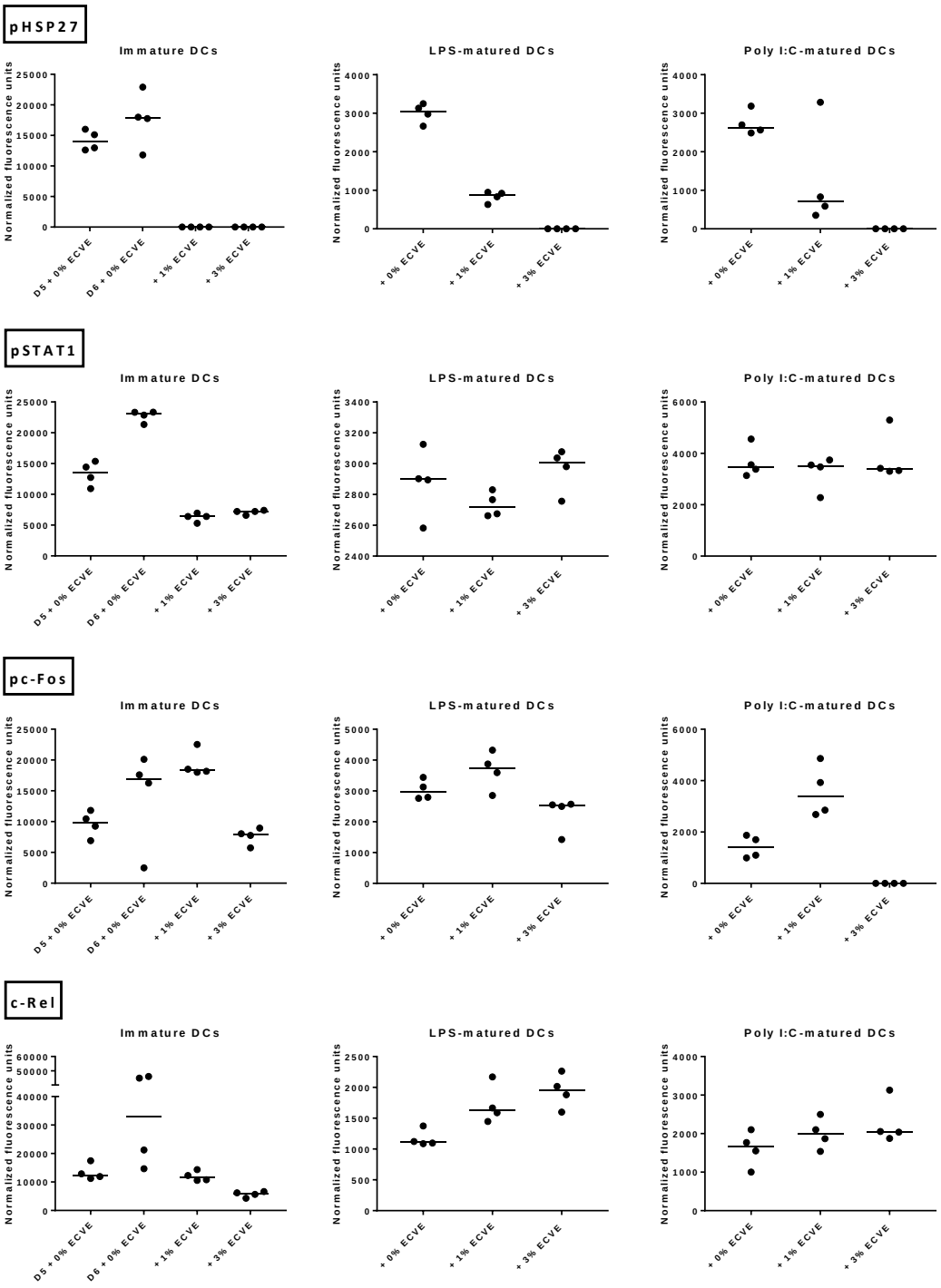
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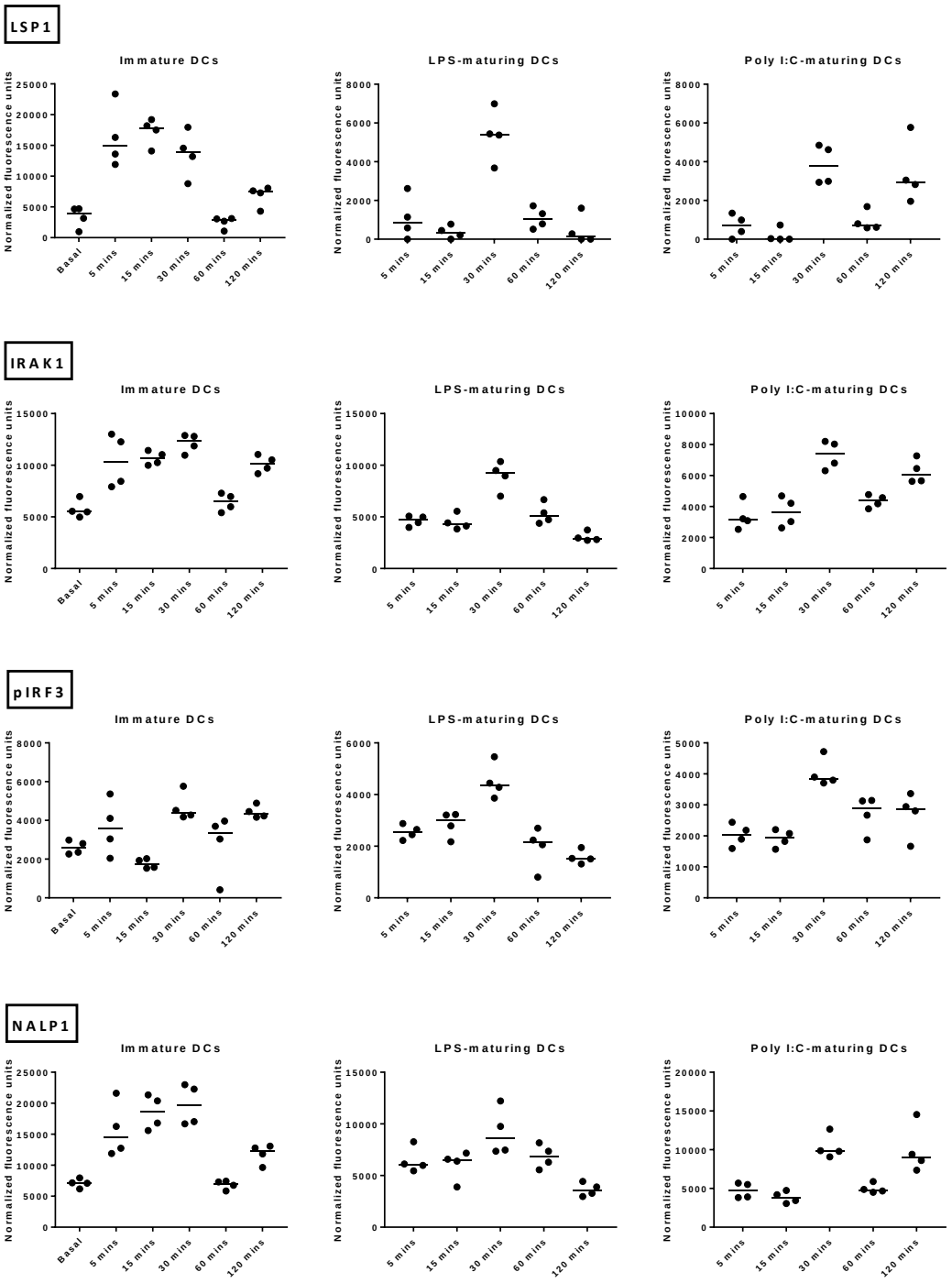
LPS-matured DCs
Poly I:C-matured DCs
pSTAT1
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
pc-Fos
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
c-Rel
Immature DCs
LPS-matured DCs
Poly I:C-matured DCs
Normalized fluorescence units
D5 + 0% ECVE
D6 + 0% ECVE
+ 1% ECVE
+ 3% ECVE

Figure 4.7. New materials improved the RPPA signals. (A) Rabbit IgG, at 8 different dilutions, was printed in 8 replicates onto nitrocellulose slides. Slides were then blocked with Super G, followed by immunostaining with biotinylated anti-rabbit secondary antibody and streptavidin. (B) Lysates of DC samples were printed as four replicates followed by immunostaining with primary antibody, biotinylated anti-rabbit secondary antibody and two rounds of streptavidin amplification. Each dot represents a single replicate. Data is from one experiment and presented as scatter plot showing the median.

4.2.2.6 The highest expression of most signalling molecules occurred by 30 minutes after stimulation.

Changes within the expression of many signalling molecules takes place within a few minutes of cell stimulation. In order to determine the peak of signalling molecule expression, a time course experiment was performed. DCs were treated with LPS/Poly I:C and 3% ECVE for the following time durations: 5, 15, 30, 60 and 120 minutes. Lysates of these DC samples were printed as 4 replicates onto nitrocellulose slides. The slides then were blocked with Super G, followed by immunostaining, signal detection and data analysis.

The expression of many signalling molecules peaked at 30 minutes, followed by a reduction of signal level in both immature and maturing DCs. Therefore, in addition to 24 h-ECVE treatment, 30 minute ECVE treatment was employed to the study the effects of ECVE treatment on signalling pathways in DCs.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Immature DCs
LPS-maturing DCs
Poly I:C-maturing DCs
IRAK1
Immature DCs
LPS-maturing DCs
Poly I:C-maturing DCs
pIRF3
Immature DCs
LPS-maturing DCs
Poly I:C-maturing DCs
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Basal
5 mins
15 mins
30 mins
60 mins
120 mins

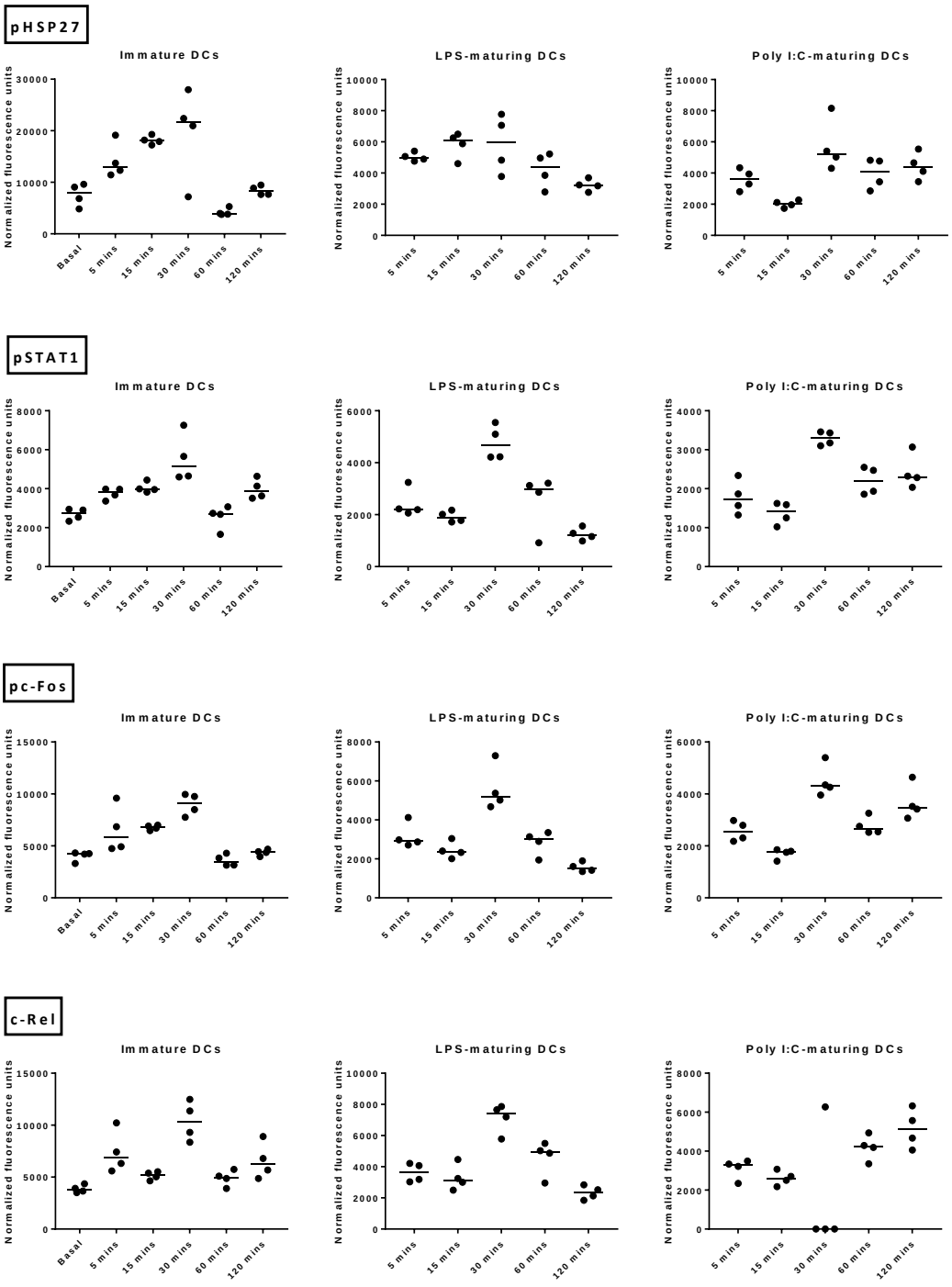


Figure 4.8. The highest expression of most signalling molecules occurred by 30 minutes after stimulation. DCs were stimulated with LPS/Poly I:C in the presence

of 3% ECVE for different durations. Lysates of DC samples were printed as four replicates followed by immunostaining with primary antibody, biotinylated anti-rabbit secondary antibody and 2-rounds of streptavidin amplification. Each dot represents a single replicate. Data is from one experiment and is presented as scatter plots showing the medians.

4.2.3 Signalling molecules analysis using RPPA

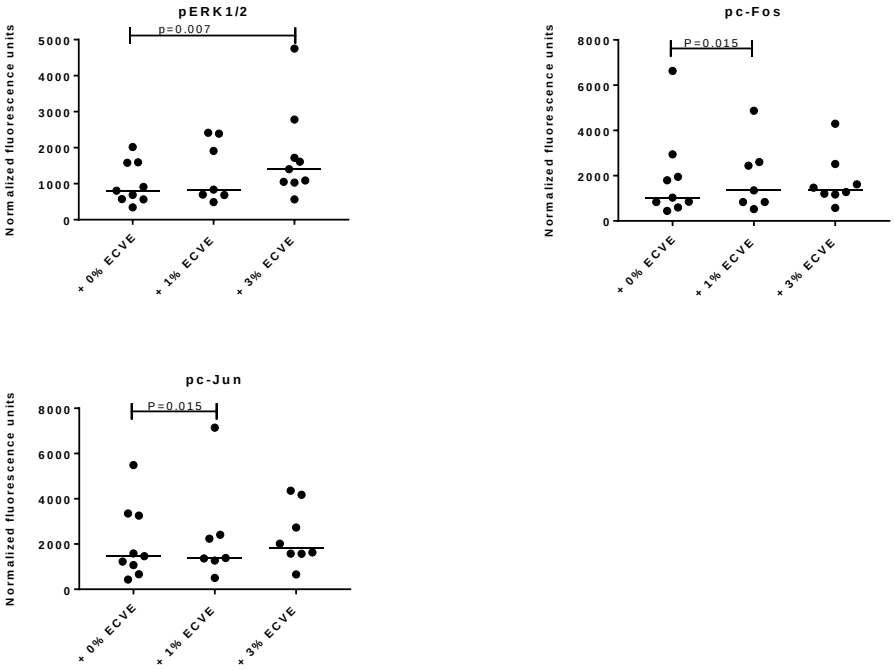
4.2.3.1 24-ECVE treatment upregulated the expression of pERK1/2, pc-Fos and pc-Jun by LPS-matured DCs

The expression of signalling molecules was examined under three different maturation conditions: immature, maturing and mature DCs, treated without or with 1% or 3% ECVE. The choice of the 29 signalling molecules analysed was based on a previous study in our group investigating the effects of CS on DCs [137]. The molecules investigated are involved in several signalling pathways including: NF-κB, MAPK, JAK/STAT, PI3K/AKT and TLR pathways.

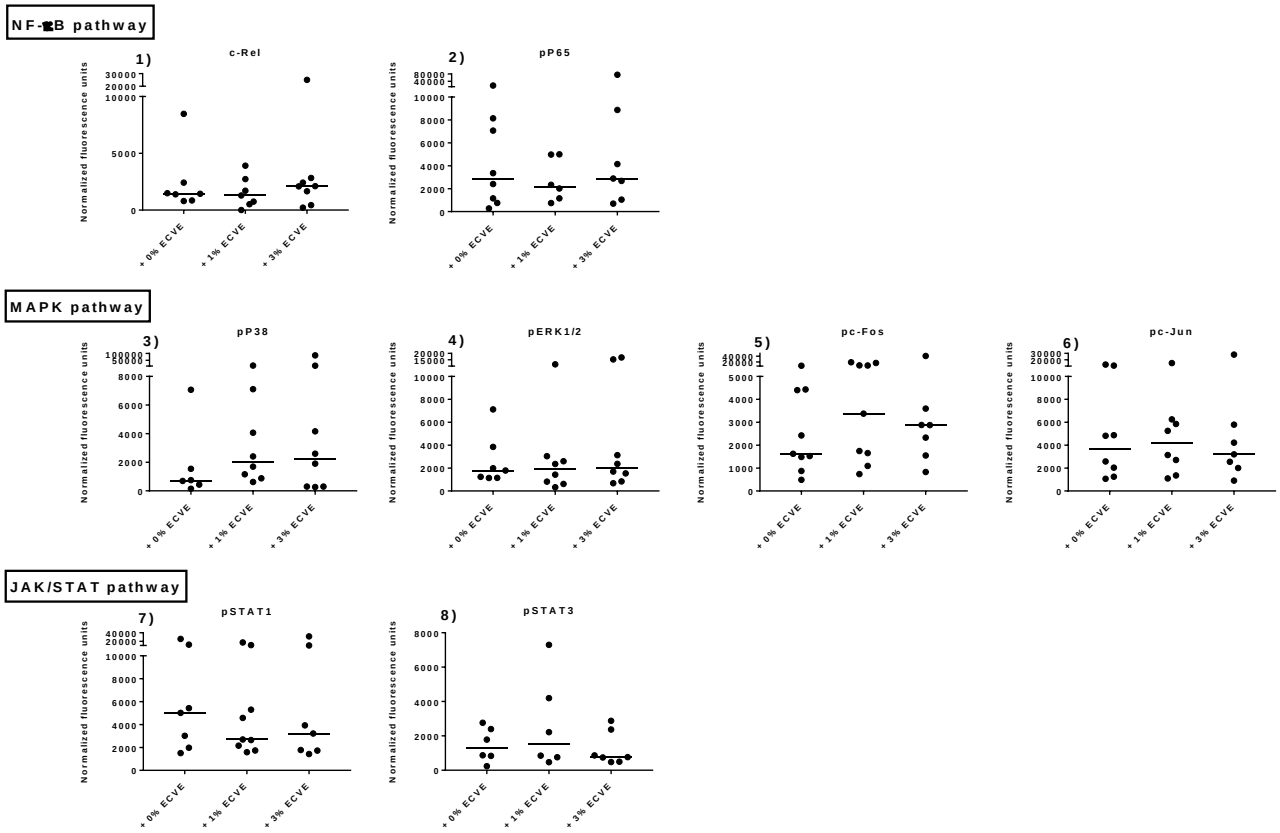
The expression of signalling molecules was comparable in both immature and LPS/Poly I:C-maturing DCs in the presence or absence of ECVE treatment for 30 minutes (Fig. 4.9B-D). Exposure to 1% ECVE for 24 h significantly increased pc-Fos ($p=0.015$) and pc-Jun levels ($p=0.015$) in LPS-matured DCs, compared to LPS-matured DCs without ECVE (Fig. 4.9A). In addition, exposure to 3% ECVE significantly increased pERK1/2 expression ($p=0.007$) in LPS-matured DCs compared to LPS-matured DCs without ECVE (Fig. 4.9A). 24 h-ECVE treatment did not have significant effects on immature (Fig. 4.9E), or Poly I:C-matured, DCs (Fig.

4.9G). Thus, for most signalling molecules in immature, maturing or matured DCs, treatment with ECVE did not show a statistically significant effect on the expression of individual molecules. This is not surprising given that previous work in our laboratory has indicated that pathological effects on the expression of individual signalling molecules can be relatively subtle [162], whereas the combined, or 'global', effects on multiple signalling molecules may be biologically significant. As described in the next section, therefore the effects of ECVE-treatment on the global expression of signalling molecules in the DCs was analysed.

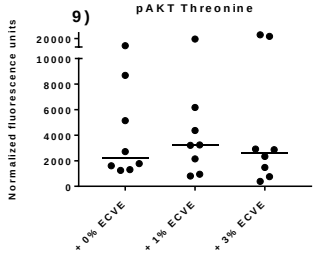
A.

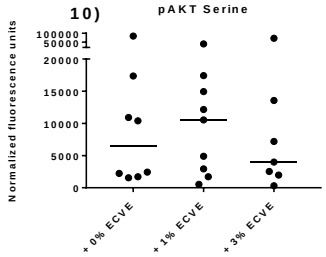


B. Immature DCs with 30 min-ECVE treatment

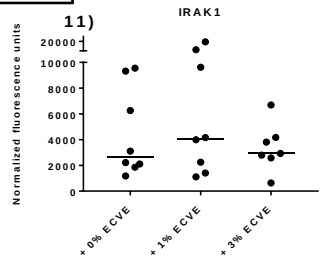


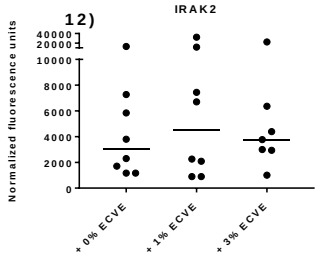
PI3K/AKT pathway

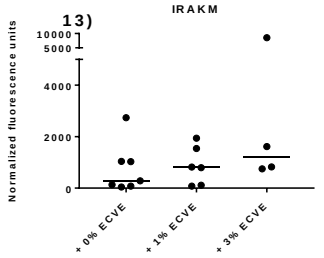


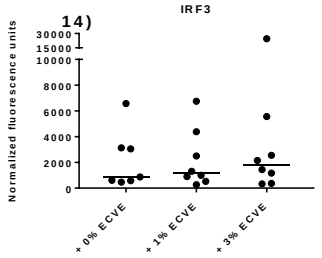


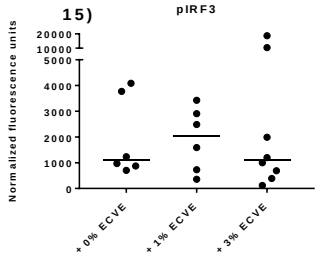
TLR pathway

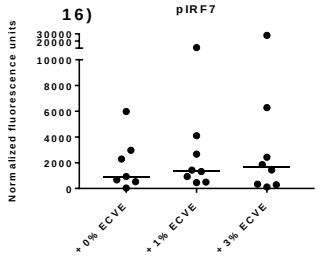


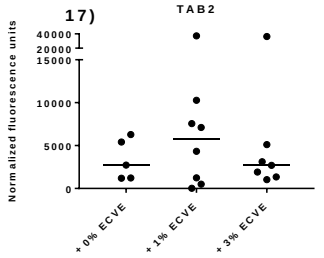


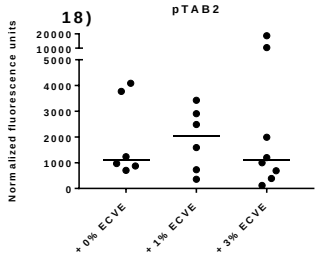












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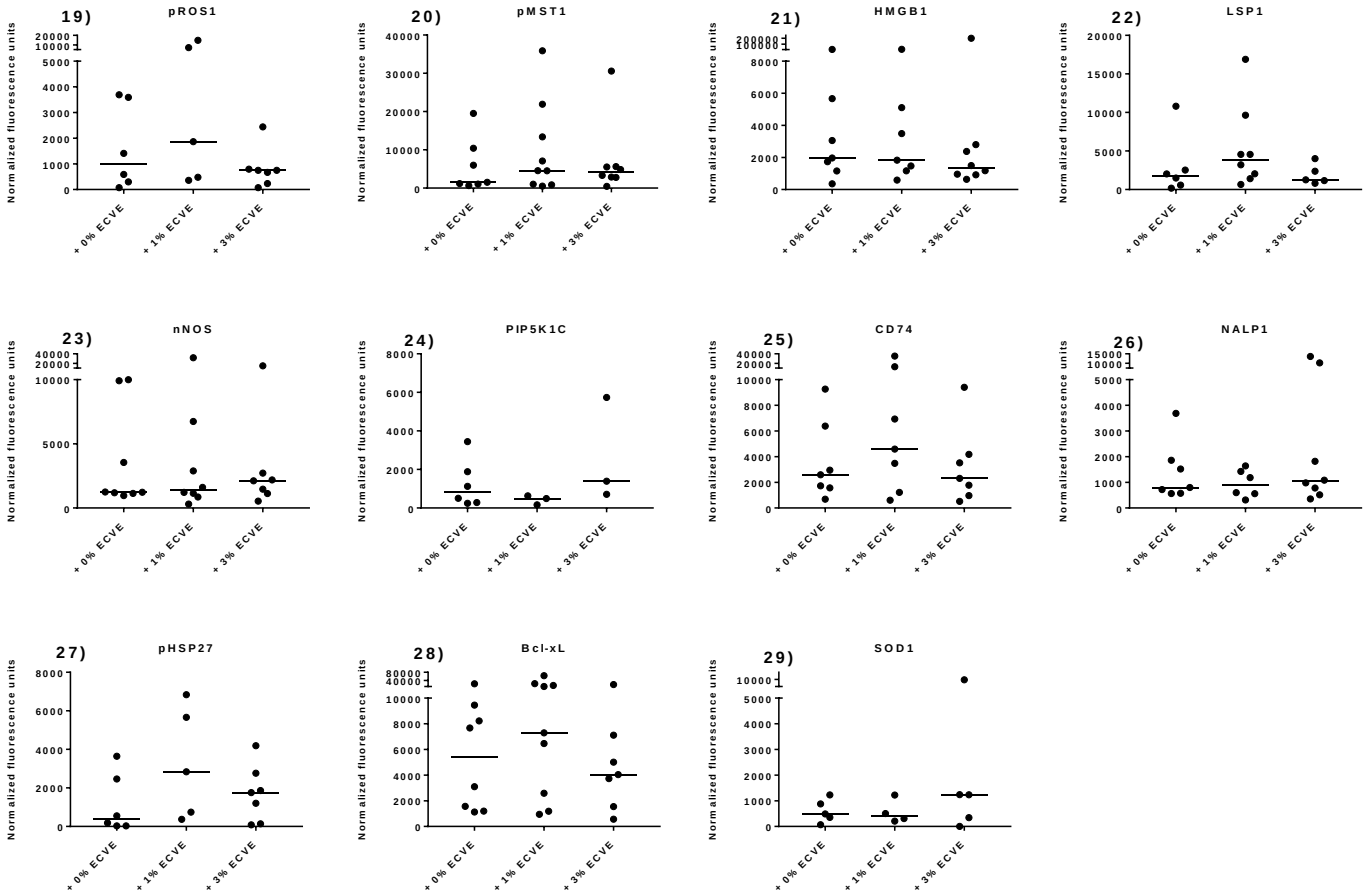
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pMST1
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HMGB1
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nNOS
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PIP5K1C
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CD74
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NALP1
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pHSP27
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Bcl-xL
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SOD1
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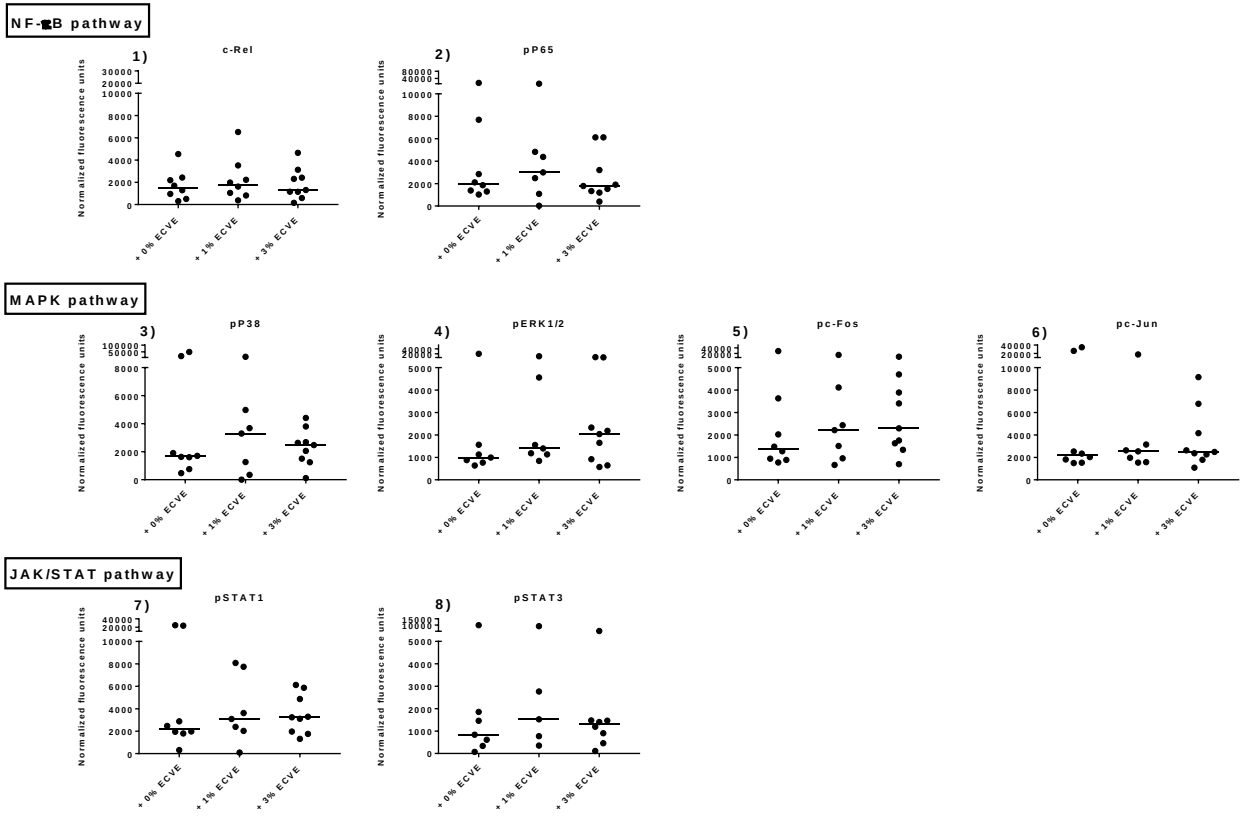
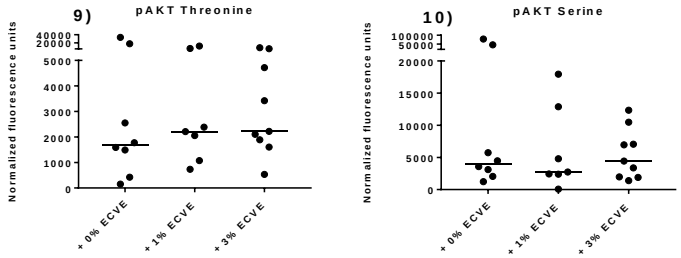
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

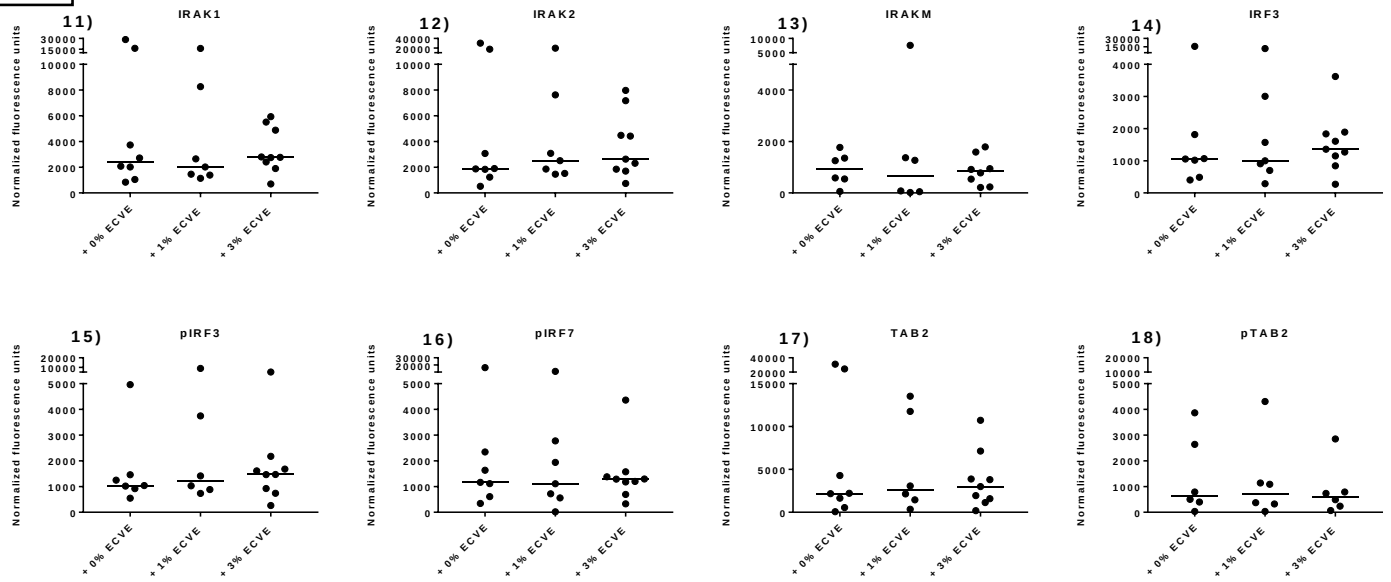
C. LPS-maturing DCs with 30 min-ECVE treatment



PI3K/AKT pathway



TLR pathway



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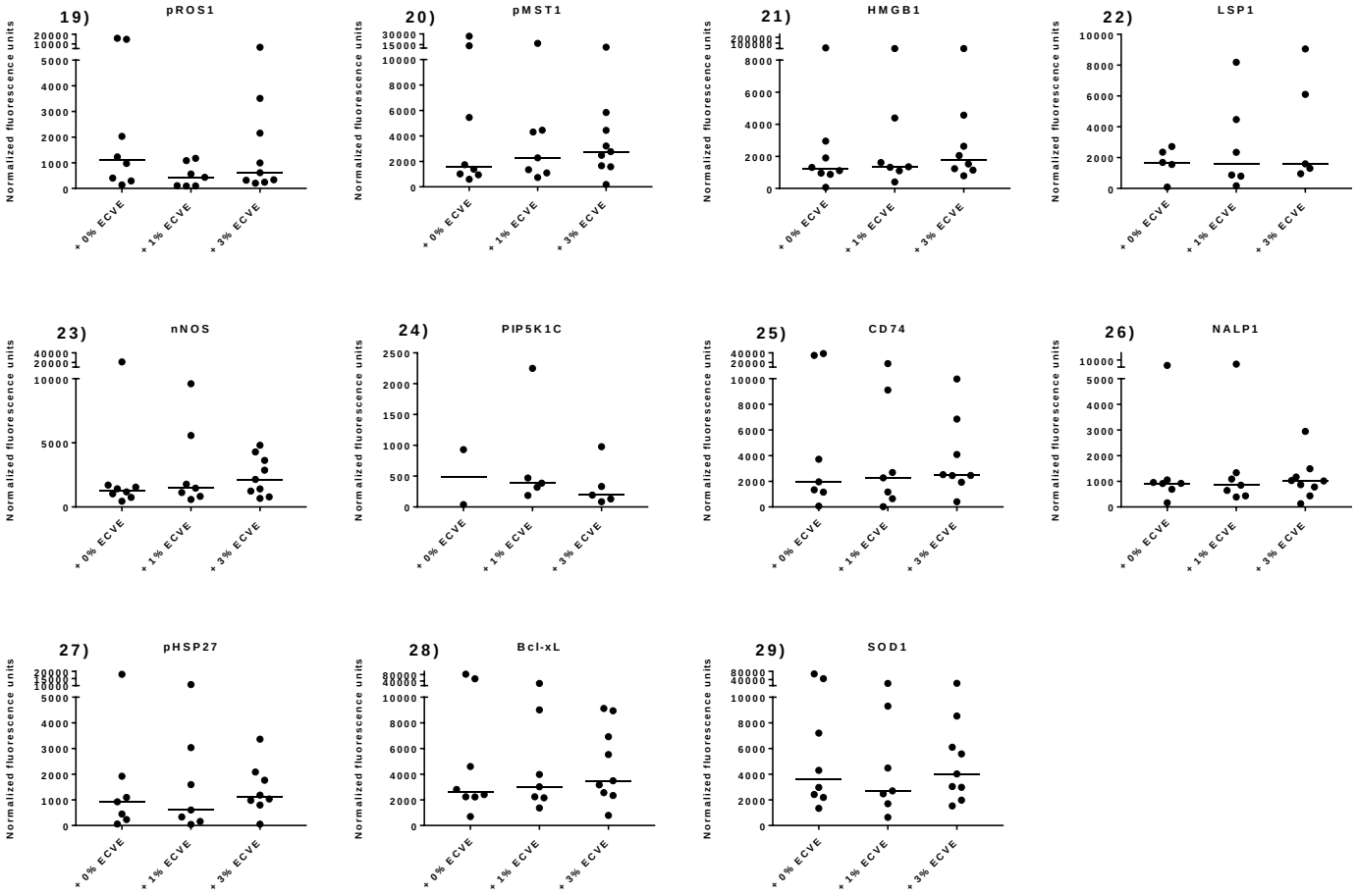
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27)
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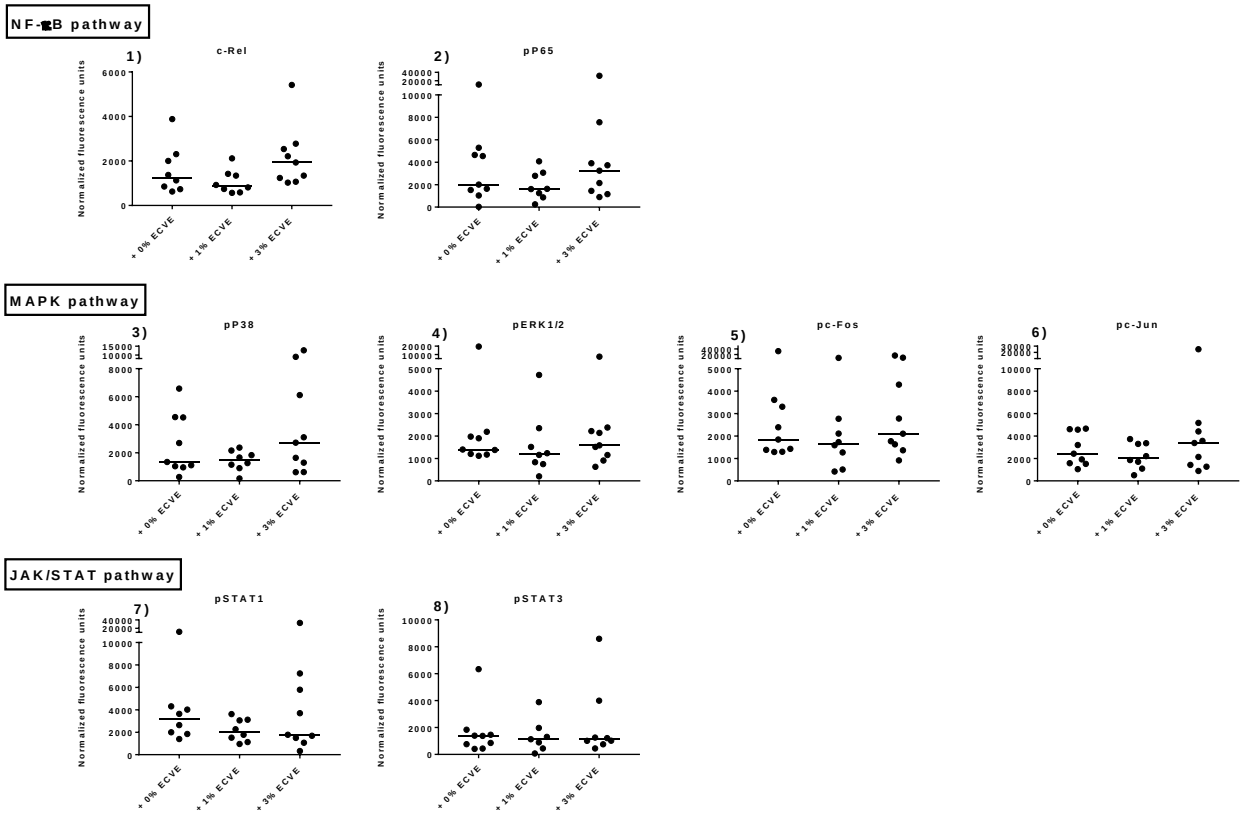
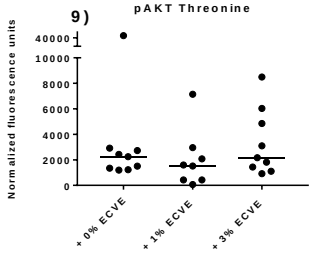
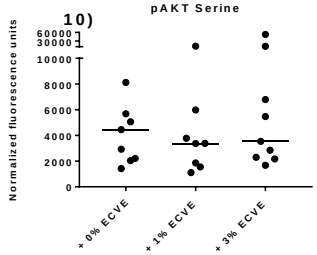
28)
Bcl-xL
29)
SOD1
Normalized fluorescence units
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

D. Poly I:C-maturing DCs with 30 min-ECVE treatment

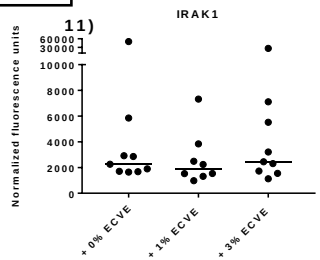


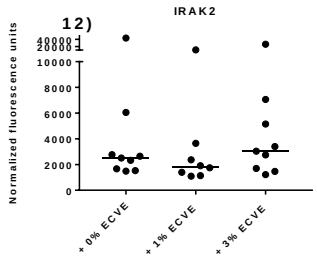
PI3K/AKT pathway

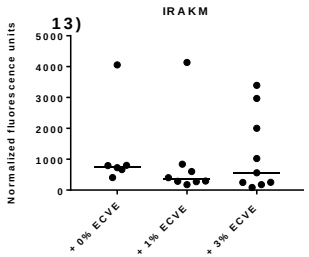


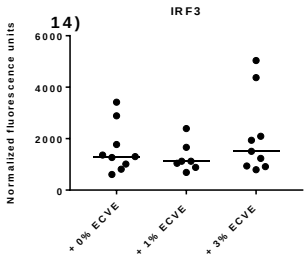


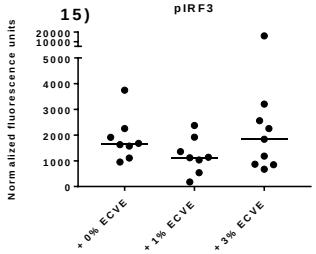
TLR pathway

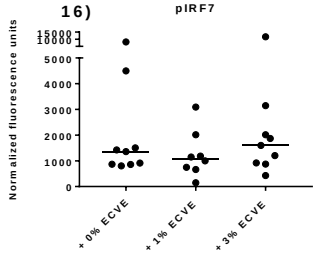


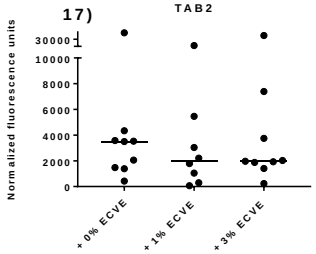


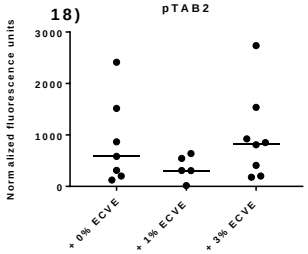












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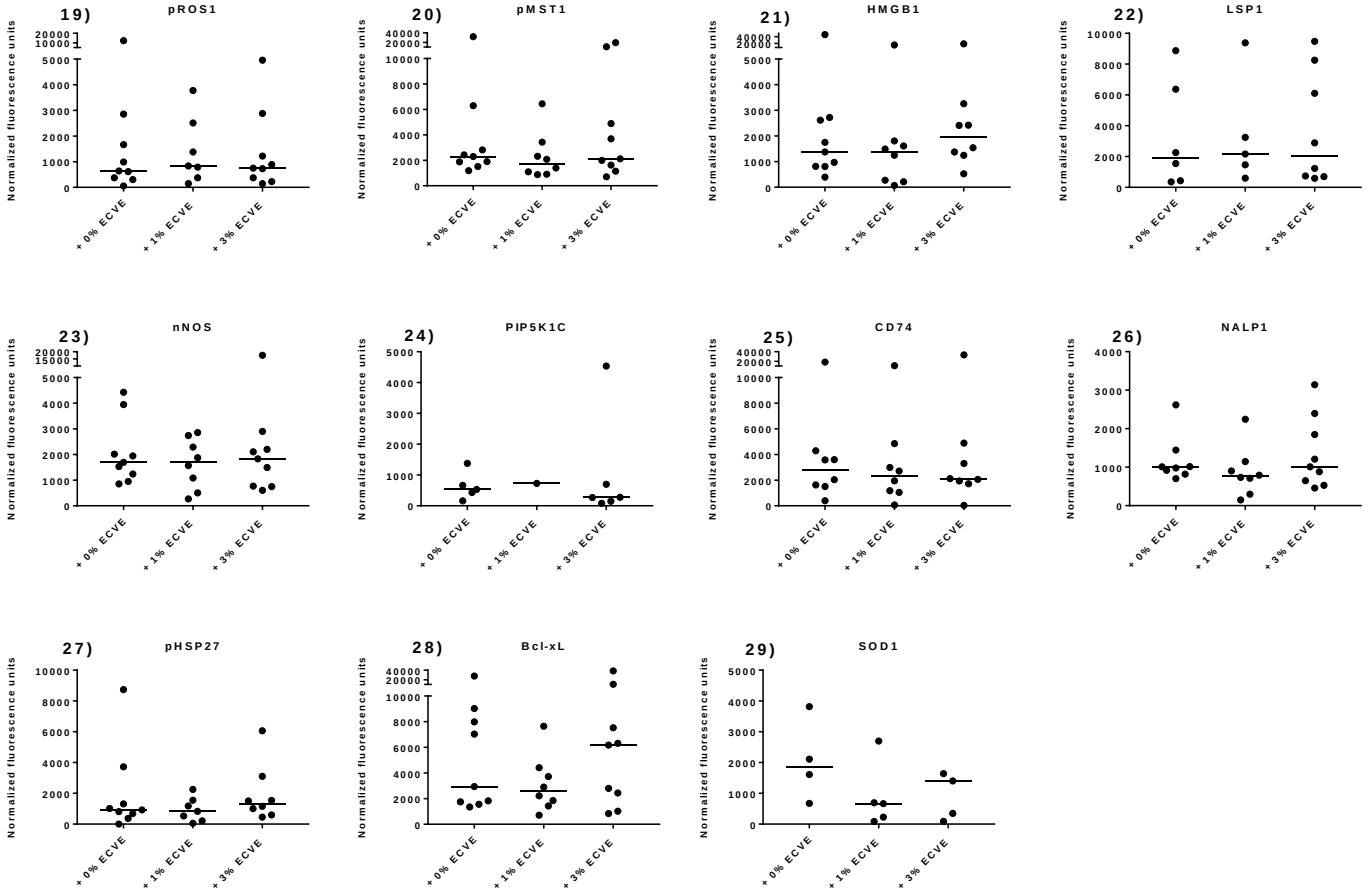
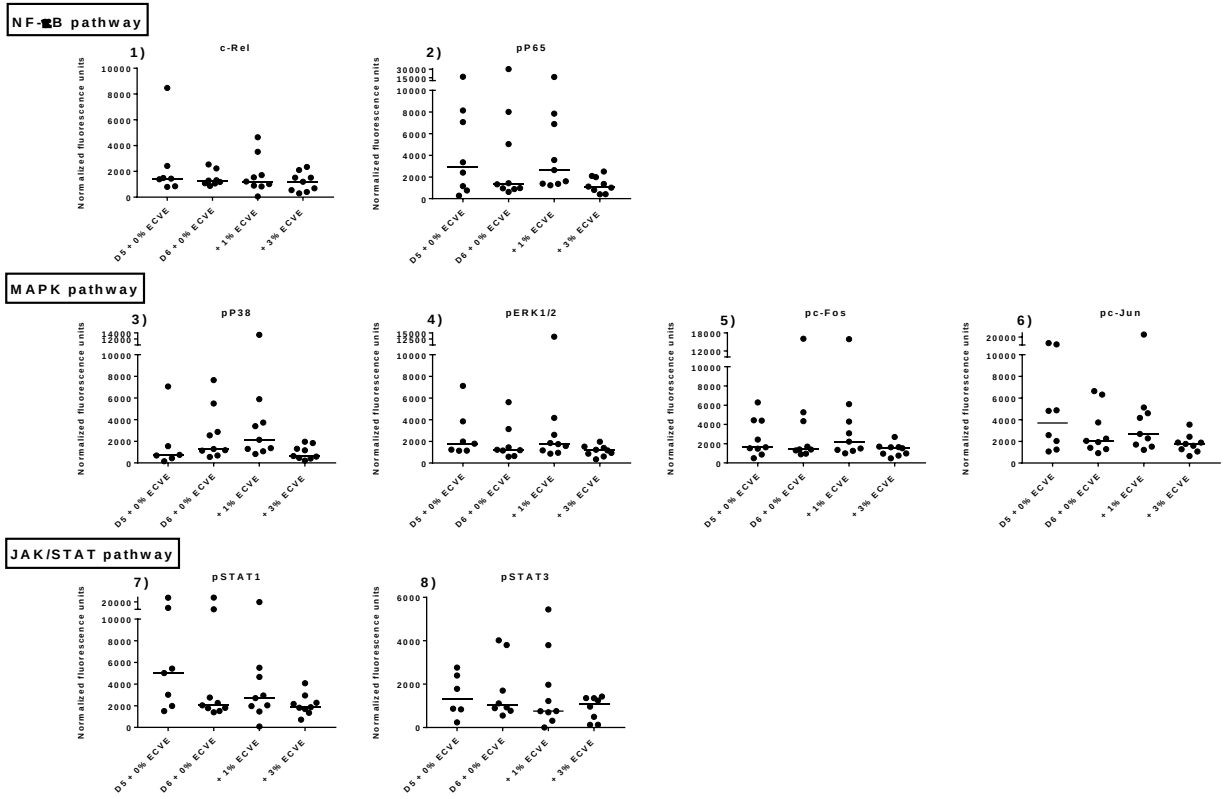
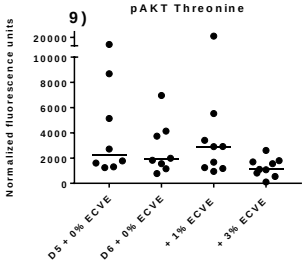
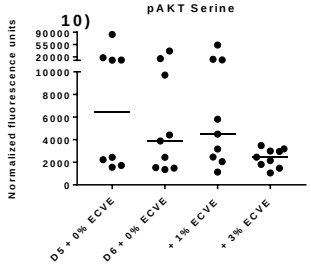

19)
pROS1
20)
pMST1
21)
HMGB1
22)
LSP1
23)
nNOS
24)
PIP5K1C
25)
CD74
26)
NALP1
27)
pHSP27
28)
Bcl-xL
29)
SOD1
Normalized fluorescence units
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

E. Immature DCs with 24 h-ECVE treatment

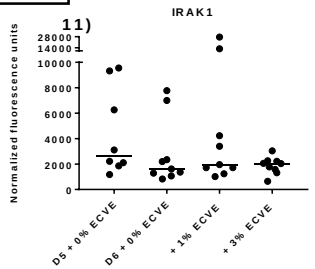


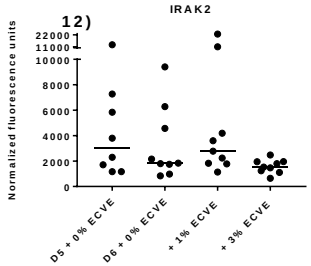
PI3K/AKT pathway

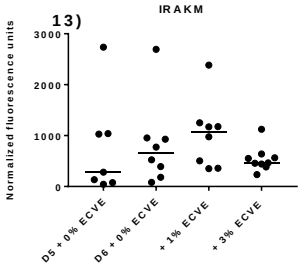


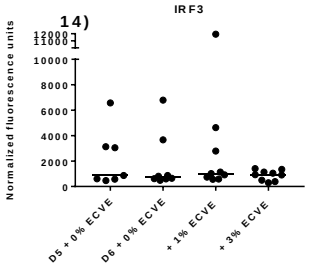


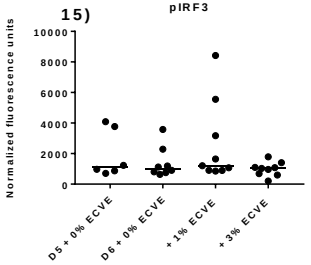
TLR pathway

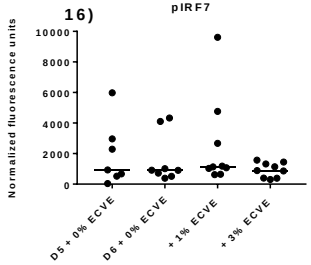


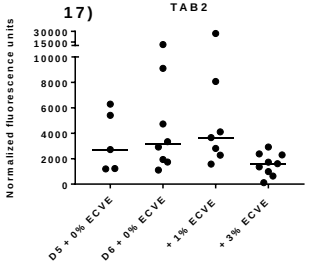


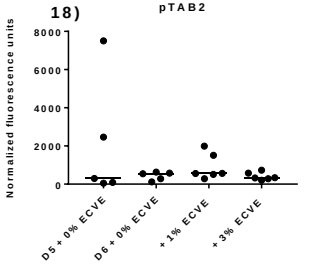












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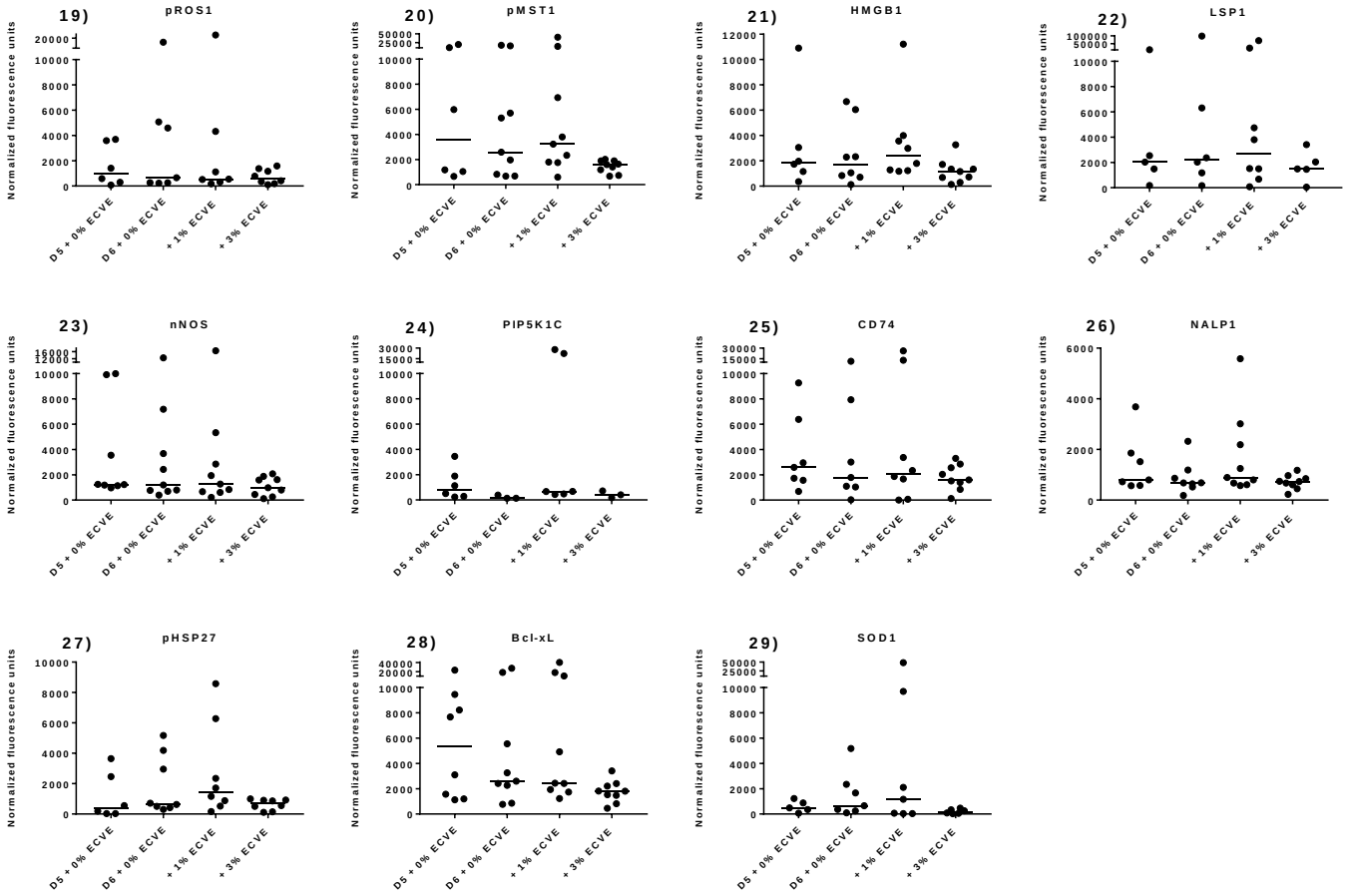
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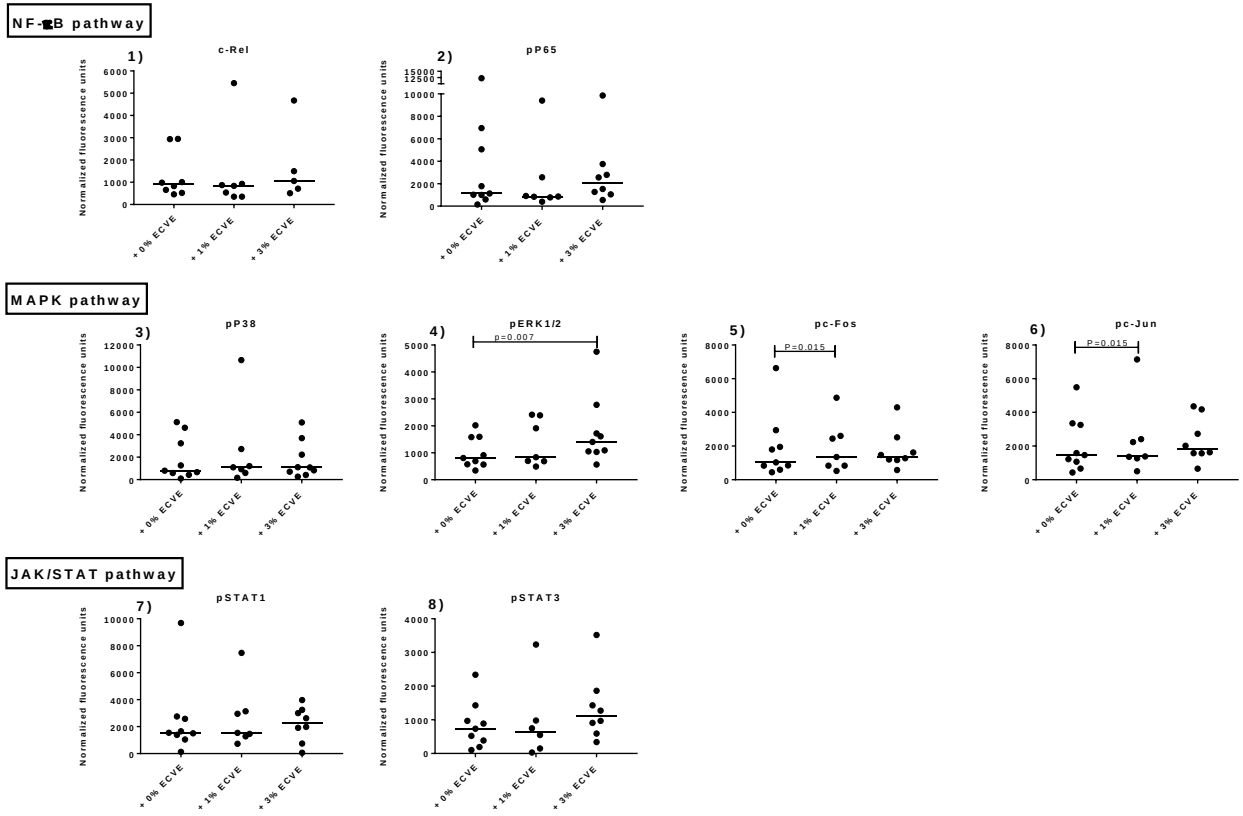
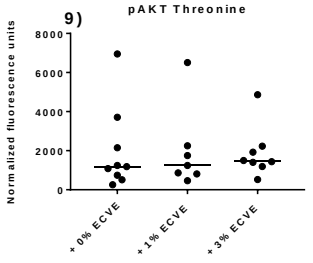
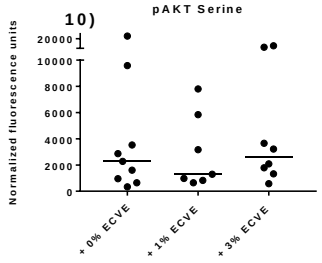
20) pMST1
21) HMGB1
22) LSP1
23) nNOS
24) PIP5K1C
25) CD74
26) NALP1
27) pHSP27
28) Bcl-xL
29) SOD1
Normalized fluorescence units
D5 + 0% ECVE
D6 + 0% ECVE
+ 1% ECVE
+ 3% ECVE

F. LPS-matured DCs with 24 h-ECVE treatment

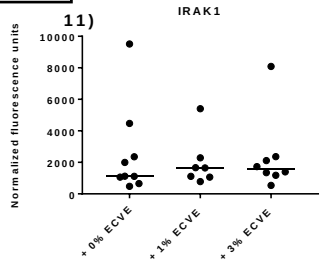


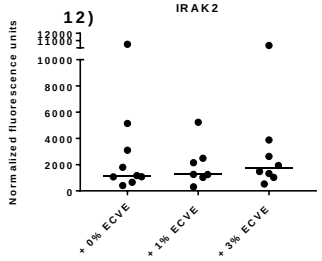
PI3K/AKT pathway

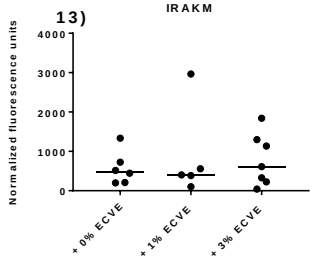


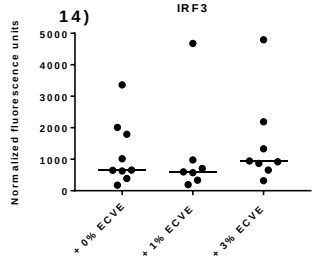


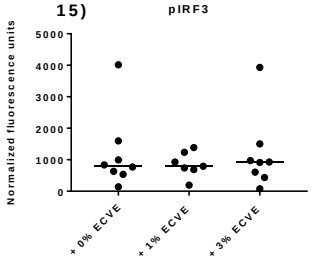
TLR pathway

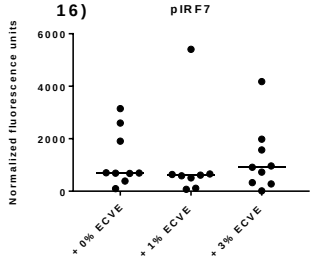


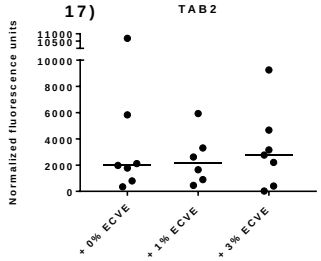


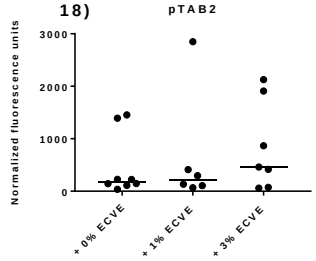












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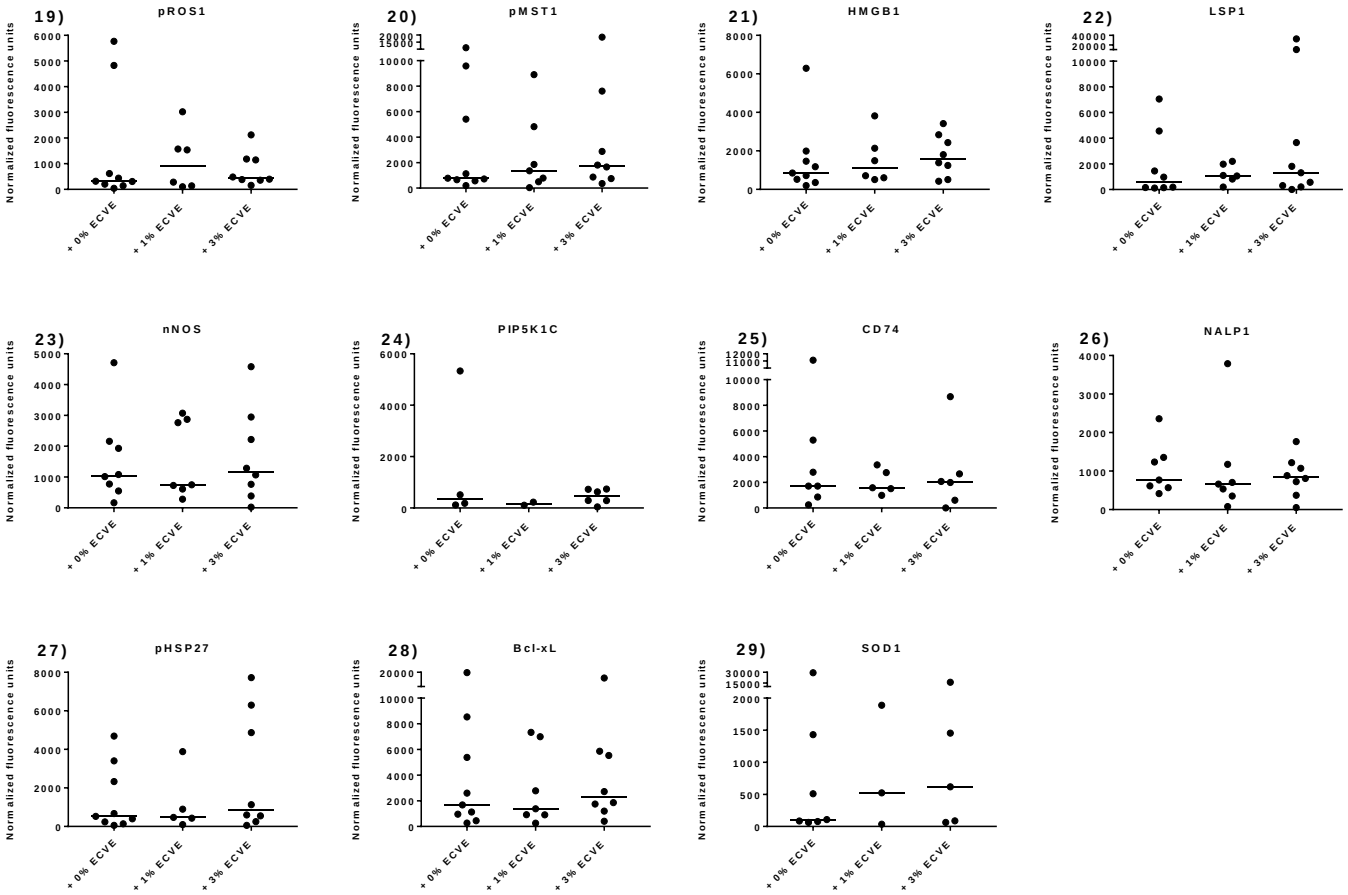
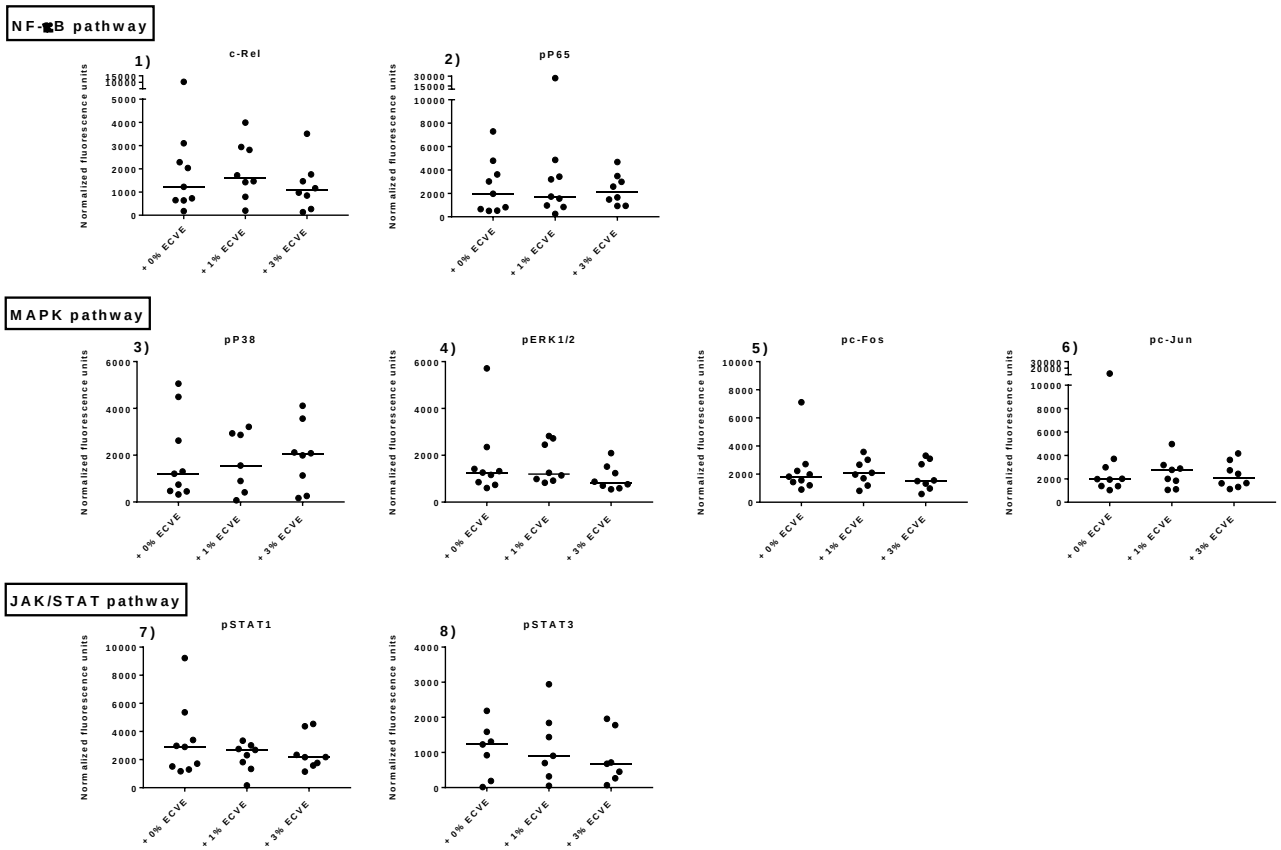
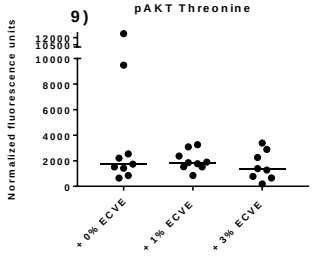
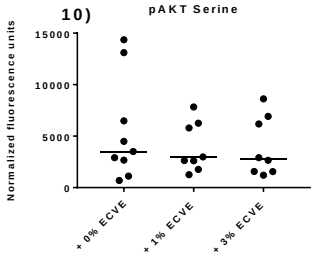

19)
pROS1
20)
pMST1
21)
HMGB1
22)
LSP1
23)
nNOS
24)
PIP5K1C
25)
CD74
26)
NALP1
27)
pHSP27
28)
Bcl-xL
29)
SOD1
Normalized fluorescence units
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

G. Poly I:C-matured DCs with 24 h-ECVE treatment

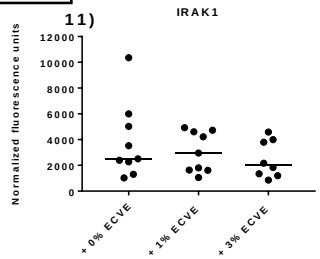


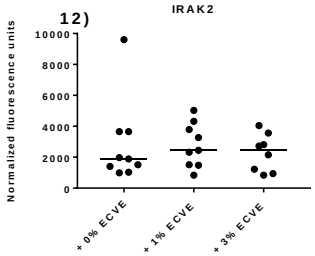
PI3K/AKT pathway

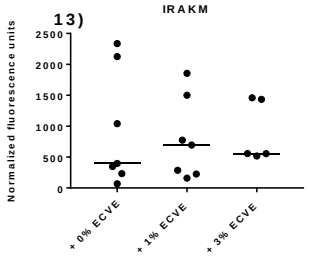


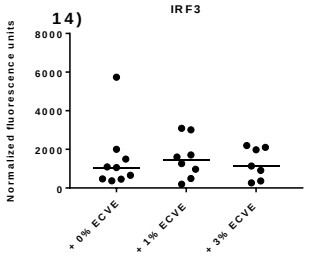


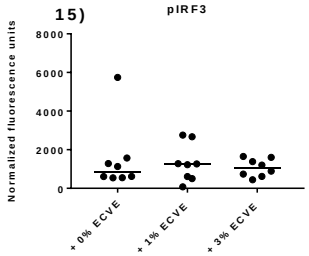
TLR pathway

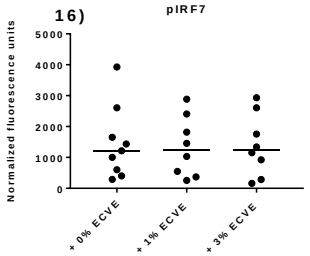


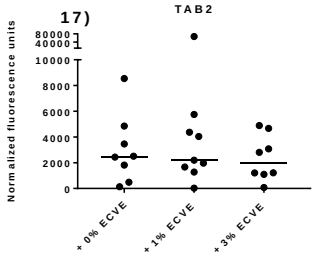


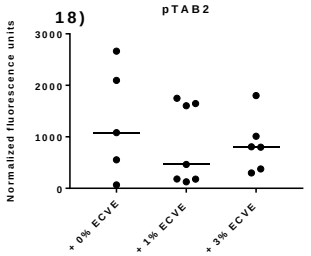




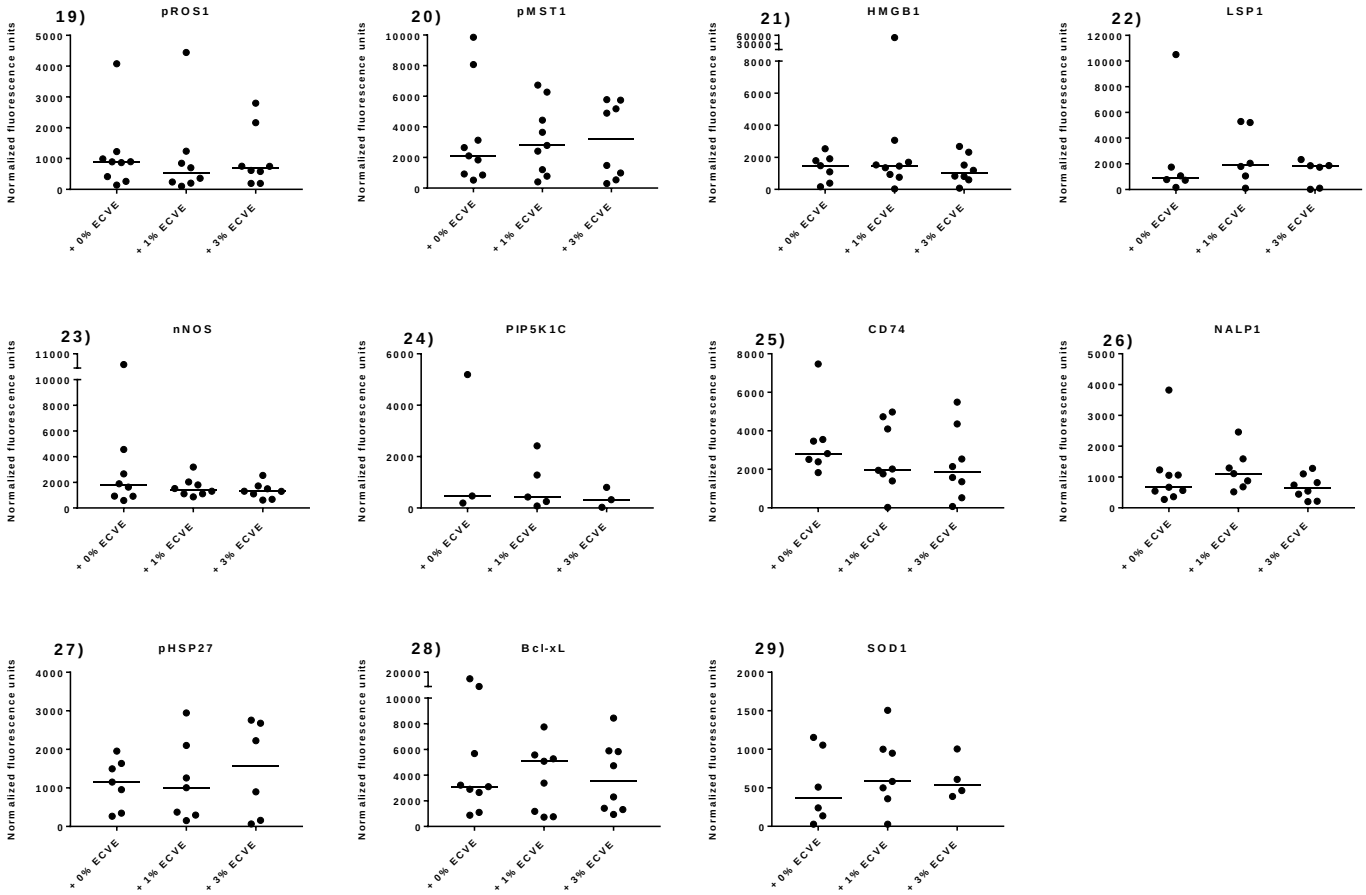








Others


19)
pROS1
20)
pMST1
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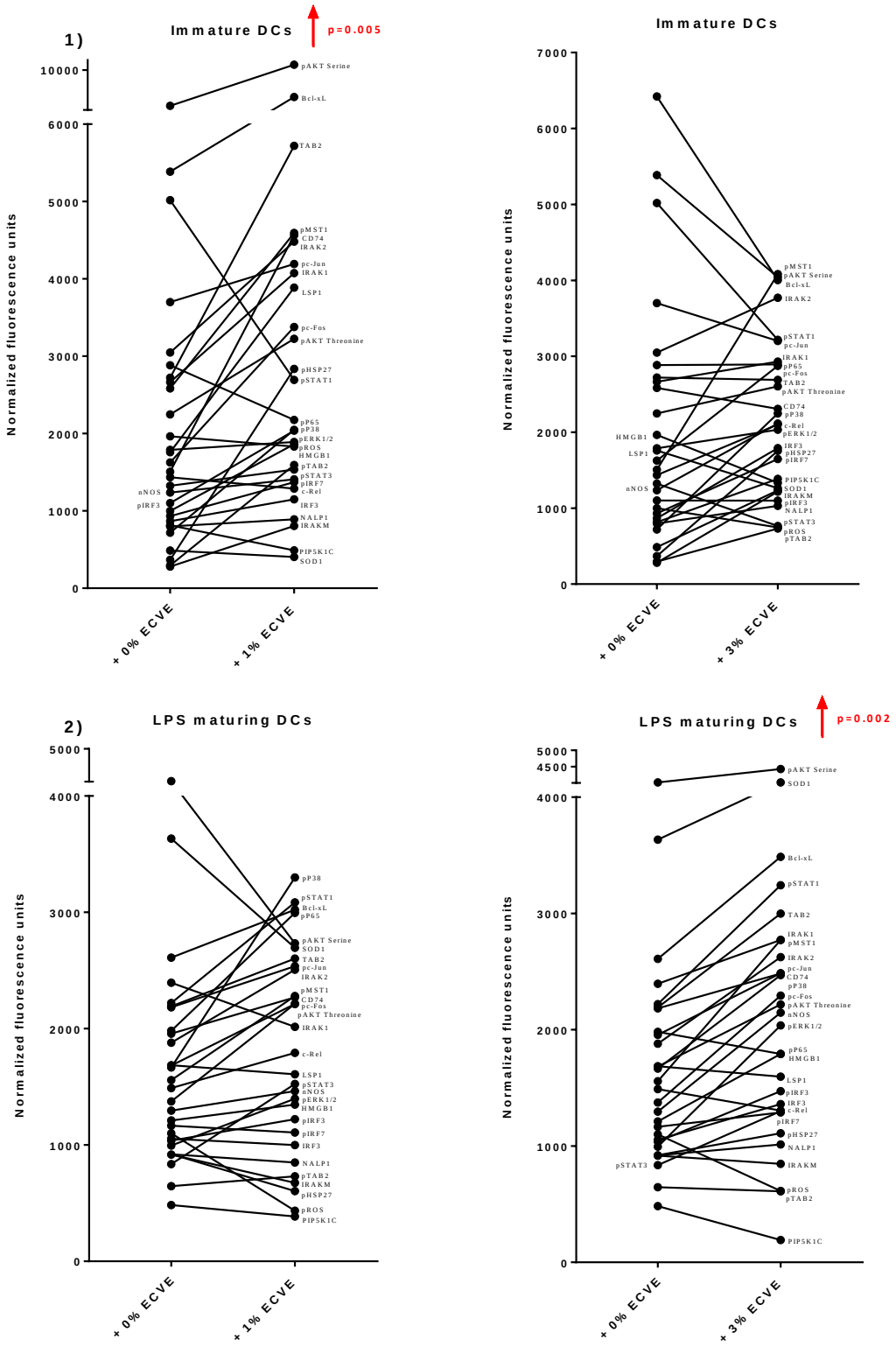
HMGB1
22)
LSP1
23)
nNOS
24)
PIP5K1C
25)
CD74
26)
NALP1
27)
pHSP27
28)
Bcl-xL
29)
SOD1
Normalized fluorescence units
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

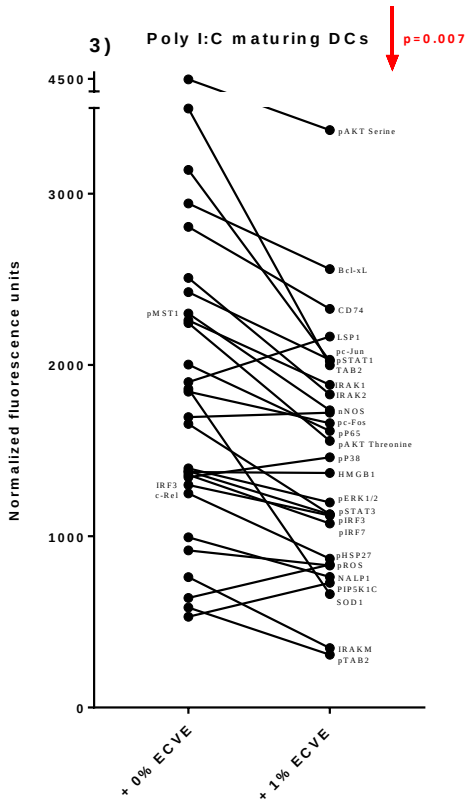
Figure 4.9. RPPA for DCs treated with ECVE. RPPA was used to analyse the expression of 29 signalling molecules which are involved in NF-κB, MAPK, JAK/STAT, PI3K/AKT pathways, amongst others. (A) 24h-ECVE treatment significantly upregulated pERK1/2, pc-Fos and pc-Jun expression of LPS-matured DCs. Expression of 29 signalling molecules in immature (B), LPS-maturing (C), and Poly I:C-maturing (D) DCs treated with ECVE for 30 minutes are shown; and expression in immature (E), LPS-matured (F), and Poly I:C-matured (G) DCs treated with ECVE for 24 h are shown. Data is presented as scatter plots of nine independent experiments (i.e. DCs generated from 9 different PBMC donors), showing the median of each group and each dot represents the mean of four replicates of the same individual. If data were normally distributed, paired-t test was used (Fig. B1 0% vs 1%, B8 0% vs 1%, B19 0% vs 3%, B24 0% vs 1% and 0% vs 3%, B25 0% vs 3%, B29 0% vs 3%, C1 0% vs 1% and 0% vs 3%, C13 0% vs 3%, C22 0% vs 3%, D1 0% vs 1%, D2 0% vs 1%, D3 0% vs 3%, D6 0% vs 1%, D14 0% vs 1%, D15 0% vs 1%, D18 0% vs 1% and 0% vs 3%, D22 0% vs 1% and 0% vs 3%, E9 0% vs 3%, E17 0% vs 3%, E18 0% vs 1% and 0% vs 3%, F6 0% vs 3%, F8 0% vs 3%, F13 0% vs 3%, F23 0% vs 3%, F26 0% vs 3%, G2 0% vs 3%, G8 0% vs 1% and 0% vs 3%, G110% vs 1% and 0% vs 3%, G16 0% vs 1% and 0% vs 3%, G17 0% vs 3%, G18 0% vs 3%, G21 0% vs 3%, G24 0% vs 1% and 0% vs 3%, G) ; otherwise Wilcoxon test was used in the rest of comparison .

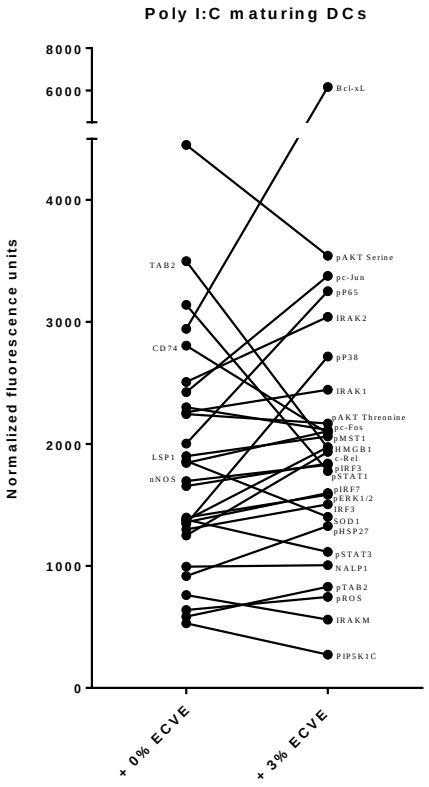
4.2.3.2 Global effects of ECVE-treatment on signalling molecules in DCs

The data presented in the previous section were re-analysed to determine the global effects of ECVE-treatment for 30 minutes or 24 h on signalling molecule expression in immature, maturing and mature DCs. This was achieved by comparing the median values of the nine independent experiments for all 29 signalling molecules with or without ECVE-treatment. 1% ECVE treatment significantly increased global expression of the signalling molecules in immature DCs after 30 minutes (Fig. 4.10A) and 24 h (Fig. 4.10B) treatment. 3% ECVE also significantly increased global expression of the signalling molecules in LPS-maturing DCs after 30 minutes (Fig. 4.10A) and in LPS-matured DCs after 24 h treatment (Fig. 4.10B). However, 1% ECVE exposure for 30 minutes had suppressive effects on signalling molecules in Poly I:C-maturing DCs (Fig. 4.10A). Changes were not statistically significant in the other conditions examined. Overall, these findings suggested that ECVE tended to have inducing, stimulatory effects on immature and LPS-maturing/matured DCs but suppressive effects on Poly I:C-maturing DCs. Table 4.2 summarizes the statistically significant global effects of ECVE on signalling molecules.

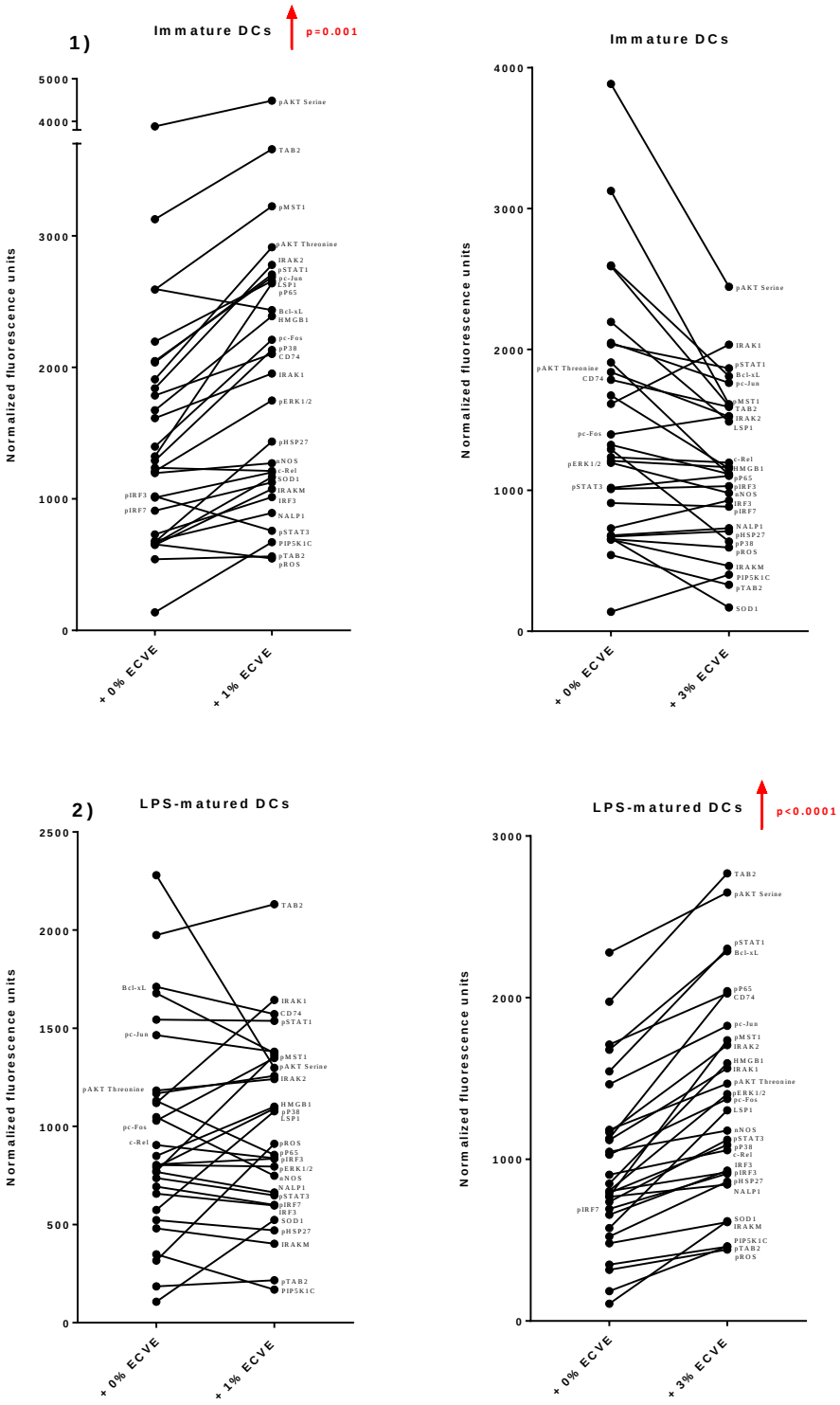
A. 30 min-ECVE treatment




3)
Poly I:C maturing DCs
p=0.007
Normalized fluorescence units
4500
3000
2000
1000
0
pAKT Serine
Bcl-xL
CD74
pMST1
LSP1
pc-Jun
pSTAT1
TAB2
IRAK1
IRAK2
nNOS
pc-Fos
pP65
pAKT Threonine
pP38
HMGB1
IRF3
c-Rel
pERK1/2
pSTAT3
pIRF3
pIRF7
pHSP27
pROS
NALP1
PIP5K1C
SOD1
IRAKM
pTAB2
+ 0% ECVE
+ 1% ECVE


Poly I:C maturing DCs
Normalized fluorescence units
8000
6000
4000
3000
2000
1000
0
Bcl-xL
pAKT Serine
TAB2
pc-Jun
pP65
IRAK2
CD74
pP38
IRAK1
pAKT Threonine
pc-Fos
LSP1
pMST1
HMGB1
c-Rel
pIRF3
nNOS
pSTAT1
pIRF7
pERK1/2
IRF3
SOD1
pHSP27
pSTAT3
NALP1
pTAB2
pROS
IRAKM
PIP5K1C
+ 0% ECVE
+ 3% ECVE

B. 24 h-ECVE treatment



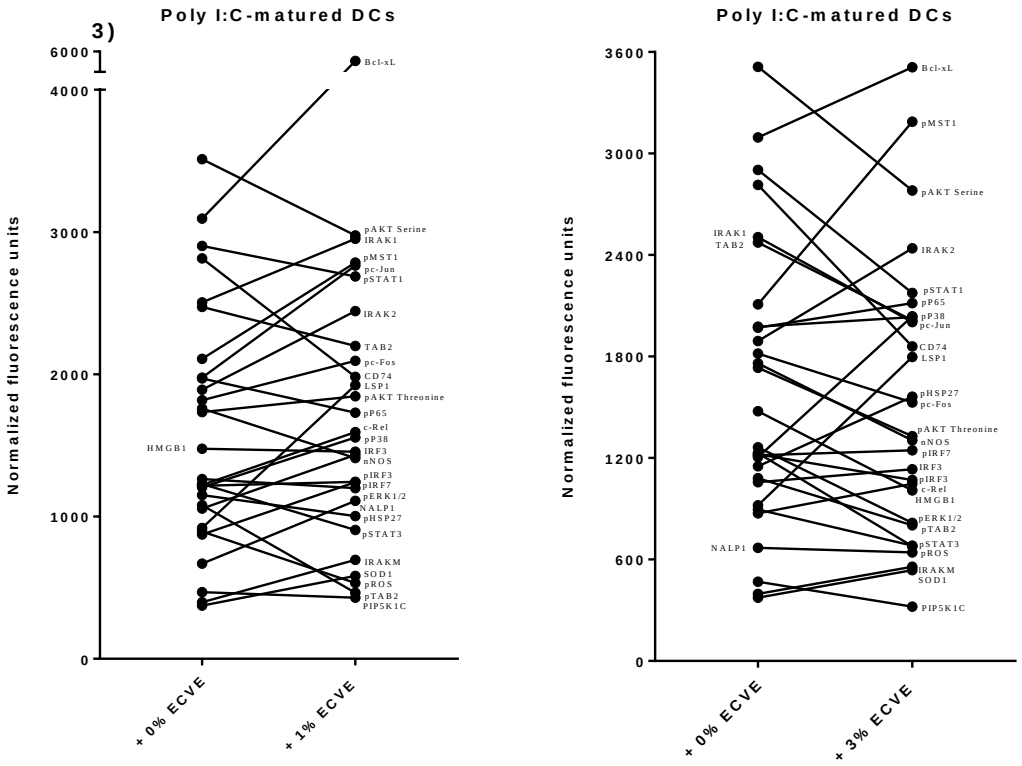


Figure 4.10. Effects of ECVE-treatment on global expression of signalling molecules by DCs. Global effects on 29 signalling molecules in DCs treated with ECVE for (A) 30 minutes and (B) 24 h. Data is presented as scatter plots and each dot represents the median expression of each signalling molecule from the nine independent experiments. If data were normally distributed, paired one-way ANOVA was used (Fig. 4.10A1-3, 4.10B1, 4.10B3); otherwise Freidman test was used (Fig. 4.10B2).

Table 4.3. Summary of global effects on signalling molecules

1% ECVE immature DCs (↑, p=0.005)		**3% ECVE LPS-maturing DCs (↑, p<0.002)**		**1% ECVE Poly I:C-maturing DCs (↓, p=0.007)**	
Increase (23)	pP38 pERK1/2 pc-Fos pc-Jun pSTAT3 pAKT-Threonine pAKT-Serine IRAK1 IRAK2 IRAKM IRF3 pIRF3 pIRF7 TAB2 pTAB2 pROS1 pMST1 LSP-1 nNOS CD74 NALP1 pHSP27 Bcl-XL	Increase (22)	pP38 pERK1/2 pc-Fos pc-Jun pSTAT1 pSTAT3 pAKT-Threonine pAKT-Serine IRAK1 IRAK2 IRF3 pIRF3 pIRF7 TAB2 pMST1 nNOS CD74 NALP1 pHSP27 Bcl-XL HMGB1 SOD1	Increase (5)	pP38 pROS1 LSP-1 nNOS PIP5K1C
Decrease/ Unchanged (6)	c-Rel pP65 pSTAT1 HMGB1	Decrease/ Unchanged (7)	c-Rel pP65 IRAKM pTAB2	Decrease/ Unchanged (24)	c-Rel pP65 pERK1/2 pc-FOS pc-Jun pSTAT1 pSTAT3 pAKT-Threonine pAKT-Serine IRAK1 IRAK2 IRAKM IRF3 pIRF3 pIRF7 TAB2 pTAB2 pMST1 HMGB1 CD74

	SOD1 PIP5K1C		 pROS1 LSP-1 PIP5K1C		NALP1 pHSP27 Bcl-XL SOD1

1% ECVE immature DCs (↑, p=0.001)		**3% ECVE LPS-matured DCs (↑, p<0.0001)**	
Increase (25)	pP65 pP38 pERK1/2 pc-Fos pc-Jun pSTAT1 pAKT-Threonine pAKT-Serine IRAK1 IRAK2 IRAKM IRF3 pIRF3 pIRF7 TAB2 pTAB2 pMST1 HMGB1 LSP-1 nNOS PIP5K1C CD74 NALP1 pHSP27 SOD1	Increase (29)	c-Rel pP65 pP38 pERK1/2 pc-Fos pc-Jun pSTAT1 pSTAT3 pAKT-Threonine pAKT-Serine IRAK1 IRAK2 IRAKM IRF3 pIRF3 pIRF7 TAB2 pTAB2 pROS1 pMST1 HMGB1 LSP-1 nNOS PIP5K1C CD74 NALP1 pHSP27 Bcl-XL SOD1
Decrease/ Unchanged (4)	c-Rel pSTAT3 pROS1 Bcl-XL	Decrease/ Unchanged (0)	None

4.3 Discussion

In this chapter, the effects of ECVE on signalling molecules involved in the NF-κB, MAPK, JAK/STAT, PI3K/AST and TLR pathways in DCs were analysed, using RPPA. 24h ECVE-treatment only significantly upregulated the individual expression of pERK1/2, pc-Jun and pc-Fos in LPS-matured DCs. Although most of signalling molecules were not significantly altered by ECVE individually, ECVE had stimulatory global effects on immature and LPS-maturing/matured DCs whilst it had inhibitory global effects on Poly I:C-maturing DCs.

RPPA is a sensitive and quantitative technique that overcomes many limitations compared to other available techniques for studying proteomics. Using RPPA, many samples can be studied simultaneously and small amounts of samples are required. Several factors could affect the performance of RPPA; therefore, it is critical to optimize the technique before applying samples. Serum starvation is a commonly performed procedure in molecular and cell biology. Serum provides an appropriate environment for cell growth but the complex make-up of serum may be a confounding factor affecting experiments. Therefore, serum starvation could remove unknowns, thereby reducing interference and providing more reproducible experimental conditions to reduce variability in results [163]. Serum starvation could reduce basal cellular activity [164] but also cause stress that triggers apoptosis [165]. As the effect of serum starvation varies between cell types, firstly the effect of serum starvation on the characteristics of DCs was analysed. DCs were cultured with or without serum for 24 h before stimulation and their surface expression of DC-SIGN and CD80 was examined. Serum starvation significantly suppressed the levels of

DC-SIGN and CD80 in immature DCs. Although medium with or without serum facilitated significant elevation of CD80 expression after maturation, serum starvation also caused DC-SIGN expression in mature DCs compared to immature DCs. Previous studies have found that DCs cultured in serum-free media were less mature, as demonstrated by suppression of costimulatory molecule expression and cytokine production, than those cultured in serum-containing media [166,167]. Moreover, much stronger T cell proliferative responses induced by mature DCs were observed in serum-containing media than co-cultures without serum [166]. Low amount/absence of cytokines could affect the characteristics and function of DCs [168], as GM-CSF and IL-4 were not supplemented when culturing DCs in serum-free media which may be the factor altering surface markers. These findings suggested serum starvation was not suitable to use in our study.

Nuclear factor-κB (NF-κB) participates in inflammatory response based its role in the expression of pro-inflammatory genes including cytokines, chemokines, and adhesion molecules [169]. The NF-κB family of transcription factors contains 5 subunits – NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB and c-Rel – which locate in the cytoplasm in inactive forms through association with the cytoplasmic inhibitor IκB. The binding of receptors of pro-inflammatory cytokines and PAMPs to their ligands leads to phosphorylation IκB by the IκB kinase (IKK) enzyme, leading to the degradation of IkB. Degradation of IkB allows the translocation of NF-κB to the nucleus and triggers transcription of various genes involved in immune regulation and inflammation. It is known that cigarette smoke induces inflammatory responses in various types of cells by triggering the NF-κB pathway via ROS, reactive nitrogen

species and aldehydes contained in cigarettes [170]. NF-κB activation is required for DC maturation as evidenced by elevation of CD86 and MHC II expression [171]. Blockage of P65 was reported to inhibit LPS-induced upregulation of CD83, CD86 and HLA-DR in MoDCs and their ability to induce T cell proliferation and polarization [172]. Deletion of c-Rel also reduced DC maturation as evidenced by reduction of CD80 [173], CD86 [173,174] and CD40 [173] expression and compromised DC stimulation of T cells [174]. CS and nicotine decreased expression of pP65 and c-Rel in Poly I:C-matured MoDCs [137], suggesting their role in inhibiting DC maturation and further altering T cell proliferation. The expression of pP65 and c-Rel in DCs were comparable after 30 min- and 24 h-ECVE treatment compared to untreated DCs.

Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signalling involved in various cellular programs including cell proliferation, differentiation, survival, death, and transformation. The mammalian MAPK family consists of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK). Phosphorylation of p38 and ERK1/2 were increased in lungs of mice exposure to CS [175]. CSE caused the loss of the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that regulates airway fluid homeostasis, allowing cilia to beat and clear pathogens in lungs. Inhibition of ERK1/2, but not p38, prevented the CSE-induced CFTR suppression [176]. ECVE was reported to induce p38 activation in neutrophils [80]. Both p38 and ERK1/2 participate in DC activation and function. Reduction of endocytosis during DC activation was suppressed by inhibition of p38, and p38 altered DC maturation as inhibition of p38 abrogated the up-regulation of CD80 [177-180], CD83 [177,178],

CD86 [177-180], CD40 [178,179] and HLA-DR [178] expression, suggesting the requirement of p38 for DC maturation. Moreover, inhibition p38 decreased production of the pro-inflammatory cytokine IL-6 [179] and the allo-stimulatory function of DCs [178]. Inhibition of ERK1/2 also suppressed the expression of costimulatory molecules CD80, CD86 and CD40, and pro-inflammatory IL-6 production by DCs [179]. CSE induced phosphorylation of p38 and ERK1/2 of conventional DCs that led to the secretion of CCL3 and CXCL2 by a ROS dependent manner [181]. CCL3 and CXCL2 induce proliferation of CD8+ T cell and infiltration and activation of neutrophils, respectively. In the current study, pERK1 level in LPS-matured DCs was significantly elevated by 3% ECVE exposure for 24 h that might in turn upregulate DC maturation, cytokine production and T cell proliferation.

c-Fos forms a heterodimer with c-Jun, resulting in the activating protein-1 (AP-1) complex that regulates cell proliferation, differentiation and survival [182]. Phosphorylation of c-Fos is induced by p38 MAPK and ERK while phosphorylation of c-Jun is induced by JNK and ERK [180,183]. CS increased expression of c-Fos but not c-Jun in terminal bronchioles and alveolar ducts in mice, related to cigarette number [184]. Expression of c-Fos was also increased in human periodontal ligament fibroblasts after nicotine treatment for 2 hour [185]. c-Fos is required for DC maturation and function, evidenced by reduction of CD86, CD40, IL-12 and antigen-specific T-cell activation by depletion of c-Fos [186]. Inhibition of c-Jun reduced CD80 and CD83 expression and inhibited the release of IL-12 and TNF-α from mature MoDCs. However, c-Jun did not alter the ability of DCs to induce T cell proliferation [178]. CSE increased phosphorylation of c-Fos and c-Jun in conventional DCs, which

was abolished by the anti-oxidant NAC [181], suggesting the contribution of CS to maturation and cytokine production of DCs via oxidants and free radicals and generation of intracellular ROS. In the current study, expression pc-Fos and pc-Jun were upregulated in LPS-matured DCs treated with 1% ECVE for 24 h, suggesting the potential activation of MAPK pathway by ECVE

Signal transducer and activator of transcription (STAT) molecules, located in the cytoplasm and activated through tyrosine phosphorylation by cytokine receptor associated kinases (JAKs) or growth factor receptor tyrosine kinases [187], are involved in cellular survival, proliferation, differentiation and apoptosis [188]. STAT1 is activated in mature DCs and upregulates their CD40 expression [189]. CpG DNA acts via TLR9 to induce autocrine IFN-β signalling, which activates STAT1 in myeloid DCs, thereby inducing maturation of DCs by increasing expression of CD80 and CD86 and production of pro-inflammatory IL-6 and TNF-α [190]. In contrast, blockage of STAT3 reduced the ability of DCs to take up soluble antigen, but promoted maturation by upregulating expression of CD86, CD40 and MHC II and stimulation of T cells, suggesting it also has a negative role in the regulation of DC activation [191]. STAT3 induced by CS may elicit protective functions in lungs of smokers by reducing inflammatory cells and cytokines and apoptosis; therefore, inhibition of STAT3 may exacerbate the development of CS-induced lung diseases [192]. In the current study, ECVE did not alter STAT1 and STAT3 expression in DCs.

The AKT pathway modulates multiple functions including cell survival, proliferation, metabolism, invasiveness and angiogenesis. In the N-terminal region of AKT, there is a threonine residue (T308) whose phosphorylation is required for AKT activation. A

secondary regulatory phosphorylation serine site (S473) is located at hydrophobic C-terminal tail. Phosphorylation of T308 and S473 occurs in response to cytokines, hormones and growth factors, thereby activating the AKT pathway [193,194]. Activation of the AKT pathway increases responses to growth factors, inhibits sensitivity to anti-proliferative signals and apoptosis and promotes production of the angiogenic factor nitric oxide; all of these properties can be related to the onset or progression of cancers [193-195].

The AKT pathway plays a critical role in DC survival [196,197]. LPS and CD40 stimulation increase levels of AKT at both phosphorylation and steady state [196], thereby promoting DC survival; however, IL-10 markedly inhibit LPS-induced AKT activation [177]. AKT activation modulates DC maturation by upregulation of the co-stimulatory molecules CD40 and CD86 [196,197]. Also, activation of the AKT pathway significantly elevates the expression of CCR7, which is required for migration of DCs into draining lymph nodes [197]. Production of cytokines by DCs (i.e. IL-12p70 and TNF-α) is also induced by AKT activation [177], which might strengthen their ability to stimulate T cells. Indeed, AKT activation enhances antigen-specific CD8+ T cell activation and proliferation in vitro [196,197].

One study reported that nicotine induces phosphorylation of AKT in MoDCs, associated with nicotine-induced expression of the costimulatory molecule CD86 [198]. In contrast, a study showed nicotine suppressed the phosphorylated AKT-threonine expression in Poly I:C-matured DCs [137]. CSE also suppressed AKT phosphorylation in pDCs induced with CpG, leading to reductions in IFN-α production [199]; this may impair host defence thereby increasing the susceptibility

to airway infections, a major cause of COPD exacerbations. CS induced phosphorylation of AKT in adenocarcinomic human alveolar basal epithelial cells in a time- and dose-dependent manner [200]. These findings suggested CS stimulated the AKT pathway, which might increase the risk of COPD exacerbations and tumor development. Hoverer, the current study did not find significant differences in the AKT pathway in ECVE-treated DCs, measured by phosphorylation of threonine and serine residues of AKT. There was no difference of AKT signalling molecule expression in DCs treated with ECVE.

Various TLR-induced signalling pathways require the adaptor MyD88, which interacts with members of the IL-1R-associated kinase (IRAK) family, which dissociate from MyD88 and bind to tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6), leading to activation of NF-κB and MAPK pathway [201]. There are three members in the IRAK family that were investigated in current study: IRAK1, IRAK2 and IRAKM. Both IRAK1 and IRAK2 are required for the initial responses in the TLR signalling cascade and lead to cytokine production, but IRAK2 is also important in late-phase TLR responses due to the sustainability of its kinase activity [202,203]. Silencing of IRAK1 and TRAF6 induced DC apoptosis but downregulated CD40. Furthermore, IRAK1 and TRAF6 complex enhanced IL-12p70, IL-6 and TNF-α production by LPS/Poly I:C-matured MoDCs [204]. In contrast, IRAKM is a negative regulator of TLR-induced signalling that inhibits formation of the IRAK1-TRAF6 complex [19]. IRAKM has been reported to inhibit the maturation of mouse BMDCs (measured by downregulated MHC II, but elevated PD-L1 levels) and cytokine production (measured by reduced pro-inflammatory MIP, but increased immuno-

modulatory IL-10 levels), but not affecting T cell skewing in response to pathogens [205]. Nicotine has been found to reduce the expression of IRAKM in poly:IC-matured DCs [137]. The current study demonstrated ECVE did not have effects on IRAK1, IRAK2 and IRAKM.

The presence of dsRNA or dsDNA in the cytosol following infection with viruses or intracellular bacteria is recognized by TLRs resulting in phosphorylation of IFN-regulatory factor 3 (IRF3) and IFN-regulatory factor 7 (IRF7), leading to their homo- or hetero-dimerization. These dimers then translocate to the nucleus and activate the transcription of type I IFN genes which are critical in the response against viral infection. IRF3 is elicited by TLR4 in the TRIF-dependent pathway while IRF7 is triggered by TLR7 and TLR9 in the MyD88-dependent pathway [206]. Phosphorylation of IRF3 [137,207-209] and IRF7 [207,210] and production of type I IFN can be decreased by CS, suggesting that CS might impair anti-viral responses and contribute to increased risk of respiratory infections. Indeed, both E-cigarettes and CS impair defence against bacterial and viral infections, resulting in increasing pathogen burden, which has been discussed in the Introduction chapter. Current study indicated there was no difference in expression of IRF3, pIRF3 and pIRF7 in DCs treated with ECVE.

TAK1-binding protein 2 (TAB2) is a TAK1- and TRAF6-binding protein that functions as an IL-1 signalling molecule. TAB2 is translocated from the membrane to the cytosol and then links TAK1 to TRAF6 in response to IL-1 stimulation, leading to phosphorylation of MAPK kinases MKK4 and MKK3/6 and NF-κB inducing kinase (NIK) , which activate JNK, p38 MAPK and the NF-κB pathway, respectively

[211,212]. In addition, TAB2 levels were elevated in breast and prostate carcinoma cell lines and contribute to increase of MMP-9 expression which is critical for matrix-degrading activity [213]. The current study indicated there was no difference in expression of TAB2 in DCs.

Genetic rearrangements in the tyrosine kinase receptor ROS1 lead to the development of fusion proteins. ROS1 fusion proteins are constitutively active and drive downstream AKT, ERK or STAT pathways, depending on the ROS1 fusion partner [214]. No significant changes in phosphorylation of ROS1 was found in DCs under ECVE exposure.

High mobility group box 1 (HMGB1) is an abundant prototypic intracellular protein released by necrotic cells; some cell types, such as macrophages and monocytes, can secrete HMGB1 actively in response to TLR challenges. Active secretion of HMGB1 involves the acetylation of the protein and its transfer to secretory endolysosomes, whose contents are released extracellularly, thereby mediating endotoxin lethality and inflammation [215]. Extracellular HMGB1 regulates maturation of DCs by elevating CD80 [216,217], CD86 [216,218], CD40 [217,218], CD83 [216-218] and MHC II [217]. Blockage of HMGB1 suppressed CCR7 and CXCR4 expression on mature DCs and migration to lymph nodes in response to the chemokines CCL19 and CXCL12 [218]. HMGB1 also regulates release of cytokines by DCs including IL-12 [216-218], IL-6 [217], IL-1a [217], IL-8 [217], TNF-α [217] and RANTES [217]. Moreover, HMGB1 induces proliferation of allogenic T cells [216,217] and leads to Th1 responses supported by elevation of IFN-γ [216,217] and IL-2 [217]. CS induced the translocation of HMGB1 from nucleus to cytosol and its release into BAL of mice.

HMGB1 enhanced CS-induced pulmonary inflammation in mice by triggering the TLR4-mediated secretion of IL-1β and TNF-α and the recruitment of neutrophils, macrophages and lymphocytes in BAL. HMGB1 also activated the phosphorylation of p38, JNK, and IκB through TLR4/MyD88 signalling in lungs exposed to the CS [219]. In contrast, a study reported the downregulation of HMGB1 by CSE and nicotine in Poly I:C-matured DCs [137].

The endogenous production of NO can be catalyzed by neuronal nitric oxide synthase (nNOS) [40]. Blockade of nNOS diminished the expression of CD80, CD83 and HLA-DR and IL-12 production by MoDCs, indicating the contribution of nNOS to DC maturation and cytokine production [41]. It has been found that nicotine decreases the expression of nNOS in Poly I:C-matured DCs, suggesting its role in inhibiting functions of DCs [137]. Phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Gamma (PIP51KC) is associated with cell migration [220] and CSE and nicotine decreased the levels of PIP51KC in poly I:C-matured DCs [137], suggesting the role of CSE and nicotine in alteration of DCs migration. Lymphocyte Specific Protein 1 (LSP1) causes polymerization and remodelling of the actin cytoskeleton, thereby modulating the motility of various leukocytes. LSP1 facilitates the HIV-1 gp120-induced DC chemotaxis which might contribute to the dissemination of this virus within the body [221]. The expression of these signalling molecules in DCs were not affected under ECVE exposure, suggesting ECVE may not alter basic function of DCs including maturation, migration and T cell activation. However, ECVE had different global effects on these molecules that vary with concentration and exposure duration so it could not conclude ECVE has no effects on DCs.

Superoxide dismutase-1 (SOD1), which promotes cell survival by destroying ROS [222], was down-regulated in poly I:C-matured MoDCs by CSE and nicotine [137], potentially leading to reduced DC viability. Heat shock protein 27 (HSP27) is a protein chaperone that facilitates refolding of damaged proteins in response to cellular stress conditions including heat shock, oxidative stress and chemical stress. HSP27 acts as an anti-oxidant that reduces ROS by increasing glutathione and also has anti-apoptotic activity. In contrast, phosphorylated HSP27, which is produced by MAPKAP kinase 2/3 via the activation of the P38 MAPK pathway, inhibits the upregulation of intracellular glutathione and promotes cell apoptosis [223]. Exposure to the pro-oxidants in CS elevated the phosphorylation of HSP27 and this increased oxidative injury to retinal epithelium, which occurs in age-related macular degeneration [224]. B-cell lymphoma extra large (Bcl-xL), which is a transmembrane molecule in mitochondria, acts as an anti-apoptotic protein by preventing the release of mitochondrial contents such as cytochrome C that leads to caspase activation and programmed cell death. Bcl-xL promotes dendritic cell survival in vivo [225] and in vitro [186], and CSE has been shown to increase Bcl-xL levels in MoDCs due to oxidative stress [226]. In contrast, a study demonstrated that both CSE and nicotine reduce Bcl-xL expression in Poly I:C-matured MoDCs [137]. ECVE did not have effects on these signalling molecules related to cell survival which was consistent with findings that the viability of DCs was not altered as determined by PI staining. Again, ECVE had different global effects on these molecules that vary with concentration and exposure duration.

NACHT leucine-rich-repeat protein 1 (NALP1), a member of the NLR (NOD-like receptor)-family, is an intracellular protein that contains an N-terminal PYRIN (PYD) domain followed by a nucleotide-binding oligomerization domain called NACHT and several leucine-rich repeat (LRR) domains, and finally a C-terminal CARD domain. Similar to TLRs, LRRs recognize pathogens, leading to activation of inflammasomes which induce the activation of the protease caspase-1 which cleaves the precursors of pro-inflammatory cytokine IL-1β and IL-18 to yield active cytokines [227,228]. CS significantly increases IL-1β [93,229] and IL-18 [229,230] secretion in mice and humans and these cytokines contribute to the development of emphysema and airway remodelling [229], which are characteristics of COPD. Mammalian sterile 20-like kinase 1 (MST1), also known as serine/threonine kinase 4, responds to stress and regulates apoptosis [231]. MST1 negatively regulates Th17 differentiation, and deficiency of MST1 in DCs promoted Th17 differentiation and disease progression in experimental autoimmune encephalomyelitis (EAE) [232], suggesting that MST1 in DCs was critical for protection against autoimmune diseases. However, MST1 deficiency in DCs promoted antifungal infection by Th17 differentiation through IL-6 which was associated with p38MAPK pathway [232]. Although no significant difference was found after ECVE exposure, global effects of ECVE on these molecules shows their potential role in respiratory diseases.

Looking into the global effects of ECVE, 1% ECVE generally enhanced expression of the signalling molecules at both 30 minutes and 24 hour. However, no significance was found in immature DCs treated with 3% ECVE, which may be because it was above optimal concentration. For LPS-treated DCs, 3% ECVE significantly induced

the expression of the signalling molecules at both 30 minutes and 24 hour, suggesting the same direction of signalling molecule change as in immature DCs, but different sensitivity to ECVE concentration. In contrast, either no significant effect or overall significant decrease with 1% ECVE was observed on Poly I:C-treated DCs, which is consistent our previous study that CSE and nicotine suppressed signalling molecule expression most significantly in Poly I:C-matured DCs. Overall, ECVE appeared to have different effects on signalling pathway in DCs, depending on their state of maturation, type of stimulation and concentration of ECVE.

4.4 Conclusion

3% ECVE treatment for 24 h significantly upregulated the individual expression of pERK1/2, pc-Fos and pc-Jun in LPS-matured DCs but no significant effects were found on immature and Poly I:C-matured DCs. Although most signalling molecules were not affected individually, ECVE had different global effects depending on the maturation status of DCs, ECVE concentration and exposure duration. Therefore, long-term exposure to ECVE may provoke an accumulation of changes to signalling pathway expression in DCs resulting in changed behaviour of these cells. This may have wide-ranging effects the on immune system. ECVE had opposite effects on global signalling molecule expression in immature and LPS-maturing/matured DCs versus Poly I:C-maturing DCs, i.e. increasing global effects in the former and decreasing it in the latter. Thus, ECVE may have different effects on the immune system in different circumstances (e.g. bacterial versus viral infection). More studies of DC function are required to obtain more thorough understanding of ECVE on DCs.

Chapter 5 ECVE treatment reduced the expression of CD83, CD86 and HLA-DR expression on DCs

5.1 Introduction

In the previous chapter, it was found ECVE had effects on signalling molecules that are related to antigen uptake, migration, maturation and cytokine production by DCs. However, the effects on these functions varied. To investigate the effects of ECVE on these functions using other techniques, expression of a wide range of surface markers was examined by flow cytometry. In addition to DC-SIGN and CD80 examined in Chapter 3, expression of another nine surface markers was examined: CD1a, CD36, HLA-DR, CD86, CD40, CD83, CD74, PD-L1 and CXCR4. At their immature stage, DC-SIGN, CD1a, CD36 and HLA-DR expression are involved in antigen uptake and presentation by DCs. After stimulation of DCs, co-stimulatory molecules CD80, CD86 and CD40 are involved in T cell activation: thus, upregulation of these co-stimulatory molecules is indicative of DC maturation, as well as expression of CD83. CD74, known as MHC class II-associated invariant chain (Ii), regulates not only antigen processing by MHC II molecules but also migration of DCs. Although Programmed Death-Ligand 1 (PD-L1) belongs to the B7 family of co-stimulatory molecules, its engagement with PD-1 on T cell induces inhibitory signals that attenuate T cell expansion [1]. CXCR4 is a chemokine receptor and engagement with its ligands, CXCL4 and CXCL12, promotes DC migration into regional lymph nodes [2].

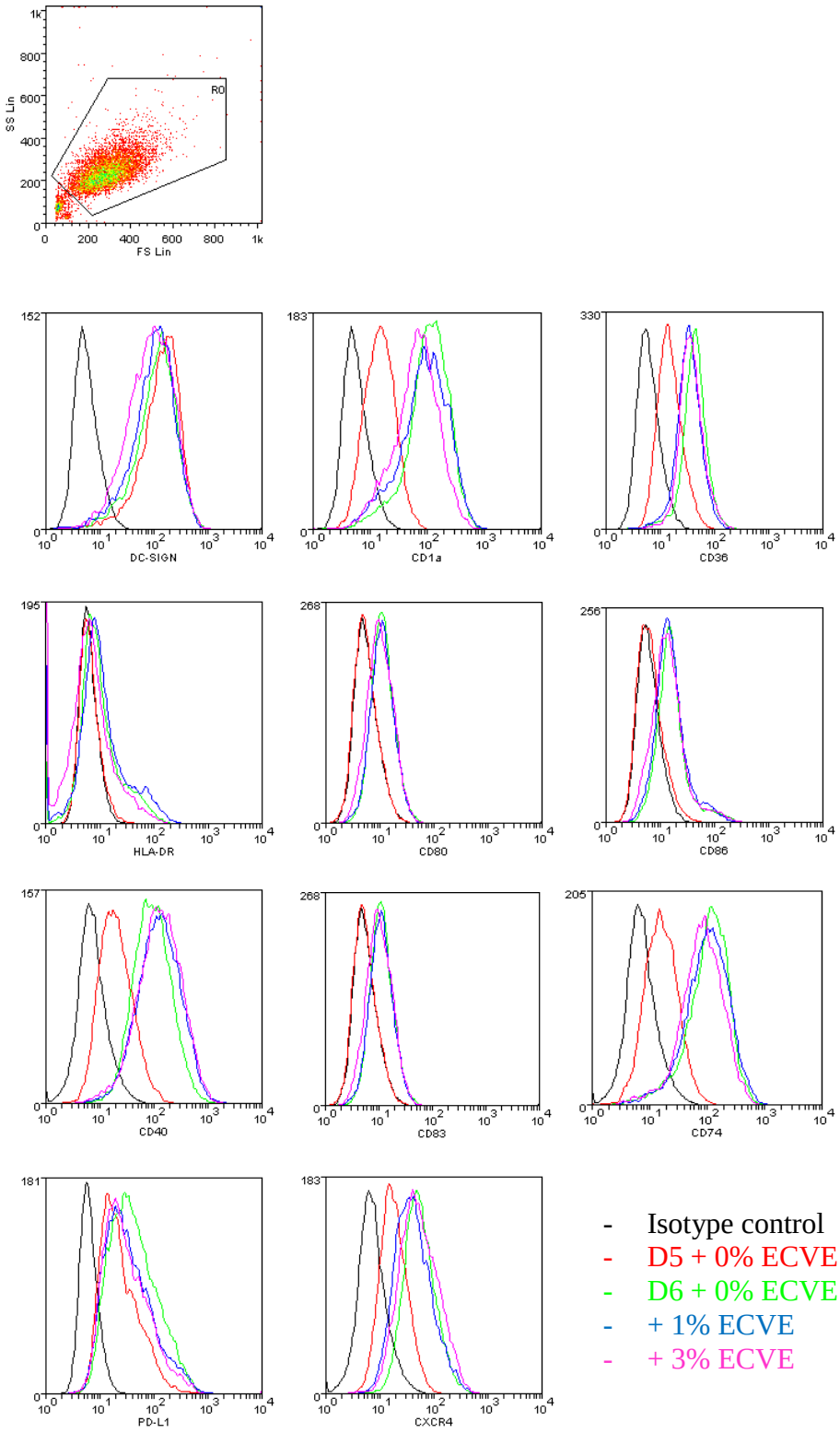
In the experiments reported in this chapter, immature and mature DCs were generated from fresh venous blood of healthy never-smokers and treated with or without ECVE for 24 h, followed by extracellular staining.

5.2 Results

Figure 5.1A shows representative dot plots and histograms of marker expression by immature DCs treated with or without different concentrations of ECVE for 24 h, compared to the matched isotype control. The results of 6 independent experiments show that 3% ECVE significantly reduced CD83 expression ($p=0.019$) on immature DCs (Fig 5.1B) but the levels of other surface molecules were comparable, regardless of ECVE exposure.

Immature DCs were further stimulated by LPS or Poly I:C in the presence or absence of ECVE for 24 h. Firstly, both LPS- and Poly I:C-matured DCs showed significant upregulation of HLA-DR, CD80, CD86, CD40, CD83 and PD-L1, but reduction of DC-SIGN, compared to immature DCs (Fig 5.2A and 5.3A). Figure 5.2B and 5.3B show dot plots and representative histograms of marker expression by LPS- or Poly I:C-matured DCs treated with or without different concentrations of ECVE, compared to the matched isotype control. The results of 6 independent experiments show that 3% ECVE decreased HLA-DR ($p=0.012$) and CD86 ($p=0.041$) expression by LPS-matured DCs (Fig 5.2C); no alterations of surface markers were found on Poly I:C-matured DCs in response to ECVE treatment (Fig 5.3C).

A.


SS Lin
FS Lin
R0
DC-SIGN
CD1a
CD36
HLA-DR
CD80
CD86
CD40
CD83
CD74
PD-L1
CXCR4
- Isotype control
- D5 + 0% ECVE
- D6 + 0% ECVE
- + 1% ECVE
- + 3% ECVE

B.

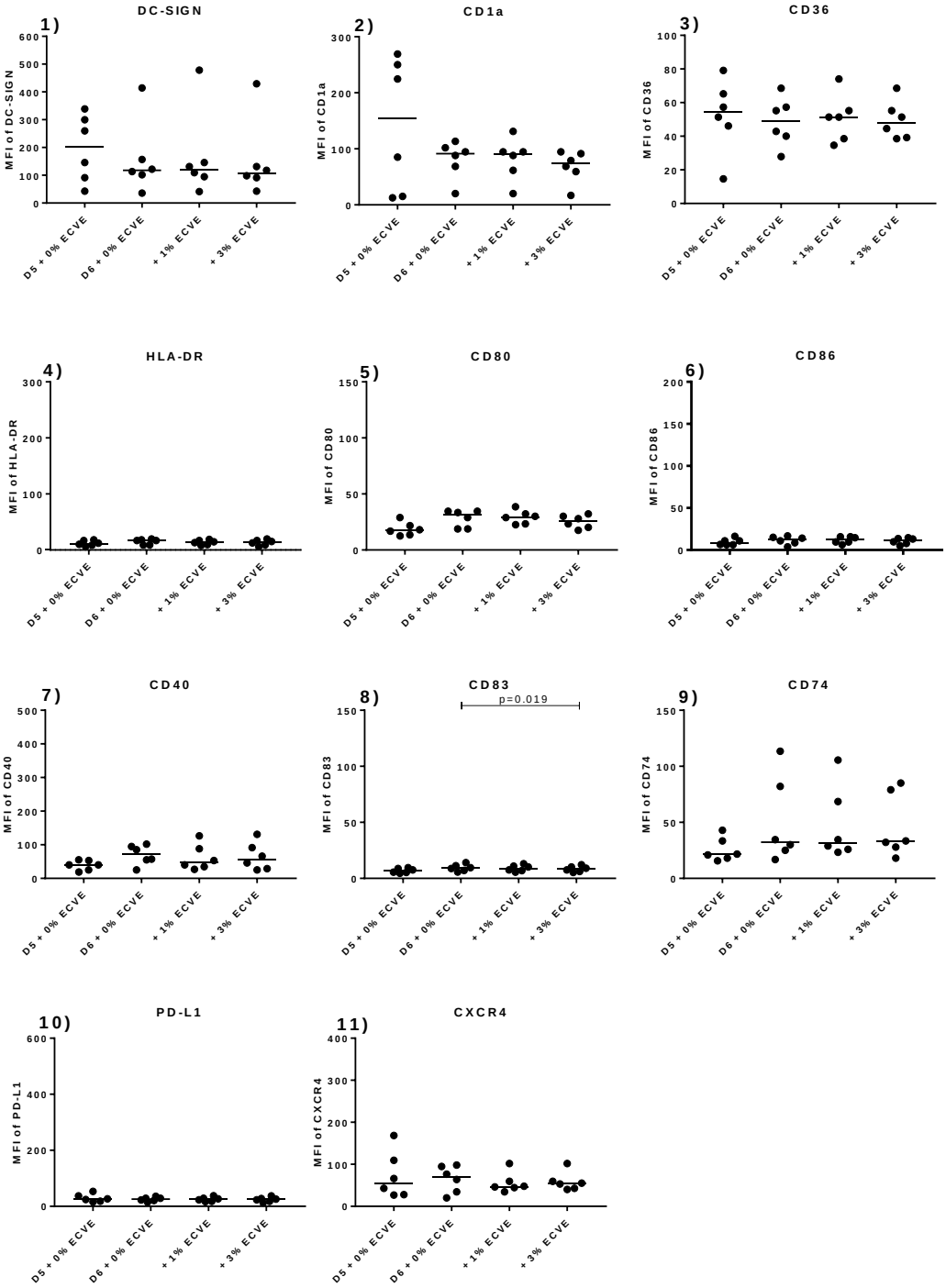
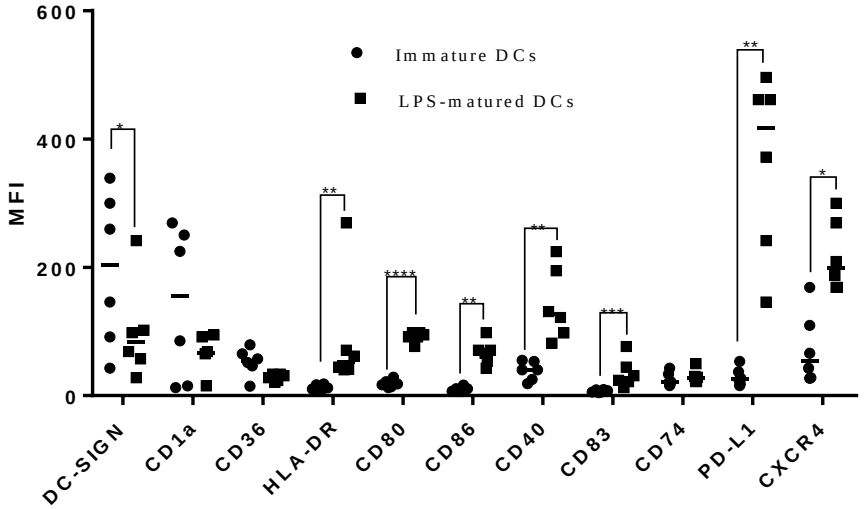
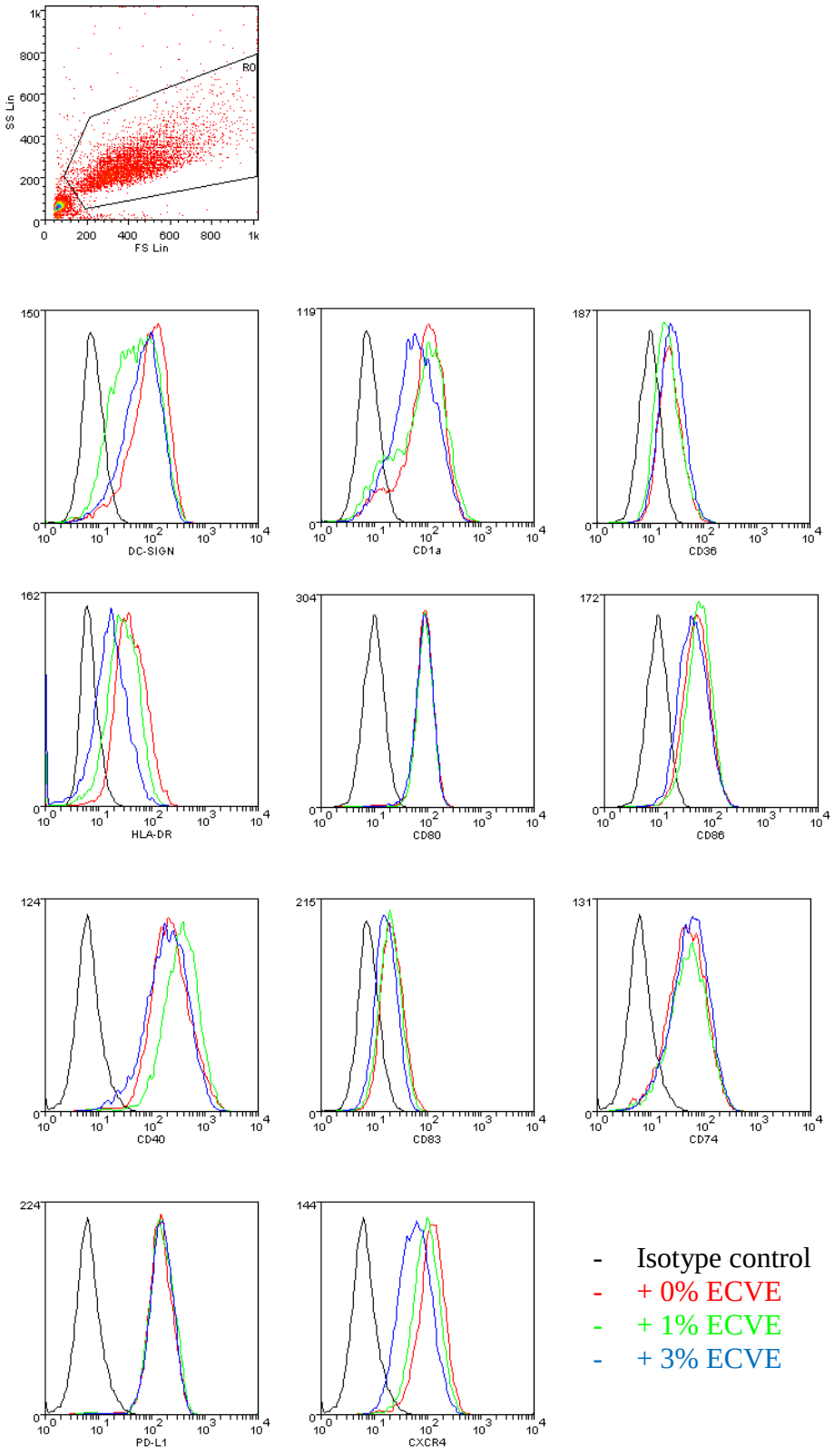

1)
DC-SIGN
MFI of DC-SIGN
2)
CD1a
MFI of CD1a
3)
CD36
MFI of CD36
4)
HLA-DR
MFI of HLA-DR
5)
CD80
MFI of CD80
6)
CD86
MFI of CD86
7)
CD40
MFI of CD40
8)
CD83
p=0.019
MFI of CD83
9)
CD74
MFI of CD74
10)
PD-L1
MFI of PD-L1
11)
CXCR4
MFI of CXCR4
D5 + 0% ECVE
D6 + 0% ECVE
+ 1% ECVE
+ 3% ECVE

Figure 5.1. 3% ECVE treatment for 24 h reduced the CD83 expression on immature DCs. Immature DCs were treated with or without ECVE for 24 h. The expression of 11 different cell surface markers was examined by flow cytometry after cell-surface staining. (A) Representative dot plot and histograms of surface markers on immature DCs. The DC population was gated and the debris were excluded (called gate R0) and this gate was applied during analysis (B) The MFI of surface markers on immature DCs after 24 h with or without ECVE treatment. Data is represented here as scatter plots and each dot represents a different individual. The horizontal bars represent the medians of six individual experiments. If data was normally distributed, paired one-way ANOVA was used, otherwise paired Friedman test was used (Fig 5.1 5.1B1, 5.1B5 and 5.1B11).

A.



B.


SS Lin
FS Lin
R0
DC-SIGN
CD1a
CD36
HLA-DR
CD80
CD86
CD40
CD83
CD74
PD-L1
CXCR4
- Isotype control
- + 0% ECVE
- + 1% ECVE
- + 3% ECVE

C.

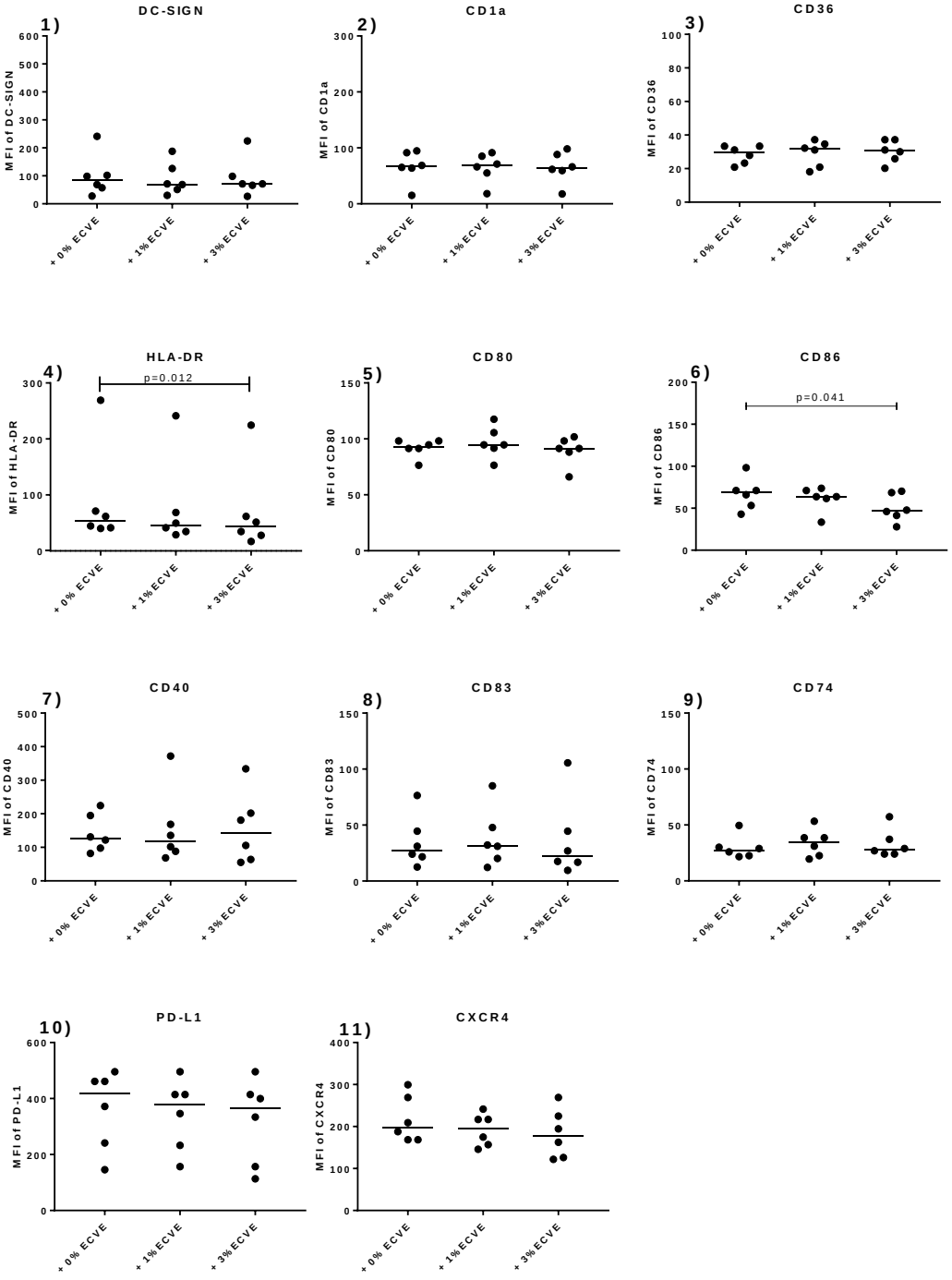
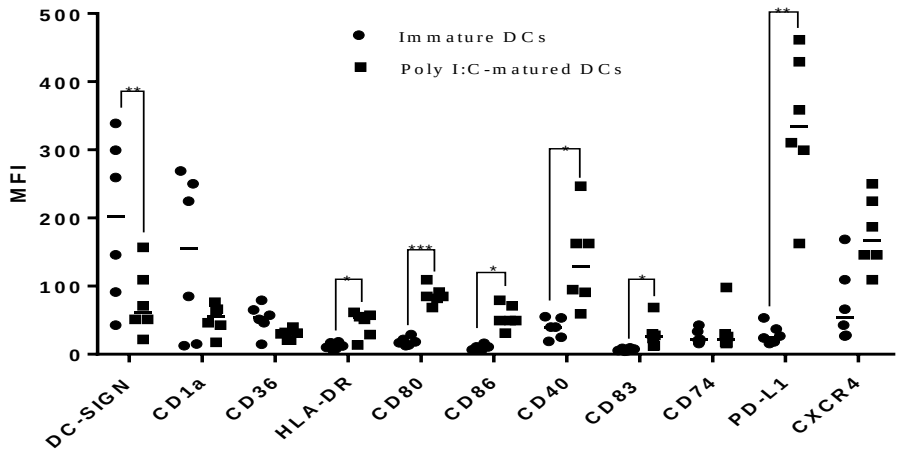
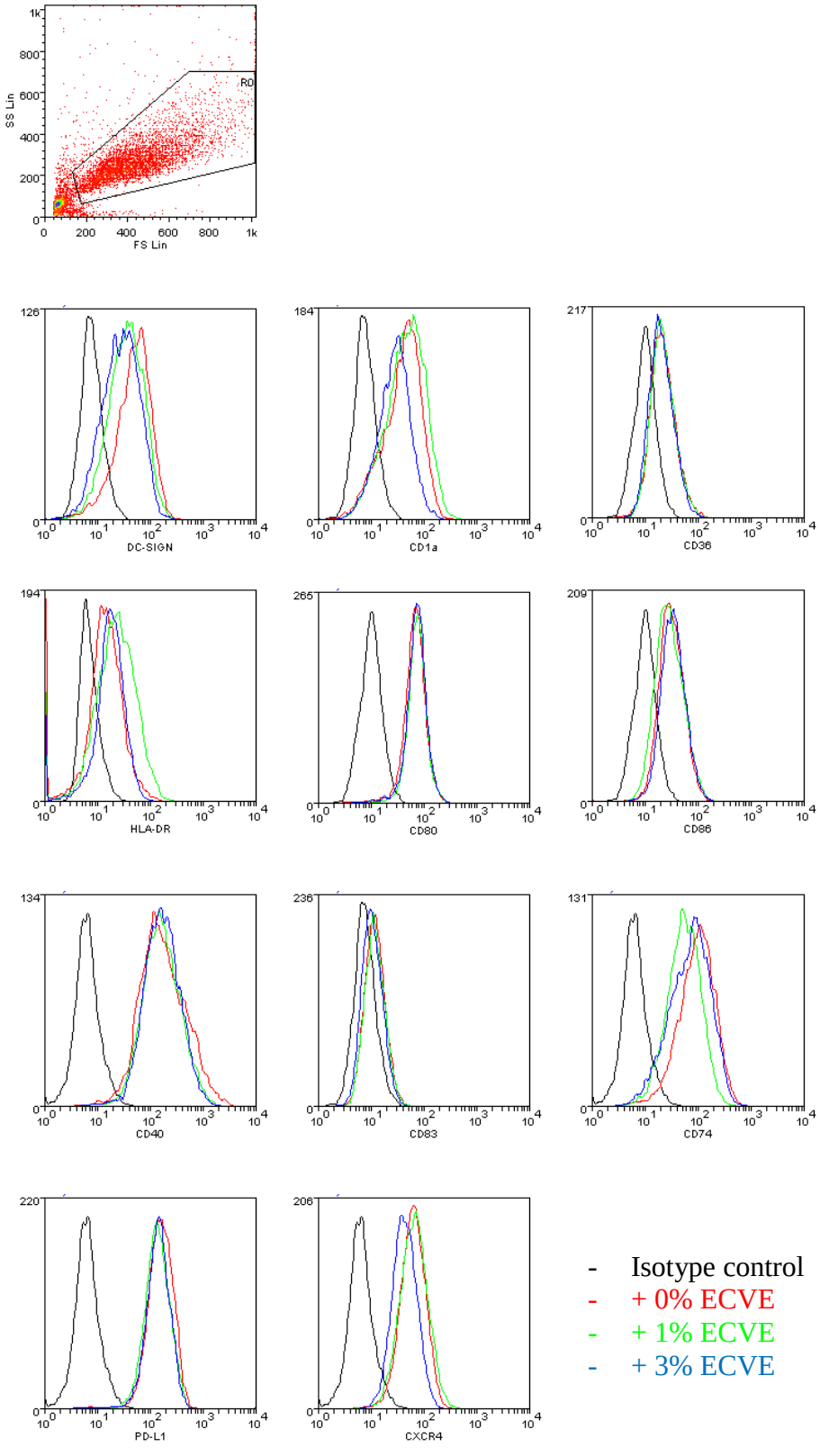

1)
DC-SIGN
MFI of DC-SIGN
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE
2)
CD1a
MFI of CD1a
3)
CD36
MFI of CD36
4)
HLA-DR
p=0.012
MFI of HLA-DR
5)
CD80
MFI of CD80
6)
CD86
p=0.041
MFI of CD86
7)
CD40
MFI of CD40
8)
CD83
MFI of CD83
9)
CD74
MFI of CD74
10)
PD-L1
MFI of PD-L1
11)
CXCR4
MFI of CXCR4

Figure 5.2. 3% ECVE treatment for 24 h reduced the HLA-DR and CD86 expression on LPS-matured DCs. Immature DCs were stimulated with LPS in the presence or absence of ECVE for 24 h. (A) Comparison of MFI value of surface markers between untreated immature and LPS-matured DCs. (B) Representative dot plots and histograms of surface markers on LPS-matured DCs after 24 h with or without ECVE treatment. The DC population was gated and the debris was excluded (called gate R0) and this gate was applied during analysis (C) MFI values of surface markers on LPS-matured DCs after 24 h with or without ECVE treatment. Data is represented here as scatter plots and each dot represents a different individual. The horizontal bars represent the medians of six individual experiments. If data was normally distributed, paired one-way ANOVA was used, otherwise paired Friedman test was used (Fig. 5.2A HLA-DR and CD74, 5.2C1, 5.2C4, 5.2C6, 5.2C7, 5.2C8, 5.2C9). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

A.



B.


SS Lin
FS Lin
R0
DC-SIGN
CD1a
CD36
HLA-DR
CD80
CD86
CD40
CD83
CD74
PD-L1
CXCR4
- Isotype control
- + 0% ECVE
- + 1% ECVE
- + 3% ECVE

C.

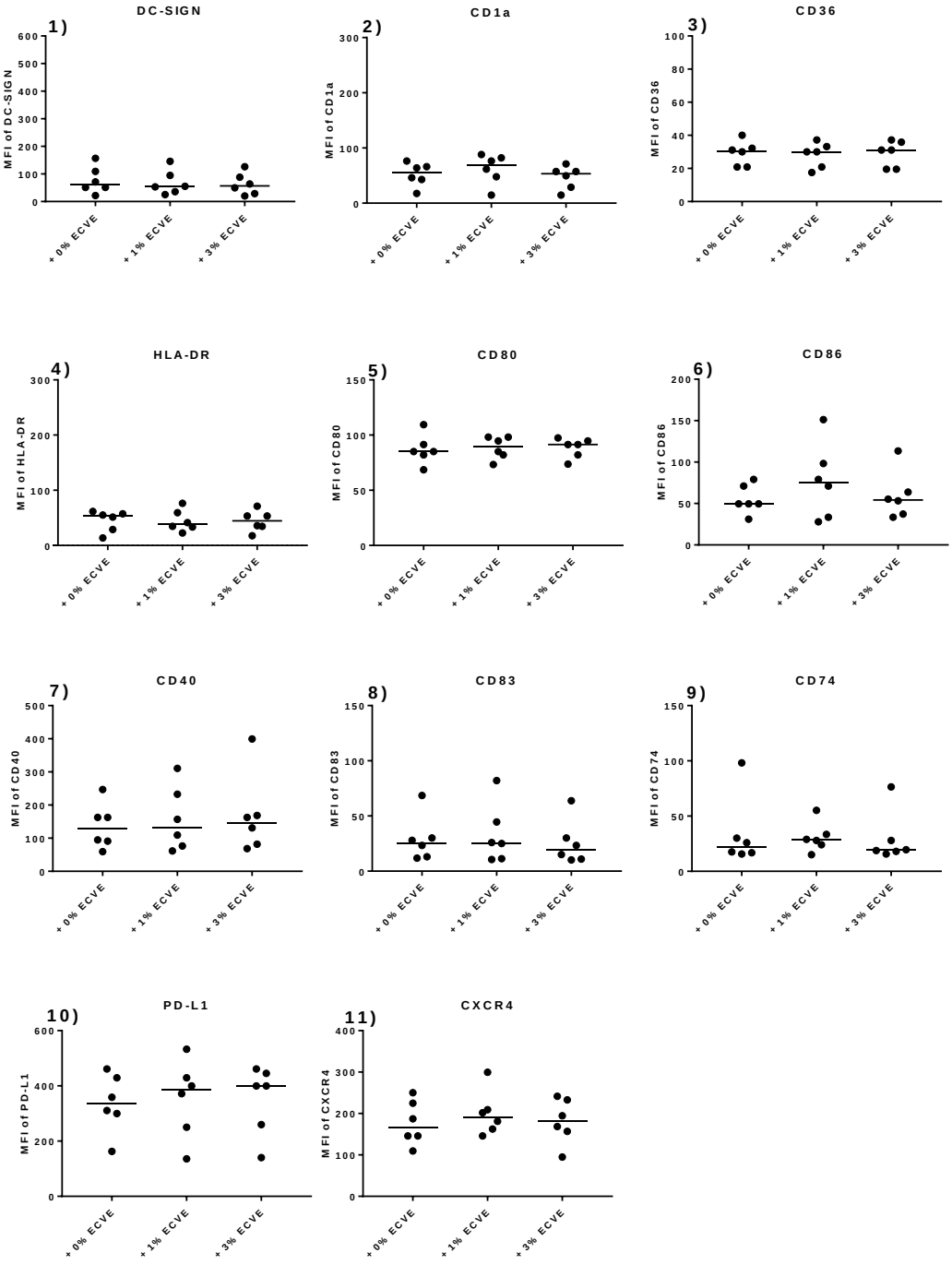

1)
DC-SIGN
MFI of DC-SIGN
2)
CD1a
MFI of CD1a
3)
CD36
MFI of CD36
4)
HLA-DR
MFI of HLA-DR
5)
CD80
MFI of CD80
6)
CD86
MFI of CD86
7)
CD40
MFI of CD40
8)
CD83
MFI of CD83
9)
CD74
MFI of CD74
10)
PD-L1
MFI of PD-L1
11)
CXCR4
MFI of CXCR4
+ 0% ECVE
+ 1% ECVE
+ 3% ECVE

Figure 5.3. 24 h-ECVE treatment did not alter surface marker expression on Poly I:C-matured DCs. Immature DCs were stimulated with Poly I:C in the presence or absence of ECVE treatment for 24 h. (A) Comparison of MFI value of surface markers between untreated immature and Poly I:C -matured DCs. (B) Representative dot plot and histograms of surface markers on Poly I:C-matured DCs after 24 h with or without ECVE treatment. The whole population was gated but the debris were excluded (called gate R0) and this gate was applied during analysis (C) MFI values of surface markers on Poly I:C -matured DCs after 24 h with or without ECVE treatment. Data is represented here as scatter plots and each dot represents a different individual. The horizontal bars represent the medians of six individual experiments. If data was normally distributed, paired one-way ANOVA was used, otherwise paired Friedman test was used (Fig. 5.2A HLA-DR and CD74, 5.3C9).

* $p<0.05$, ** $p<0.01$,

5.3 Discussion

In this chapter, the effects of ECVE on the expression of a broad range of cell surface markers on DCs was investigated. Both immature and mature DCs were treated with or without 1% and 3% ECVE for 24 h and their surface markers were examined by flow cytometry. The current findings showed 3% ECVE inhibited CD83 expression on immature DCs compared to immature DCs without ECVE. Moreover, 3% ECVE reduced the levels of CD86 and HLA-DR on LPS-matured DCs. There was no significant effect of ECVE on the expression of other markers in any conditions.

DC-SIGN (CD209), which is expressed at higher levels by immature than by mature DCs, recognizes mannose-containing carbohydrates of pathogens, leading to phagocytosis. In the present study, decreased expression of DC-SIGN was observed after LPS or Poly I:C stimulation and ECVE did not alter DC-SIGN expression by either immature DCs or mature DCs, consistent with the findings in Chapter 3 using MoDCs generated from anonymous buffy coats purchased from the Blood Transfusion Service.

CD1a is a type 1 CD1 membrane protein that can be used as a marker of human DCs in their early development [233]. CD1a is an MHC class I-like molecule whose function is to capture and present self-derived or pathogen-derived lipids to activate T cells [233,234]. As humectants of E-liquids could contain lipids, it was interesting to investigate whether ECVE exposure can alter the expression of CD1a of DCs thereby affecting presentation of lipids coming from E-liquid. In addition to lipid presentation, CD1a+ DCs are able to produce high amounts of IL-12 upon simulation, suggesting their association with Th1 responses [235,236]. It has been reported that conventional cigarette smokers had increased levels of CD1a on mDCs in BALF [237,238] while nicotine inhibited CD1a expression on MoDCs [239]. However, no significant difference of CD1a expression was found on either immature or mature DCs after ECVE exposure in the current study.

CD36 functions as a scavenger receptor that participates in phagocytosis of apoptotic cells in corporation with $\alpha_v\beta_3$ and $\alpha_v\beta_5$,and cross presentation to cytotoxic T cells [240,241]. ECVE did not affect CD36 expression on either immature or mature DCs. However, others reported that nicotine decreased the level of CD36 on MoDCs but

increased phagocytosis of apoptotic cells [239]. This suggests the involvement of other receptors such, as $\alpha_v\beta_3$ and $\alpha_v\beta_5$, in phagocytosis.

HLA-DR, a MHC II molecule, is constitutively expressed on APCs. HLA-DR binds to peptides generated in intracellular vesicles of APCs and then presents peptides to $CD4^+$ T cells through interaction with the T cell antigen receptor, thereby eliciting T cell responses. This process is known as 'signal 1' for DC-T cell interaction. In the present study it was found that ECVE did not have effects on HLA-DR expression on immature DCs. The expression of HLA-DR was upregulated in both LPS- and Poly I:C-matured DCs, compared to immature DCs. However, 3% ECVE suppressed HLA-DR expression on LPS-matured DCs. These results contrast with a previous study in which no difference in HLA-DR expression on immature and LPS-matured MoDCs was observed when they were treated with CSE for 24 h [242]. Reduction of HLA-DR was also reported in murine lung DCs [150] and bone-derived DCs [243] after long-term CS exposure. However, short-term exposure increased the levels of HLA-DR on murine bone-derived DCs [243].

The co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) deliver the 'signal 2' to T cells, which is required for their activation via the binding to CD28 on T cells. Upregulation of co-stimulatory molecule levels is characteristic of mature DCs, as seen in the current study when stimulating immature DCs with LPS or Poly I:C. ECVE did not affect the levels of CD80 and CD86 on immature DCs, consistent with findings using CS and nicotine. However, both CD80 and CD86 were decreased by CS on LPS-matured DC [242]. Interestingly, 3% ECVE attenuated CD86 expression on LPS-mature DCs, but not CD80 expression. Although both CD80 and CD86 are recognized

as co-stimulatory molecules, there are some differences between their structure and function. CD80 is a bivalent dimer whereas CD86 is a bivalent monomer [244]. The expression of CD80 is more abundant than CD86 on immature DCs while CD86 expression increases more rapidly and strongly than CD80 in response to stimuli causing DC maturation [244]. Although both CD80 and CD86 can bind to CD28, CD80 also can interact with CTLA-4 [244] which inhibits T cell activation and proliferation [245]. Compared to CD80, CD86 is a more important co-stimulatory molecule for T cell activation [246] and blockade of CD86 inhibited T cell proliferation in MLR [153,247], whereas blockade of CD80 promoted it [153]. In the current study, levels of CD86 but not CD80 was decreased by 3% ECVE in LPS-matured DCs suggesting a potential role of ECVE in attenuating T cell proliferation.

CD40 is the member of tumour necrosis factor receptor superfamily and is expressed on DCs, B cells, monocytes, macrophages and other non-hematopoietic cells [248]. CD40 also functions as a costimulatory molecule on DCs via engagement of CD40L on T cells and levels of CD40 increase during DC maturation, which helps to trigger T cell activation and differentiation. CD40 on DCs stimulates kinase activation, including the MAPK and NF-κB pathways, resulting in cytokine and chemokine production, induction of co-stimulatory molecules, survival and prolonged antigen presentation [249,250]. Neither CS nor nicotine changed CD40 expression on immature MoDCs, whereas CD40 expression was suppressed on LPS-matured MoDCs, suggesting the effects of CSE on DCs may be related to their state of activation [242]. However, we did not observe any changes of CD40 expression in

either immature or mature DCs after ECVE exposure. No differences in CD40 was found in murine lung DCs after CS exposure [150].

CD83, which is an established maturation marker for DCs, binds to CD83L on T cells thereby enhancing T cell activation. The level of CD83 is low in immature DCs but significantly increased after stimulation, which is consistent with our findings in both immature and LPS- and Poly I:C-matured DCs. CD83 expression in immature DCs was downregulated by 3% ECVE exposure for 24 h in the current study. A significant reduction of CD83 in mDCs in BALF was found in current smokers with COPD but not in former smokers with COPD [238]; a similar scenario was also found in current smokers regardless of the presence of COPD [251], raising the possibility that E-cigarettes might inhibit DC maturation as do conventional cigarettes. Conversely, an upregulation of CD83 was found using murine bone-derived DCs exposed briefly to CS, indicating CS might drive DC maturation [243]. In contrast, acute 4-h CS exposure did not alter CD83 expression on human mDCs in BALF in vivo [252]. It is well known that CS is involved in the pathogenesis of COPD by initiating excessive inflammatory processes which recruit immune cells into airways and alveoli. Indeed, the total number of CD83+ cells was increased in lung biopsies from COPD patients [226] and increased CD83 expression in both lung mDC1 and mDC2 was correlated with the disease severity [157]. In contrast, another study found that the number of DCs expressing CD83 was lower in the small airways and alveoli in patients with COPD, compared to smokers without COPD and healthy never-smokers. Also, there was no difference between current and former smokers with COPD [253], indicating

that COPD per se was associated with impairment of DC maturation. There are thus contrasting results on the effects of COPD and smoking per se on CD83 expression.

CD74 (invariant chain; Ii) associates with the groove of newly synthesized MHC class II molecules in the endoplasmic reticulum to prevent the binding of peptides from the endogenous processing pathway or misfolded proteins. Ii directs MHC class II molecules to the cytosol and then binds to endosomes containing endocytosed and degraded proteins, followed by Ii cleavage by proteases called cathepsins. After the release of Ii fragment, MHC class II molecules bind to antigenic peptides and migrate to the cell surface. Lack of Ii causes retention of MHC class II molecules in the endoplasmic reticulum with misfolded proteins [254]. In addition to its role in antigen processing, CD74 regulates migration of DCs in time and space [255]. We found the levels of CD74 on immature and mature DCs were comparable before and after ECVE exposure, suggesting that ECVE did not affect CD74-dependent processes.

PD-L1 belongs to the B7 family of co-stimulatory molecules and its engagement with PD-1 on T cell induces inhibitory signals that attenuate T cell expansion [256]. PD-L1/PD-1 interactions also promote regulatory T cell differentiation and function [257] but inhibit self-reactive T cell activation [258]. Expression of PD-L1 was upregulated upon stimulation by LPS or Poly I:C but not affected by ECVE on either immature or mature DCs, suggesting ECVE did not alter the role of PD-L1 in regulating the balance between stimulatory and inhibitory signals needed for effective immunity and the maintenance of self-tolerance.

CXCR4 is a chemokine receptor for its ligand CXCL12 and the engagement of CXCR4 and CXCL12 promotes DC migration into regional lymph nodes [259],

maturation, survival and DC-dependent antigen-specific T cell proliferation [260]. The expression of CXCR4 has been reported to be upregulated on human MoDCs by LPS, TNF-α or CD40L [261]. A significant reduction of CXCR4 expression on bone marrow mesenchymal stem cells was detected in COPD [262] and acute exacerbation of COPD resulted in recruitment of blood fibrocytes via a CXCR4-dependent pathway [263]. In the current study, the level of CXCR4 was elevated on LPS-matured DCs in comparison to immature DCs. However, CXCR4 expression was not alter by ECVE in either immature or mature DCs.

The contradictory findings of different studies on the effects of CS on surface markers of DCs might be due to a number of factors, including differences in study participants (e.g. genetic background and socio-economic status), the source of DCs (e.g. lung or blood), types of DCs and patterns of cigarette exposure (e.g. duration and frequency). There may be even more limitations that need to be considered when studying E-cigarettes, such as types of E-cigarette devices and E-liquids used (remembering that there are also thousands of different flavourings available). The use of E-cigarettes varies between individuals, making it difficult to build up a comprehensive system to replicate the diversity of vaping patterns. Thus, the experiments undertaken here can only reflect facets of the overall picture.

Inhibition of DC maturation could diminish T cell activation, in turn altering their ability to respond to infection. Impaired host defence of other cell types discussed in section 1.2.6. and inhibition of DC maturation suggest a possible negative role of E-cigarettes on immune responses to bacterial and viral infections, which are major factors in COPD exacerbations.

5.4 Conclusion

Expression of costimulatory molecules and of the maturation marker CD83 by MoDCs was upregulated upon maturation induced by either LPS or Poly I:C. However, CD83 expression on immature DCs and HLA-DR and CD86 expression on LPS-matured DCs was suppressed by 3% ECVE, suggesting that ECVE attenuated not only DC maturation but also the potential for T cell activation. In addition, 3% ECVE inhibited surface makers related to maturation, without altering antigen uptake-related surface markers. In conclusion, higher concentration of ECVE had stronger inhibitory effects on DC maturation.

Chapter 6 ECVE induced pro-inflammatory cytokine production by DCs

6.1 Introduction

After capturing and processing pathogens, immature DCs undergo a complex process of maturation and undertake their communication with T cells. Three signals induced by DCs are required for T cell activation and polarization: recognition by the TCR of antigenic peptides presented by MHC molecules, co-stimulatory molecule interaction and cytokine production. Cytokines secreted against invading pathogens may affect the DC balance between tolerance and immunity, which could contribute to the occurrence of inflammatory and autoimmune diseases [264].

The influence of ECVE on the first and second signals delivered by DCs has been evaluated in the previous chapter by analysing the surface markers on DCs, including HLA-DR and co-stimulatory molecules. Here, the effects of ECVE on cytokines produced by DCs, the third signal, were investigated. In the experiments reported in this chapter, immature and LPS/Poly I:C-matured DCs were generated from fresh venous blood of healthy never-smokers and treated with or without ECVE for 24 h, followed by collection of supernatants. ELISA then was performed to measure the levels of the cytokines IL-6, IL-8, IL-10, IL-12 and TNF-α, which are commonly produced in response to a wide range of environmental conditions that may affect the downstream adaptive immunity.

6.2 Results

Details of the cytokine ELISAs employed are given in section 2.91. Figure 6.1 shows the dose-titration curves for the cytokine standards IL-6, IL-8, IL-10, IL-12 and TNF-α, demonstrating validation of the ELISA. Figures 6.2 and Figure 6.3 present the concentrations of these five cytokines in the culture supernatants of LPS- or Poly I:C-matured DCs treated with or without ECVE for 24 hours, compared to immature DCs. The DCs were cultured at a concentration of $5x10^5$ cells/ml in 24 well tissue culture plates (i.e. $5x10^5$ cells per well). Both LPS/Poly I:C-matured DCs without ECVE produced significantly higher levels of IL-6, IL-8, IL-10, IL-12 and TNF-α than immature DCs without ECVE . Moreover, 1% ECVE significantly induced the secretion of IL-6 in LPS-matured DCs ($p=0.017$) while the levels of the other four cytokines were comparable, in comparison to LPS-matured DCs without ECVE (Fig 6.2). However, no alterations of all cytokines caused by 24 h-ECVE treatment was observed in Poly I:C-matured DCs (Fig 6.3).

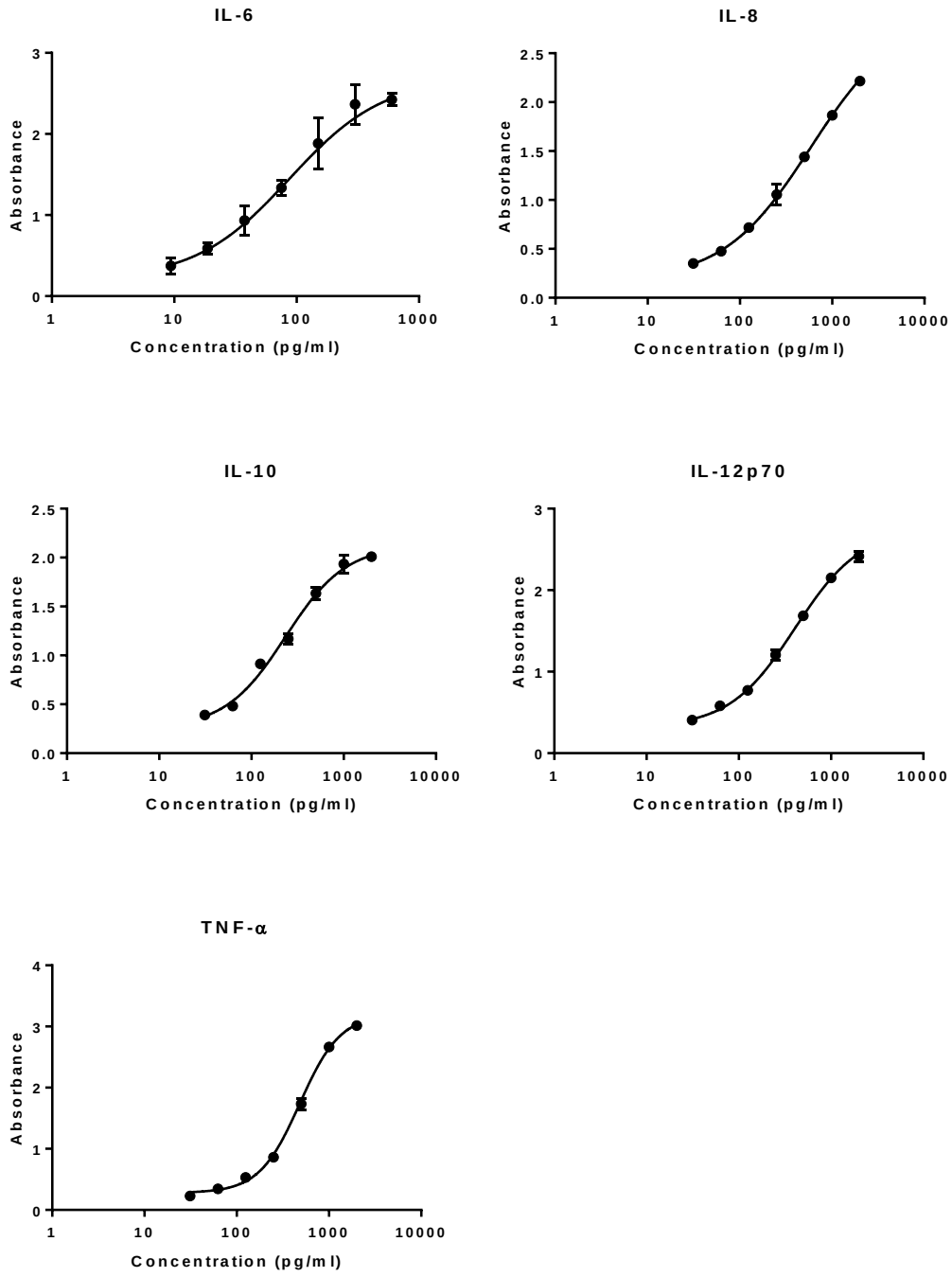


Figure 6.1. Standard curves of cytokines. A standard curve of 7-fold serial dilutions of each of the cytokines to be detected was generated.

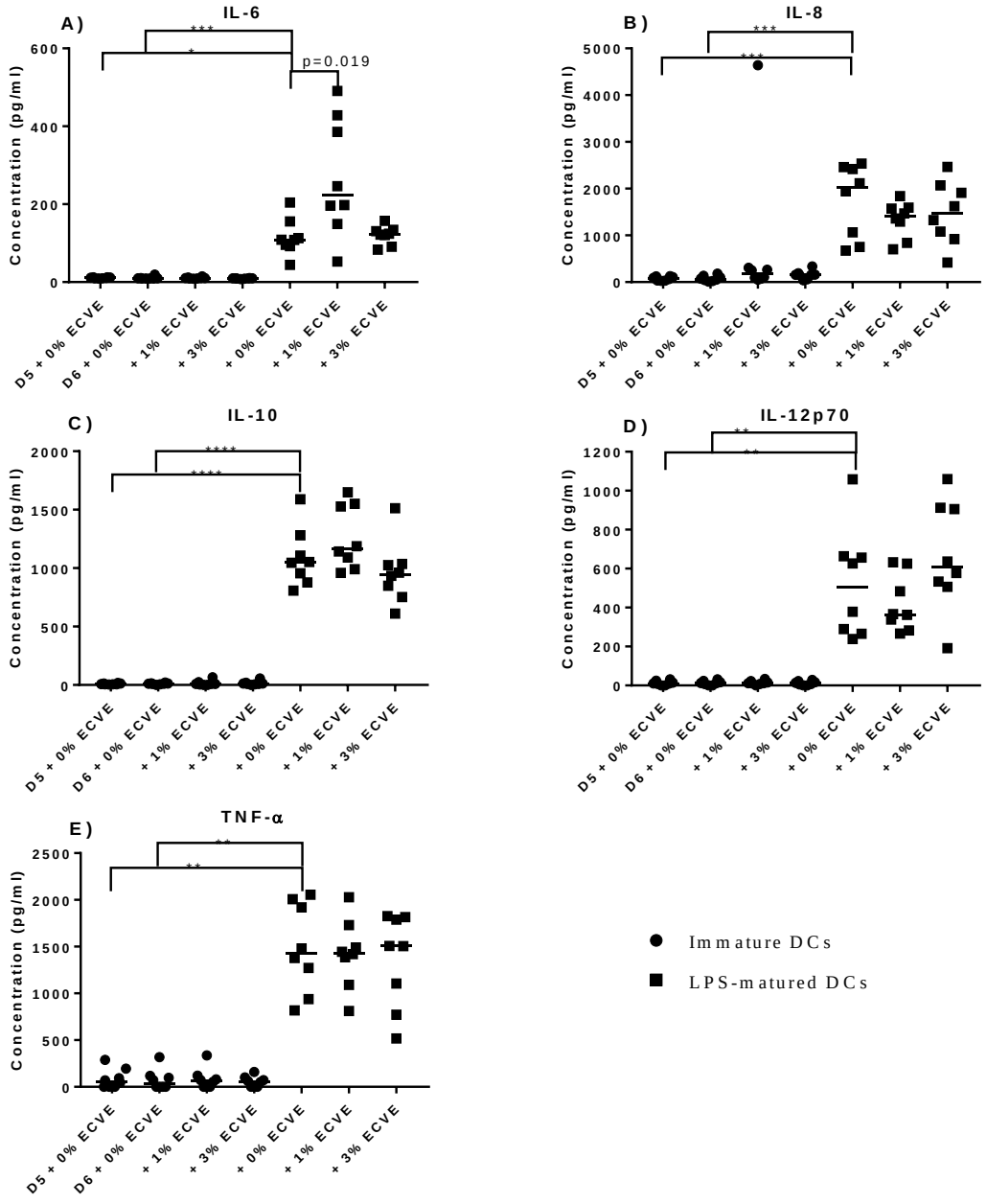


Figure 6.2. 1% ECVE treatment for 24 h induced IL-6 production by LPS-matured DCs. The supernatants of immature and LPS-matured DCs, in the absence or presence of ECVE for 24 hours, were collected and measured by ELISA to determine levels of IL-6, IL-8, IL-10, IL-12 and TNF-α. Data is presented as scatter

plots of eight independent experiments and their medians. If data is normally distributed, paired one-way ANOVA was used, otherwise paired Friedman test was used (Fig 6.2A immature vs mature, immature + 0% ECVE vs 1/3% ECVE, 6.2B , immature + 0% ECVE vs 1/3% ECVE, 6.2C immature + 0% ECVE vs 1/3% ECVE, 6.2E immature vs mature, immature + 0% ECVE vs 1/3% ECVE) * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $P<0.0001$

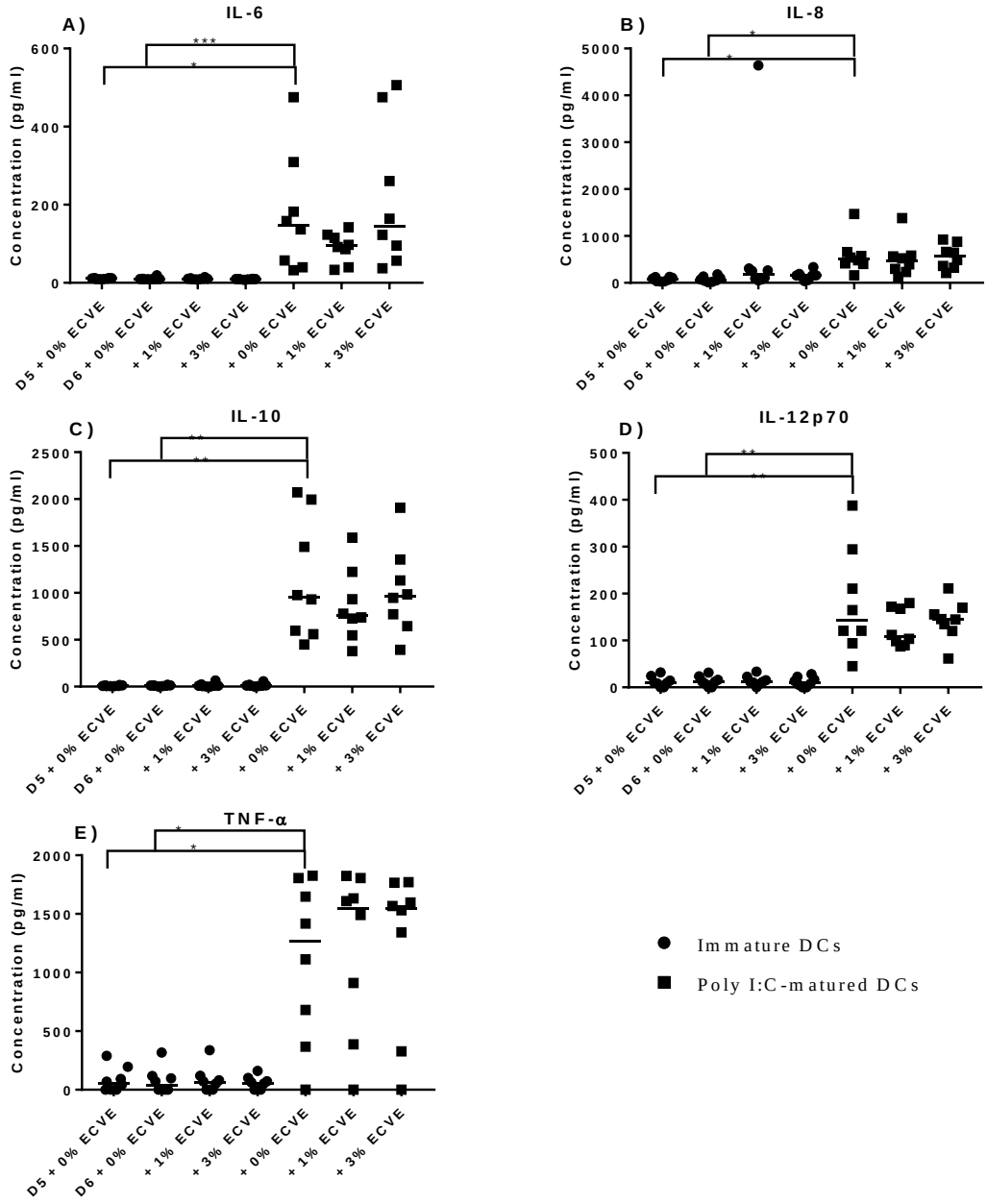


Figure 6.3. 24 h-ECVE treatment did not alter cytokine production by Poly I:C-matured DCs . The supernatants of immature and Poly I:C-matured DCs in the absence or presence of ECVE for 24 hours were collected and applied on ELISA in

two replicates to evaluate levels of IL-6, IL-8, IL-10, IL-12 and TNF-α. Data is presented as scatter plots of eight independent experiments and their median. If data is normal distributed, One-way ANOVA was used, otherwise Friedman test was used (Fig 6.3A. immature vs mature, 6.3B immature vs mature, immature + 0% ECVE vs 1/3% ECVE, 6.3C immature vs mature, 6.3E immature vs mature) * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $P<0.0001$

6.3 Discussion

Depending on the signals received, DCs secrete a variety of cytokines that drive downstream immune responses. LPS binds to cell-surface TLR4 that signals via MyD88 or TRIF while Poly I:C binds to endosomal TLR3 that signals via TRIF, leading to activation of the NF-κB and MAPK pathways thereby stimulating inflammatory cytokine production [265]. The aim of this chapter was to examine the effects of ECVE on cytokine production by DCs. Both immature and LPS/Poly I:C-matured DCs were treated with or without 1% and 3% ECVE for 24 h and the concentrations of five cytokines in the culture supernatants were assessed by ELISA. The current study showed that LPS/Poly I:C-matured DCs produced significantly higher concentrations of all these cytokines than immature DCs; furthermore, 1% ECVE treatment significantly elevated IL-6 secretion by LPS-matured DCs. However, there was no significant effects of ECVE on cytokine secretion by Poly I:C-matured DCs in any conditions.

IL-12 produced by DCs mediates differentiation and expansion of Th1 cells while IL-10 promotes Th2 responses by supressing Th1 responses. Activation of Th1 cells produces pro-inflammatory cytokines including IL-2 and IFN-γ, responsible for killing intracellular pathogens and anti-tumour activity. In contrast, Th2 cells produce IL-4, IL-5, IL-10, and IL-13 which are involved in antibody production and eosinophil activation. CSE strongly induce IL-12 production from DCs [243,266], in agreement with findings in BALF of CS-exposed mice [267]. In contrast, a study showed that CSE suppressed IL-12 and induced IL-10 release by LPS-matured MoDCs, suggesting that CSE inhibited Th1 responses but triggered Th-2 responses [242]; this may help to explain why CS is a risk factor for the development of Th-2-mediated diseases like asthma [268] and is associated with defects in host defence against infection and cancer. However, the presence of nicotine decreased the levels of IL-12 and IL-10 produced by LPS-matured MoDCs [269], suggesting an inhibitory effect of nicotine on DCs. This was contradicted in a study showing the capability of nicotine to induce DC activation and cytokine production [198]. In the current study, secretion of IL-12 and IL-10 from DCs was comparable after ECVE treatment compared to untreated DCs consistent with a study using cDCs [181], proposing that ECVE did not alter polarization of CD4+ T cells.

IL-6 is a pleiotropic cytokine involved in various biological activities including immune regulation, hematopoiesis, inflammation, and oncogenesis. IL-6 not only acts as a pro-inflammatory cytokine that induces acute-phase protein synthesis in response to pathogens, but also promotes the differentiation of B cells into plasma cells, thereby increasing antibody production. In addition, IL-6 is implicated in the differentiation of

Th17 cells from naïve T cells, together with TGF-β. Th17 cells, which secrete the pro-inflammatory cytokines IL-17 and IL-21, promote host defence against infection by bacteria and fungi by recruiting neutrophils and macrophages to infected tissues and inducing other pro-inflammatory cytokines; they are also linked to the development of autoimmune diseases [270]. Several studies show that CS enhances IL-6 production by various types of cells including DCs [243,271,272], and in BALF of mice [267]. In the current study, 1% ECVE significantly stimulated IL-6 secretion by LPS-matured DCs, suggesting E-cigarette use may promote an inflammatory environment in humans.

TNF-α, can be produced by a variety of cell types including neutrophils, macrophages, T cells, monocytes and DCs. It is a pro-inflammatory cytokine that also contributes to the acute phase reaction. Large amounts of TNF are released in response to inflammatory stimuli, resulting in the activation of vascular endothelium and increased vascular permeability. TNF-α secretion in lungs increases the levels of cytokines, chemokines and adhesion molecules, leading to activation and recruitment of neutrophils and macrophages and promoting lung tissue destruction. Indeed, levels of TNF-α are higher in smokers, with or without COPD, compared to non-smokers [273,274], which is consistent with a CS-exposed mouse model [275]. In vitro, CSE enhanced the secretion of TNF-α from DCs [243] and macrophages [276] but the secretion of TNF-α by nicotine-treated DCs was diminished [269], suggesting that nicotine could exert immunosuppressive effects on DCs. ECVE did not alter TNF-α production by DCs in response to either LPS or Poly I:C.

IL-8 (CXCL8) is a pro-inflammatory chemokine that recruits leukocytes including neutrophils, basophils and T cells to sites of infection or tissue injury. Increases in IL-8 and neutrophils have been found in patients with of COPD [277,278] and they may form a positive feedback loop in the disease. CSE significantly induced IL-8 production by plasmacytoid dendritic cells [199] and LPS-matured MoDCs due to oxidative stress [266], suggesting a role for oxidative stress in CS-mediated neutrophilic airway inflammation. No changes in levels of IL-8 secreted by immature and mature DCs exposed to ECVE were observed in the current study, but ECVE has been found to augment the release of IL-8 from neutrophils [279]; therefore, it remains possible that ECVE may interfere the production of IL-8 by other cell types.

6.4 Conclusion

DCs produced high levels of IL-6, IL-8, IL-10, IL-12 and TNF-α upon LPS or Poly I:C stimulation. 1% ECVE increased the level of IL-6 secreted by LPS-matured DCs, suggesting that E-cigarette use may induce inflammatory environments in humans and could be a potential factor for development of inflammatory and autoimmune diseases in long term.

Chapter 7 ECVE-treated DCs promote the proliferation of T cells

7.1 Introduction

After taking up antigens, DCs mature, migrate to lymph nodes and present antigen fragments to naïve T cells thereby activating adaptive immune responses: this demonstrates the critical role of DCs in linking innate and adaptive immunity [280]. DCs present exogenous antigens via MHC II, leading to activation of CD4+ T cells. CD4+ T cells then differentiate into effector cells which include: Th1 cells, which activate macrophages to kill intra-cellular pathogens; Th2 cells, which activate B cells to produce antibodies. Polarization of CD4+ T cells depends upon cytokines that are present at the time of T-cell activation [281]. DCs also cross-present exogenous antigens via MHC I to activate CD8+ T cells and their cytotoxic reaction [282].

In Chapter 5, 3% (but not 1%) ECVE exposure for 24 hours decreased the levels of HLA-DR ($p=0.012$) and CD86 ($p=0.041$) expression by LPS-matured DCs (see Fig 5.2). These molecules are associated with DC maturation, antigen presentation and T cell activation. Conversely, in Chapter 4, ECVE treatment tended to upregulate pro-inflammatory signalling molecules in DCs, which might therefore be associated with enhanced antigen presenting activity. Therefore, in this part of the study, whether ECVE further alters the communication between DCs and T cells was investigated using an allogeneic mixed lymphocyte reaction (MLR) assay as a model system. The MLR assay is a well-known *in vitro* tool to study T-cell activation and proliferation. The allogenic MLR is based on a proportion of the responder T cells expressing TCRs that make functional interactions with allogenic HLA/antigen peptide complexes

displayed by allogeneic DCs, which also provide costimulatory signals. The method of detecting T cell proliferation has been described in section 2.8. Briefly, to examine the proliferation of T cells, CD3+ T cell were stained with CFSE (which enables labelled cells to be followed through multiple rounds of proliferation by dye dilution) and then co-cultured with allogeneic DCs (which had been matured with LPS in the absence or presence of ECVE for 24 h) in different DC/T cell ratios for 5 days. After co-culturing, cells were stained with CD3, CD4 and CD8 antibodies to discriminate CD3+CD4+ or CD3+CD8+ T cells. The cell phenotypes and the expression of CFSE were determined using an FC500 flow cytometer.

7.2 Results

Cells were single-stained for the expression of CD3, CD4 or CD8 after co-culturing with allogeneic DCs for five days. Figure 7.1 showed the dot plots of the CD3+, CD4+ and CD8+ populations. Subsequently, co-staining meant that the gate of CD3+CD4+ or CD3+CD8+ cells could be applied when analysing CFSE staining to investigate the proliferation of CD4+ and CD8+ T cells.

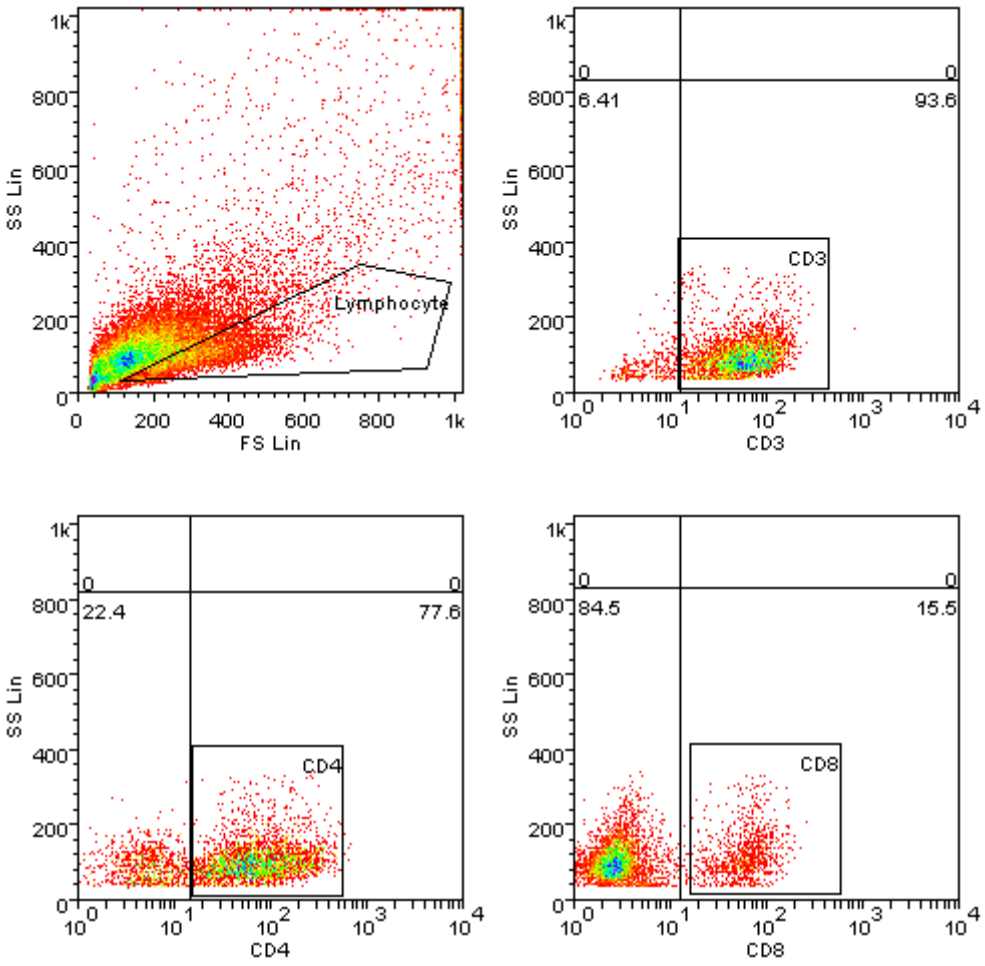
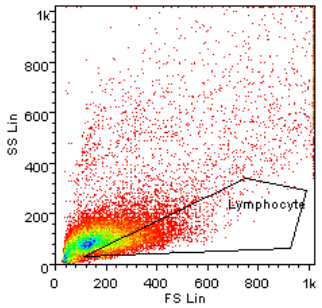


Figure 7.1. Dot plots of lymphocytes and their expression of CD3, CD4 and CD8. After co-culturing with allogeneic DCs for five days with DC/T cell rations at 1:10, cells were single stained with anti-CD3-ECD, anti-CD4-PE-Vio770 or anti-CD8-PE to discriminate the population expressing these markers. The lymphocyte population was gated. This is an example of three independent replicates analysed using Weasel software.

MLR was performed using CD3+ T cells co-cultured with allogeneic DCs (i.e. the T cells and DCs were from different, unrelated healthy donors). The DCs has been matured by treatment with LPS for 24 h in the absence or presence of 1% or 3% ECVE. The DCs and T cells were cultured for 5 days at DC:T cell ratios of 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 (see section 2.8 in Chapter 2 for details). The experiment was repeated three times with different pairs of allogeneic donors for each experiment. Figure 7.2 and figure 7.3 show the dot plot and histograms of CFSE staining of CD4+ and CD8+ T cells, respectively. The percentages of T cells proliferating in the three independent experiments are presented in Figure 7.4. Generally, the percentage of T cell proliferation decreased with increasing T cell/DC ratios. This varied between the three experiments, possibly reflecting the extent of the HLA allogeneic differences between the stimulator and responder cells. Moreover, ECVE-treated DCs stimulated more proliferation of T cells than untreated DCs, particularly at the higher T cell/DC ratios, and this was more apparent for CD4+ T cells than for CD8+ T cells. In addition, the LPS-matured DCs treated with 1% ECVE induced more T cell proliferation compared to those treated with 3% ECVE (Fig 7.4).

A.



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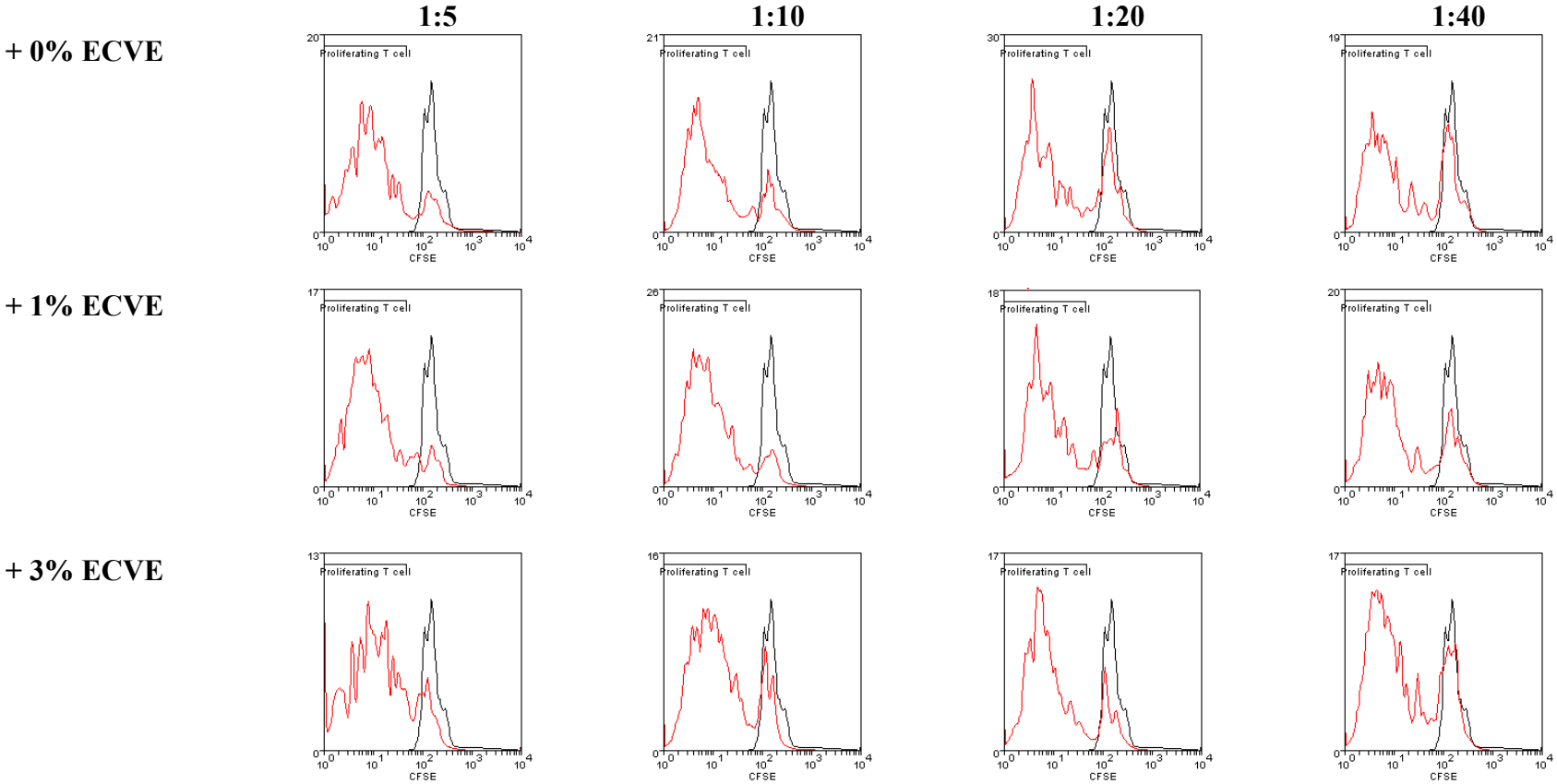

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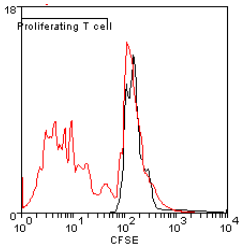
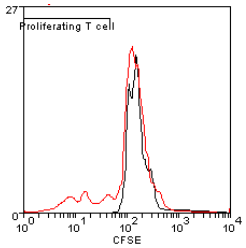
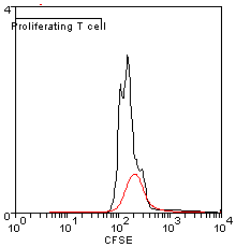
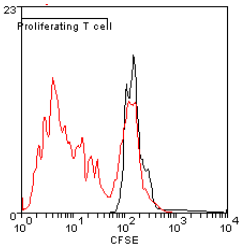
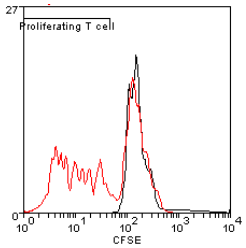
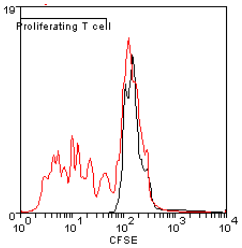
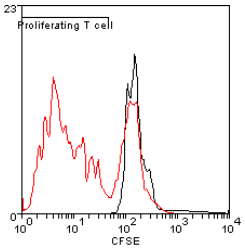
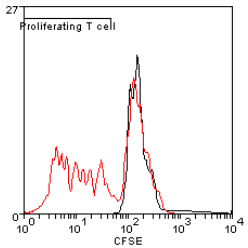
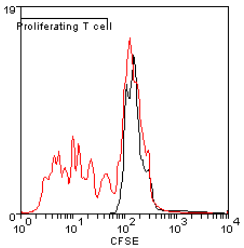
+ 0% ECVE			
+ 1% ECVE			
+ 3% ECVE			

Figure 7.2. ECVE-treated DCs promoted the proliferation of CD3+CD4+ T cells.

CD3+ T cells were co-cultured with allogeneic DCs that had been matured by treatment with LPS for 24 hours in the absence or presence of 1% or 3% ECVE. The DCs and T cells were cultured at different ratios for five days. Dot plot and histograms of CFSE staining of CD3+CD4+ T cells are shown. The dot plot represents the co-culture of CD3+ cells with DCs and the lymphocyte population was gated The black line represents the CFSE expression of CD3+CD4+ T cells cultured alone (i.e. without DCs) for 5 days. The red line represents CFSE expression of CD3+CD4+ T cells cultured with DCs for 5 days. This is an example representative of three independent experiments.

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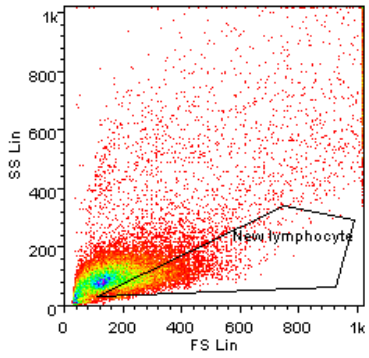

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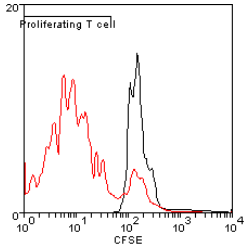
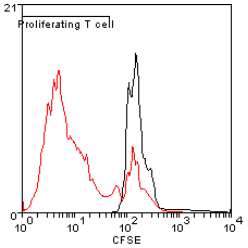
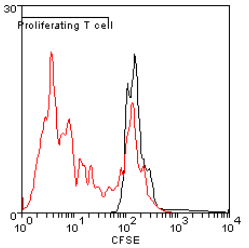
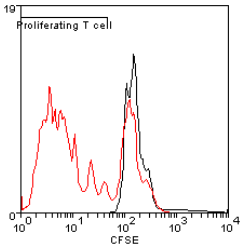
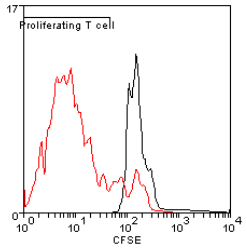
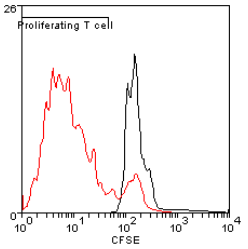
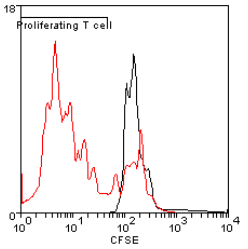
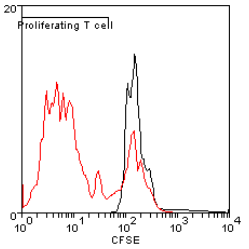
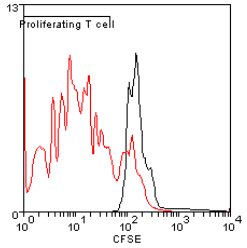
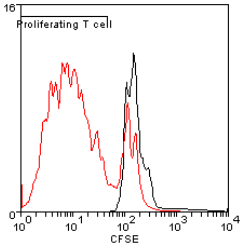
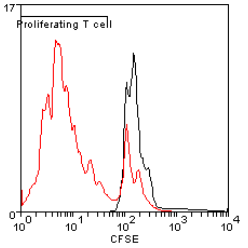
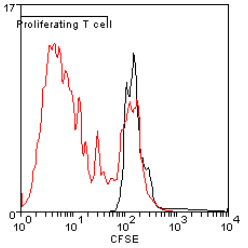
+ 0% ECVE				
+ 1% ECVE				
+ 3% ECVE				

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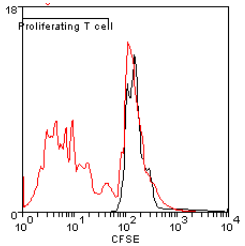
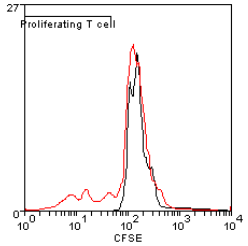
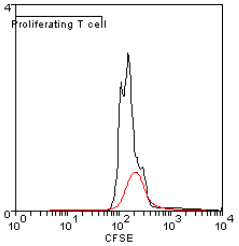
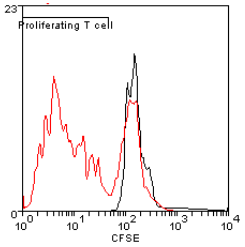
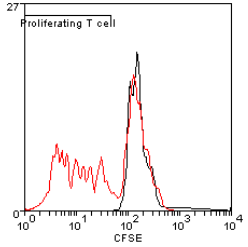
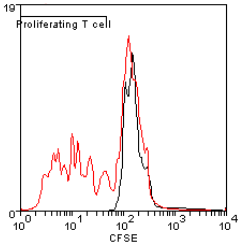
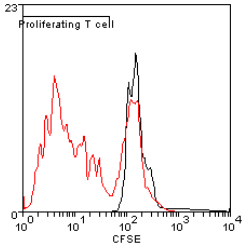
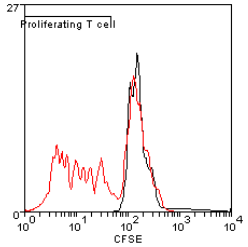
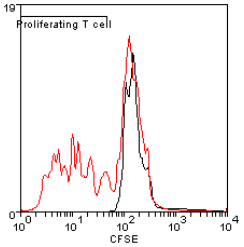
+ 0% ECVE			
+ 1% ECVE			
+ 3% ECVE			

Figure 7.3. ECVE-treated DCs promoted the proliferation of CD3+CD8+ T cells.

CD3+ T cells were co-cultured with allogeneic DCs that had been matured by treatment with LPS for 24 hours in the absence or presence of 1% or 3% ECVE. The DCs and T cells were cultured at different ratios for five days. Dot plot and histograms of CFSE staining of CD3+CD8+ T cells are shown. The dot plot represents the co-culture of CD3+ cells with DCs and the lymphocyte population was gated. The black line represents the CFSE expression of CD3+CD8+ T cells cultured alone (i.e. without DCs) for 5 days. The red line represents CFSE expression of CD3+CD8+ T cells cultured with DCs for 5 days. This is an example representative of three independent experiments.

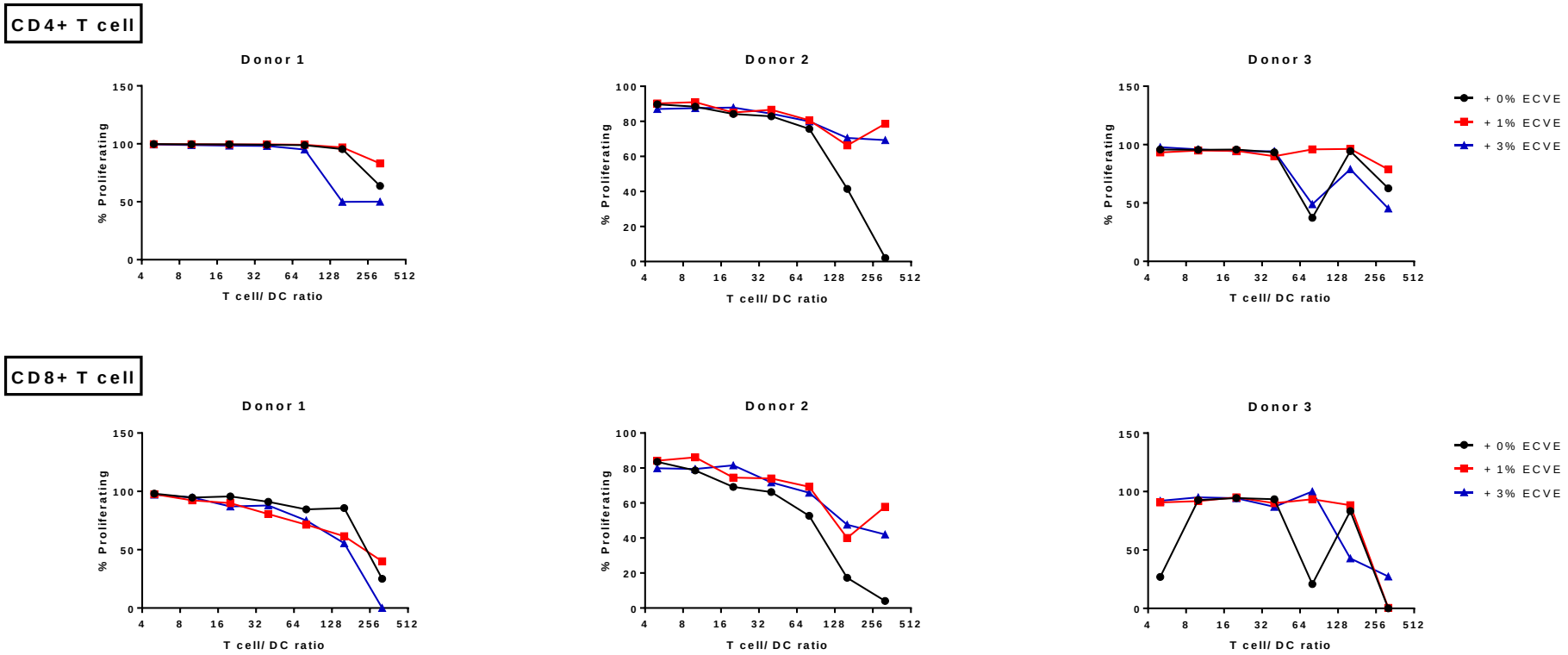


Figure 7.4. Proliferation of CD4+ and CD8+ T cells in three independent experiments using MLR assay.

7.3 Discussion

24 h-ECVE treatment promoted the ability of DCs to stimulate T cell proliferation in an allogenic MLR assay, particularly at the higher T cell/DC ratios, and this was more evident for CD4+ T cells than CD8+ T cells. Therefore, the present chapter provides evidence that ECVE can modulate not only the maturation, but also function of DCs.

DCs are potent APCs that express MHC class I/II and costimulatory molecules, and that activate T cells both in vivo and in vitro. Activation of T cells generates a variety of immune responses, not only to eliminate pathogens, but also to maintain immune homeostasis. According to our previous findings (Chapter 4), 3% ECVE treatment significantly unregulated pro-inflammatory signalling pathways in LPS-treated maturing and matured DCs, (1% ECVE also upregulated 13/29 signalling molecules in LPS-matured DCs, but this did not reach statistical significance). Conversely, as reported in Chapter 5 that 3% (but not 1%) ECVE treatment significantly reduced the levels of HLA-DR and CD86 in LPS-matured DCs – molecules which are critical in maturation and antigen presentation ability of DCs. Therefore, it was hypothesised that the effects of ECVE treatment might both potentiate and suppress the T cell-stimulatory activity of DCs, and that the balance of these effects might depend on ECVE concentration (1% versus 3% in our experiments). The allogeneic MLR assay is commonly used for the evaluation of DC function in inducing proliferation of T cells in vitro by co-culturing responder T cells with stimulator DCs from allogeneic donors. Here LPS-matured DCs were treated with different concentrations of ECVE for 24 h followed by co-culturing with allogenic T cells for 5 days.

Other research found that the proliferation of allogenic T cells was suppressed by 2% CSE, and this effect was not exclusively mediated by nicotine [242]. However, another study reported diminished capacity of DCs to stimulate T cells when nicotine was present during the co-culture for 5 days [269], which was consistent with significant impairment of T cell proliferation in animals treated chronically with nicotine [283]. Furthermore, CSE-treated cDCs inhibited proliferation of CD4+ T cells in vitro [181], which was in agreement with findings in vivo [284]. Similar findings were observed in LPS-stimulated murine bone-marrow derived DCs treated with CSE for 10 days [243] and CS-exposed animals in vivo [150]. The decrease in CD4+ T cell proliferation indicates that CS exerts suppressive effects on immune responses, thereby promoting the susceptibility of tobacco smokers to infections, which are critical factors in COPD exacerbations.

Increasing of total CD3+ cells includes both CD4+ and CD8+ T cells occured in the alveolar walls of smokers with emphysema [285] and with COPD [286], and abnormal T cell numbers has also been detected in autoimmune diseases [287,288]. Therefore, the adverse effects on human health caused by ECVE could not be eliminated. On the other hand, CSE significantly induced the cDC-mediated proliferation of CD8+ T cells but decreased proliferation of CD4+ T cells [181] which may explain the reduction of CD4+ T cells, and an increase in CD8+ cells with a lower CD4+/CD8+ T cell ratio in BAL in smokers [289]. CD8+ T cells are cytotoxic to cells infected by virus and release granzyme B and perforins that promote cell apoptosis and destruction of lung parenchyma, leading to the development of emphysema [290]. Increasing number of CD8+ T cells is predominant role in pathology of COPD and related to airflow limitation which reflects progression of the disease [284].

In the present study, three independent MLR experiments using different allogeneic pairs of LPS-matured stimulator DCs and responder T cells all indicated that ECVE-treated DCs stimulated more proliferation of T cells than untreated DCs, particularly at the higher T cell/DC ratios. (This was more evident for the proliferation of CD4+ T cell than for CD8+ T cells.) This is consistent with the activating effects of ECVE on immature and LPS-treated DC pro-inflammatory signalling molecule expression reported in Chapter 4. The precise level of the ECVE effect differs between the three experiments – for example, it is strongest in Experiment 2; this may be dependent on the nature and extent of the HLA allogeneic differences between the stimulator DCs and the responder T cells.

It is also apparent that the 1% ECVE-treated DCs were better stimulators than those treated with 3% ECVE – this is most apprent in Experiment 3. This may reflect the reduction of HLA-DR and CD86 by 3% ECVE in LPS-matured DCs. Indeed, consistent with this, there is evidence that CD86 is more important in the activation of pro-inflammatory T cells than is CD80 [153].

The limitation of this study is the small sample size; therefore, more volunteers are needed which may help to get clearer insights into the effects of ECVE on DC function.

7.4 Conclusion

ECVE-treated LPS-matured DCs stimulated more proliferation of T cells than LPS-matured DCs without ECVE, particularly at the higher T cell/DC ratios, and this was more evident for CD4+ T cells than CD8+ T cells. In addition, the 1% ECVE-treated DCs were stronger stimulators compared to those treated with 3% ECVE, which may

reflect the reduction of HLA-DR and CD86 by 3% ECVE in LPS-matured DCs. These findings are constistent with the previous observation that 1% ECVE treatment stimulated production of the pro-inflammatory cytokine IL-6 by DCs. A bigger sample size is needed to confirm these findings .

Chapter 8 A clinical case report of hypersensitivity pneumonitis following E-cigarette use

8.1 Introduction

The work reported in this thesis has demonstrated that ECVE can have pro-inflammatory and immune-activating effects on DCs. Clinical evidence consistent with this is, as reported in section 1.2.6, several clinical case reports of pathology caused by E-cigarette, including hypersensitivity pneumonitis (HP). A patient who was diagnosed as having acute severe HP after E-cigarette use was treated at Queen's Medical Centre, Nottingham. Here, laboratory techniques were used to measure antibody levels, which are related to the development of HP, and might help to explain the clinical, radiographic and physiological findings.

HP is a complex syndrome caused by repetitive exposure to particles (< 5um) which trigger exaggerated immune responses in the small airways and lung parenchyma. Numerous antigens from various environments have been recognized to be associated with HP (Table 8.1) [291]. HP is diagnosed by a combination of clinical, radiological, functional, cytological, histological and biological criteria, and classically categorized as acute, subacute, or chronic forms [291]. Acute HP occurs 4-8 hours after substantial exposure to antigens and is characterized by flu-like symptoms including fever, cough, myalgia and arthralgia. Acute HP is non-progressive with improvement after antigen avoidance. Subacute HP is a progressive disease, with persistent coughing and dyspnoea, due to repeated low-level exposure to antigens. Chronic HP is characterized

by progressive dyspnoea, cough, fatigue, malaise, and weight loss [291,292]. Since this classification considers the mode of exposure and the evolution the disease, a new classification system has been proposed: type 1, substantial and intermittent exposure which may lead to emphysema with chronic airflow obstruction; and type 2, chronic exposure to a low level of antigens which may lead to fibrosis with restrictive patterns [293,294]. Early diagnosis and antigen avoidance are key actions in the management of HP.

Table 8.1. Causative antigens of HP. The table is derived from reference [291].

Disease	Antigen	Source
Fungal and bacterial		
Farmer's lung	Saccharopolyspora rectivirgula	Moldy hay, grain, silage
Ventilation pneumonitis; humidifier lung; air conditioner lung	Thermoactinomyces vulgaris, Thermoactinomyces sacchari, Thermoactinomyces candidus, Klebsiella oxytoca	Contaminated forced-air systems; water reservoirs
Bagassosis	T. vulgaris	Moldy sugarcane (i.e., bagasse)
Mushroom worker's lung	T. sacchari	Moldy mushroom compost
Enoki mushroom worker's lung	Penicillium citrinum	Moldy mushroom compost
Suberosis	Thermoactinomyces viridis, Aspergillus fumigatus, Penicillium frequentans, Penicillium glabrum	Moldy cork
Detergent lung; washing powder lung	Bacillus subtilis enzymes	Detergents (during processing or use)
Malt worker's lung	Aspergillus fumigatus, Aspergillus clavatus	Moldy barley
Sequoiosis	Graphium, Pullularia, richoderma spp., Aureobasidium pullulans	Moldy wood dust
Maple bark stripper's lung	Cryptostroma corticale	Moldy maple bark
Cheese washer's lung	Penicillium casei, A. clavatus	Moldy cheese
Woodworker's lung	Alternaria spp., wood dust	Oak, cedar, and mahogany dust, pine and spruce pulp
Hardwood worker's lung	Paecilomyces	Kiln-dried wood

Paprika slicer's lung	Mucor stolonifer	Moldy paprika pods
Sauna taker's lung	Aureobasidium spp.	Contaminated sauna water
Familial HP	B. subtilis	Contaminated wood dust in walls
Wood trimmer's lung	Rhizopus spp., Mucor spp.	Contaminated wood trimmings
Composter's lung	T. vulgaris, Aspergillus	Compost
Basement shower HP	Epicoccum nigrum	Mold on unventilated shower
Hot tub lung	Mycobacterium avium complex	Hot tub mists; mold on ceiling
Wine maker's lung	Botrytis cinerea	Mold on grapes
Woodsman's disease	Penicillium spp.	Oak and maple trees
Thatched roof lung	Saccharomonospora viridis	Dead grasses and leaves
Tobacco grower's lung	Aspergillus spp.	Tobacco plants
Potato riddler's lung	Thermophilic actinomycetes, S. rectivirgula, T. vulgaris, Aspergillus spp.	Moldy hay around potatoes
Summer-type pneumonitis	Trichosporon cutaneum	Contaminated old houses
Dry rot lung	Merulius lacrymans	Rotten wood
Stipatosis	Aspergillus fumigatus, T. actinomycetes	Esparto dust
Machine operator's lung	Mycobacterium immunogenum, Pseudomonas fluorescens	Aerosolized metalworking fluid
Residential provoked pneumonitis Amebae	Aureobasidium pullulans	Residential exposure
Humidifier lung	Naegleria gruberi, Acanthamoeba polyphaga, Acanthamoeba castellani, Bacillus sp., others	Contaminated water from home humidifier, ultrasonic misting fountains
Shower curtain disease	Phoma violacea	Moldy shower curtain
Animal proteins		

Pigeon breeder's or pigeon fancier's disease	Avian droppings, feathers, serum	Parakeets, budgerigars, pigeons, chickens, turkeys
Pituitary snuff taker's lung	Pituitary snuff	Bovine and porcine pituitary proteins
Fish meal worker's lung	Fish meal	Fish meal dust
Bat lung	Bat serum protein	Bat droppings
Furrier's lung	Animal fur dust	Animal pelts
Animal handler's lung; laboratory worker's lung	Rats, gerbils	Urine, serum, pelts, proteins
Insect proteins		
Miller's lung	Sitophilus granarius (i.e., wheat weevil)	Dust-contaminated grain
Lycoperdonosis	Puffball spores	Lycoperdon puffballs

HP development is associated with a combination of type III and IV hypersensitivity. Acute HP development is associated with immune complex formation with high serum levels of precipitating antibodies (usually IgG but also can be IgM or IgA) but normal levels of IgE and eosinophils [295]. Immune complexes fix complement and bind to, and activate, leukocytes bearing Fc receptors and complement receptors, leading to the accumulation of fluid, protein and immune cells in the alveolar wall and decreasing blood-gas interchange and lung function. DCs present antigens to T cells, together with expression of IL-12, thereby leading to Th1 dominant immune responses which play a major role in development of subacute and chronic HP. Furthermore, apoptosis of lung epithelial cells and immune cells induce maturation and chemokine production of $CD11c^+$ DCs in BALF, thereby promoting HP [296]. Only a few individuals exposed to causative antigens develop HP, while the majority develop immune tolerance instead, which may be explained by genetic variability. MHC class II has been recognized as the primary susceptibility locus and polymorphisms in HLA-DR and DQ are associated with the risk of HP [297]. Fibrosis of lung might eventually developed, characterized by Th2 immune responses [298] and accumulation of matrix metalloproteinases [299].

8.2 A case report

8.2.1 Clinical information of the patient

A 16-year-old previously healthy boy presented with shortness of breath for five days before admission to the hospital. He had been vaping regularly for the past year, using exclusively shop bought E-liquids. He purchased two types of E-liquids, named here ‘E-liquid 1’ and ‘E-liquid 2’ prior to symptoms appearing; however, the relative use

of each of these two E-liquids was not defined. Examination revealed wide spread wheeze, increased work of breathing and type 2 respiratory failure. He continued to deteriorate inexorably and progressively required non-invasive, invasive mechanical ventilation, high frequency oscillatory ventilation (HFOV), and finally three days of Veno-venous extracorporeal membrane oxygenation (VV ECMO) for uncontrolled hypercapnia. He responded slowly to the intensive support and high dose steroids. High resolution computed tomography (CT) showed bilateral mid to upper zone peribronchial ground-glass changes. Serial lung function testing showed restrictive, followed by normal then mixed picture during deterioration with consistently normal transfer factor. A skin prick test (SPT) showed E-liquids caused deterioration in the patient. E-liquids were suspected as the causative agents of the HP in this case, based on the clinical course, lung function, imaging, response to steroids and deterioration on re-exposure to the likely inciting agent.

The patient was slow to improve and had fluctuating symptoms even on inhaled bronchodilator and corticosteroids. He subsequently required intra-venous methylprednisolone for complete resolution of symptoms. Two years after the episode, he remains on inhaled steroids but very well with good lung function.

8.2.2 Immunoglobulin detection using ELISA

A serum sample was obtained from the patient during hospitalisation for the acute attack of HP. A further serum sample was obtained from the patient 6 weeks later following treatment with intra-venous methylprednisolone and abstinence from E-

cigarette use. Sera were also obtained from five healthy donors (median age 21 years old, range 20-23; 3 male and 2 female) who had never smoked tobacco or E-cigarettes.

The method of ELISA has been described in section 2.92. Figure 8.1A shows a standard curve of human IgG demonstrating validation of the assay. Normal range of total IgG in serum is 6-16 g/L (i.e. 600-1600 mg/dL); on this basis, two of healthy controls had levels of total IgG slightly above the normal range. The concentrations of total IgG were very similar in the first and second samples from the patient and were similar to those measured in the healthy controls (Fig. 8.1B). Interestingly, similar levels of IgG specifically binding to either E-liquid 1 (Fig. 8.1C) or E-liquid 2 (Fig. 8.1D) were detected in the serum samples from both the patient and the controls. However, the level of IgG specific to E-liquid-1 dropped 36% between the first to the second serum sample from the patient (Fig. 8.1C). This might be a consequence of suppression of IgG levels by the methylprednisolone treatment given to the patient. By contrast, the level of IgG specific to E-liquid 2 was higher in the second sample than in the first (Fig. 8.1D), despite the immunosuppressive treatment.

Figure 8.2A shows a standard curve of human IgM demonstrating validation of the assay. Three of the five control sera, but neither of the patient's serum samples, contained detectable levels of IgM antibodies specific to E-liquid1 (Fig. 8.2B). By contrast, just one of the control samples and both the patient's serum samples, contained detectable IgM antibodies specific for E-liquid 2 (Fig. 8.2C); furthermore, the concentration of IgM anti-E-liquid 2 in the patient's second serum sample (taken over 6 weeks after quitting E-cigarette use) was about twice as high as the

concentration in the first serum sample, taken during the acute episode of HP (Fig. 8.2C).

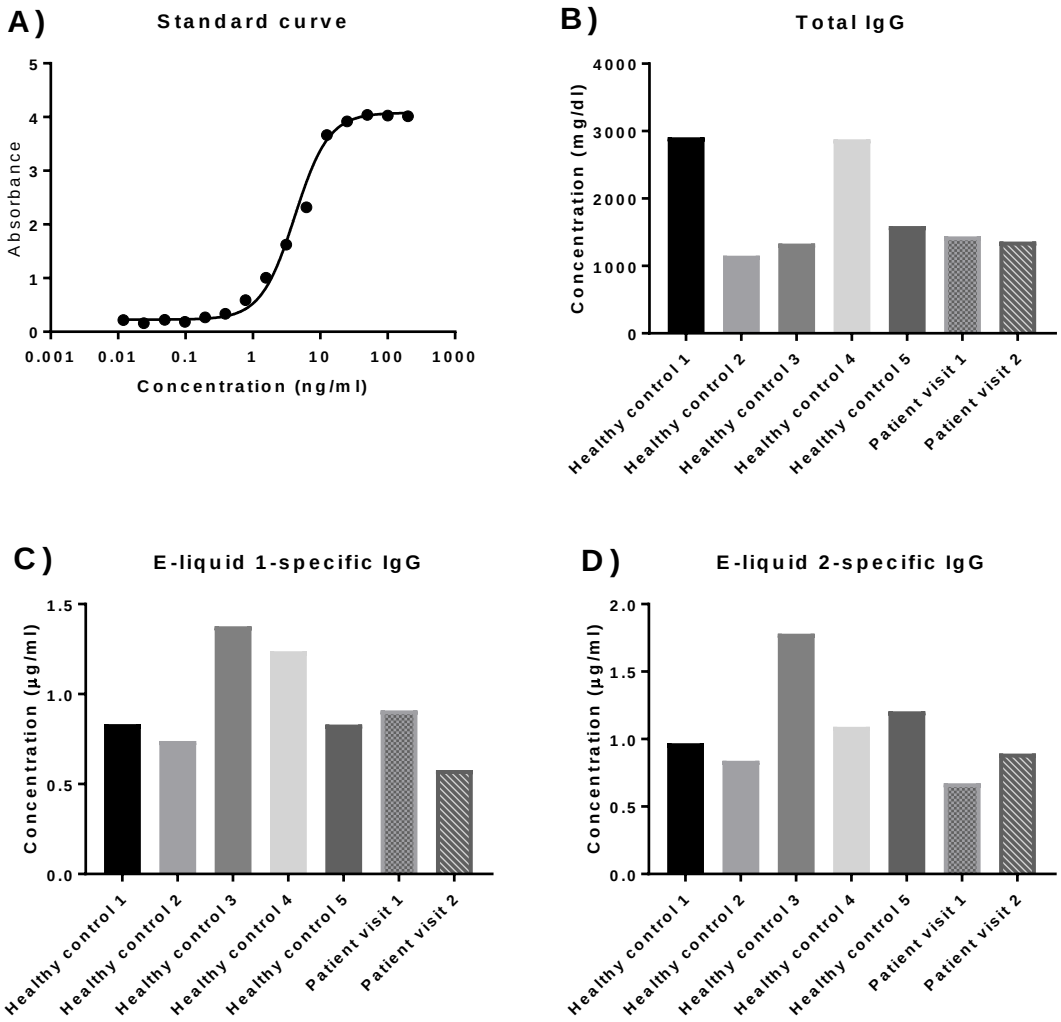


Figure 8.1. Concentration of total IgG and E-liquid-specific IgG, determined by ELISA, in serum samples from the patient and the healthy donors. (A) Standard curve of human IgG. (B) Concentration of total IgG. (C) Concentration of E-liquid 1-specific IgG. (D) Concentration of E-liquid 2-specific IgG.

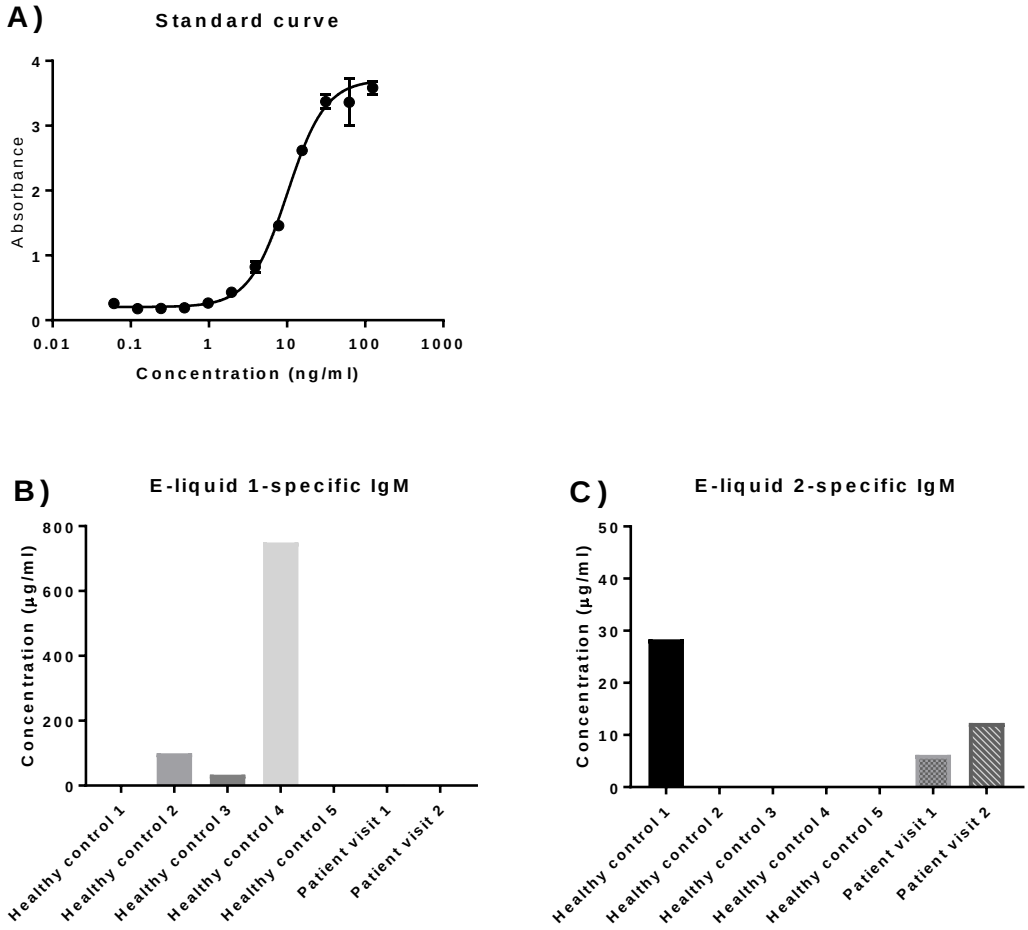


Figure 8.2. Concentration of E-liquid-specific IgM, determined by ELISA, in serum samples from the patient and the healthy donors. (A) Standard curve of human IgM. (B) Concentration of E-liquid 1-specific IgM. (D) Concentration of E-liquid 2-specific IgM.

8.3 Discussion

HP indicates a group of inflammatory disorders characterized mainly by pulmonary injury and is associated with repeated exposure to a wide variety of extrinsic antigens. Elevation of antigen-specific IgG in sera has been detected in HP [300-302] and the determination of antigen-specific IgG is important in diagnosis of HP and may help to identify causal agents. Furthermore, although antibodies associated with HP are usually of IgG class, IgM class antibodies can also be involved [303,304]. However, some HP patients are negative for specific antibodies and conversely, specific antibodies can occur in asymptomatic individuals [305]; therefore, HP cannot be defined solely on expression of specific antibodies.

In the present case, it may seem unexpected that the serum samples from both the patient and all healthy controls contained IgG antibodies to E-liquid 1 and 2. To our knowledge, none of the healthy controls had a history of exposure to E-liquids, suggesting that there are common environmental antigens that promote the production of cross-active antibodies. Also, it is well-recognised that antibodies specific to antigens associated with HP are found in a high proportion of exposed, but clinically asymptomatic individuals [303,306]. The patient had IgM antibodies specific to E-liquid 2 but not to E-liquid 1, and only one of the five healthy controls had IgM antibodies to E-liquid 2, suggesting IgM antibodies cross-reactive with E-liquid 2 are uncommon. Furthermore, IgM is expressed by naïve B cells; thus, the presence of IgM antibodies to E-liquid 2 in the patient's serum samples may indicate E-liquid 2 acted as a recent immunogenic agent that activated naïve B cells rather than memory B cells.

Overall, the most significant observation in the patient appears likely to be the production of IgM and IgG antibodies to E-liquid 2, both of which showed higher levels in the patient's second serum sample compared to the first (6-week interval) despite the patient being given potent immunosuppressive therapy (intra-venous methylprednisolone). E-liquid 2-specific antibodies may be sequestered from the circulation to the lung tissues during vaping to form immune complexes, and this tissue sequestration ameliorates following sustained cessation of vaping, leading to increased circulating concentrations of these antibodies that may continue to be produced by mature plasma cells. The fall in the level of IgG antibodies cross-reactive with E-liquid from the first to the second serum sample may be due to the immunosuppressive effects of the methylprednisolone.

8.4 Conclusion

E-liquid 2 was thought to be the immunogenic agent that triggered the occurrence of HP in this patient. The elevation of E-liquid 2-specific antibodies in the second serum sample may be related to the cessation of vaping that reduced sequestration of these antibodies to the lung. There also may be environmental antigens that induce antibodies that are cross-reactive with E-liquid flavourings in humans. It may be interesting to measure cytokine levels in serum owing to their critical roles in the development of HP [307,308], Indeed, the induction of pro-inflammatory cytokine production by ECVE was shown in Chapter 6 . However, due to the limited amount of serum from the patients, cytokine detection was no possible in the current study.

Chapter 9 General discussion and conclusion

9.1 General discussion

Electronic cigarettes are relatively recently invented smoking devices and, in the last decade, have gained rapidly in popularity around the world. They are promoted as a safe alternative to conventional cigarettes and a tool to help tobacco smoking cessation. It is well documented that conventional cigarettes have deleterious effects on humans and there is also growing evidence for the adverse effects of E-cigarette use. However, the effects of E-cigarettes on the human immune system are not fully clear. The current study provides evidence that E-cigarettes can alter both maturation and function of DCs, demonstrated by down-regulation of maturation markers but up-regulation of cell signalling molecule expression, cytokine production and T cell activation, using an in vitro model (Fig 9.1)

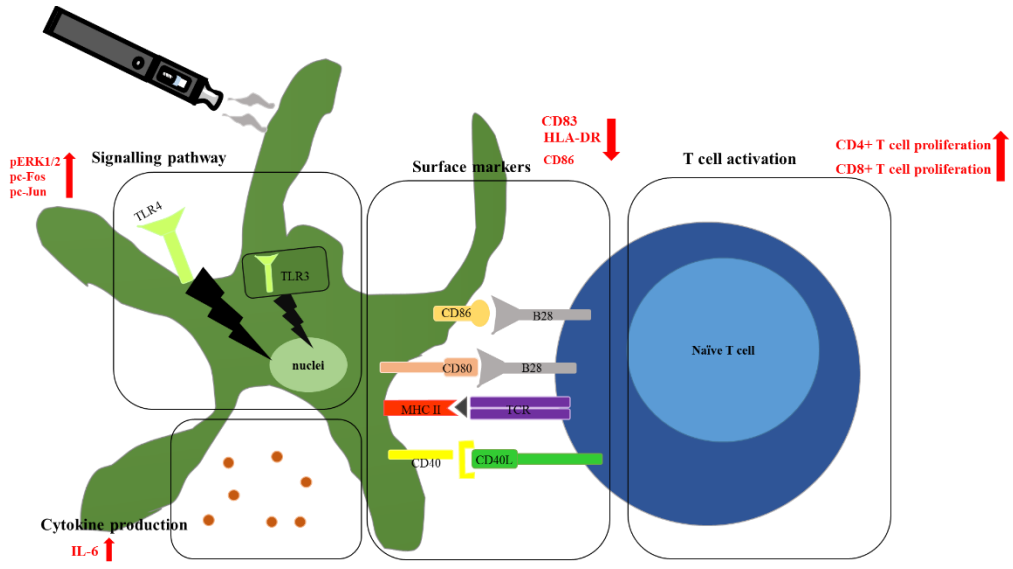


Figure 9.1. Summary of the effects of ECVE on DCs

DCs are professional antigen presenting cells that link innate and adaptive immunity. Due to the importance of DCs within the immune system, the aim of this study was to investigate the effects of ECVE on the biological behaviour of DCs in vitro using human monocyte-derived DCs. Monocyte-derived DCs were generated from positively selected CD14+ monocytes and cultured with GM-CSF and IL-4 for five days. DCs were then matured with LPS or Poly I:C, which binds to TLR4 and TLR3, respectively, to stimulate different pathways causing DC maturation, in the presence or absence of different concentrations of ECVE.

RPPA is a miniaturised dot-blot platform that allows measurement of protein expression in a large number of biological samples simultaneously in a quantitative manner using small volumes of sample. Firstly the effects of ECVE on 29 singling molecules in DCs at different maturation stages were investigated. ECVE generally did not cause a statistically significant change in the expression of individual signalling molecules in immature, maturing and matured DCs except exposure to 1% ECVE for 24 h significantly increased pc-Fos and pc-Jun levels and exposure to 3% ECVE significantly increased pERK1/2 expression in LPS-matured DCs compared to LPS-matured DCs without ECVE, suggesting the role of ECVE in triggering the MAPK pathway, which was consistent with the findings showing activation of MAPK pathway proteins in animal bone marrow-derived cDCs by CSE [181] . However, although effects on individual signalling molecules may seem relatively small, the combined or 'global' effects on multiple signalling molecules may be biologically significant. In this context, ECVE had statistically significant global effects on DCs: thus, 30 minutes and 24 h treatment with 1% or 3% ECVE significantly upregulated

signalling molecule expression in immature and LPS-stimulated DCs, respectively. 30 minute treatment with 3% ECVE downregulated signalling molecule expression in Poly I:C-maturing DCs. ECVE therefore appears to have differential effects on signalling pathways in DCs, depending on the DCs' state of maturation, type of stimulation (e.g. the TLR4 ligand LPS or the TLR3 ligand Poly I:C), and the concentration of ECVE. Due to the signalling molecules involved in regulating multiple functions in DCs, including maturation and cytokine production, we were interested in studying the effects of ECVE on these functions.

Investigating the expression of surface markers on DCs, 3% ECVE significantly reduced the level of maturation marker CD83 on immature DCs and decreased the expression of HLA-DR and CD86 on LPS-matured DCs, suggesting a role in inhibiting maturation of DCs. Since DCs present antigen peptides via MHC II to CD4+ T cells, and DCs' co-stimulatory molecule CD86 induces signals for T cell activation by interaction with CD28, reduction of HLA-DR and CD86 expression caused by ECVE may indicate its potential role in hindering T cell activation by DCs. Both expression of HLA-DR and CD86 have been shown to be inhibited by CS [150,243,256]; however, the effects of CS on DCs were highly variable, which may be due to the different types of DCs, concentrations of CSE and duration of incubation.

The cytokines produced by DCs affect the immunological environment and therefore the types of T cell responses activated by DCs. The production of IL-6, IL-8, IL-10, IL-12 and TNF-α by DCs was assessed using ELISA. Maturation with LPS and Poly I:C stimulated production of all these cytokines compared to immature DCs. ECVE did not affect the production of these cytokines, except for IL-6 secreted from LPS-

matured DCs, which was increased by 1% ECVE treatment. IL-6 not only acts as a pro-inflammatory cytokine, but also promotes Th17 responses. Other work has shown that CS induced production of pro-inflammatory cytokines in BAL and from DCs [199,243,266,267,271,272,275]; the present study shows that ECVE may also promote production of some pro-inflammatory cytokines.

Since the ECVE-induced alterations in signalling pathways, surface markers and cytokine secretion of DCs could affect activation and priming of T cells, the effects of ECVE treatment of DCs on T cell activation were further assessed. 3% ECVE induced the upregulation of signalling molecules in LPS-maturing and matured DCs, but suppressed HLA-DR and CD86, therefore it was hypothesised that the effects of ECVE treatment might both potentiate and suppress the T cell-stimulatory activity of DCs, and that the balance of these effects might depend on ECVE concentration (1% versus 3% in our experiments). Allogenic MLR assay and CFSE staining were performed to evaluate the activation-induced proliferation of T cells. LPS-matured DCs treated with ECVE stimulated more proliferation of T cells compared to DCs without ECVE, especially at the higher T cell/DC ratios. This scenario was more pronounced in CD4+ T cell proliferation than in CD8+ T cell proliferation. In addition, 1% ECVE-treated DCs were better simulators than DCs treated with 3% ECVE, which may be explained by the decreased HLA-DR and CD86 expression caused by 3% ECVE treatment of LPS-matured DCs. Furthermore, 1% ECVE stimulated significant elevation of certain signalling molecules individually in LPS-matured DCs and induced a trend of global upregulation of signalling molecules (although this did not reach statistical significance). Although some studies showed CS [242] and nicotine

[269,283] had capability to impair T cell proliferation which may increase the susceptibility of tobacco smokers to infections, the current study revealed that ECVE tended to enhance T cell proliferation, which may potentially induce excessive immune responses.

In addition, a clinical case of a patient who developed hypersensitivity pneumonitis after using certain E-liquids for vaping was also investigated. Since the pathogenesis is associated with immune complex formation with antibodies, levels of antibodies specific to E-liquid constituents in serum of the patient during the acute attack and six weeks later were measured by ELISA. It appeared that one of the E-liquids used by the patient was the causal agent and cessation of vaping may have reduced the sequestration of E-liquid-specific antibodies from the circulation to the lung tissues so that levels of these specific antibodies were higher in the second serum sample than the first. However, the E-liquid-specific antibodies detected could not be used as a specific marker of hypersensitivity pneumonitis because allergen-specific antibodies can also occur in asymptomatic individuals [303,305,306]. In the current study, specific antibodies were detected in some healthy controls who had never used E-cigarettes or conventional cigarettes: this may be due to cross-reactivity of E-liquid constituents with other environmental antigens. Another clinical case report also revealed that a healthy 18-year old woman developed hypersensitivity and acute respiratory distress syndrome after E-cigarette use, although without immunological evidence provided [309]. Therefore, these cases raise concerns of allergic reactions caused by E-cigarettes and presenting with atypical respiratory diseases in young people.

It is well known that conventional cigarettes are associated with inflammatory processes [132], including COPD and with autoimmune diseases [310]. However, long-term effects of E-cigarette use are still not clear because they have been available for no more than 15 years. Findings here showed that ECVE promoted activation of DCs and T cell-stimulation which raises the possibility that ECVE, in the long term, could also promote lung-related inflammation and/or autoimmunity. Already there is evidence, be it rare, of allergic responses caused by E-cigarette use, as seen in the patient with hypersensitivity pneumonitis in the current study and in a previous case report [309].

There are numerous studies of the effects of CS on DCs but, to our knowledge, this is the first study focusing on the effects of E-cigarettes on DCs. The current study has presented evidence concerning the effects of ECVE on the phenotype and functionality of DCs and there are some aspects we are interested to investigate in the future. Firstly, it is clear the effects of ECVE on DCs vary depending on the type of stimulation (i.e. TLR4 ligand LPS or TLR3 ligand Poly I:C) which triggers different downstream of signalling pathways. Signalling pathways are very complex and contain abundant signalling molecules which interact with each other: thus, more signalling molecules could be studied using RPPA to gain more a comprehensive insight into changes in each signalling pathway caused by ECVE treatment. Furthermore, ECVE treatment of LPS-matured DCs facilitated stimulatory effects on T cell proliferation. The cytokine secretion of allogenic T cells co-cultured with ECVE-treated DCs could further be detected to investigate the effects of ECVE on the polarising ability of DCs, since CSE was found to inhibit the production of Th1-related cytokines, but tended to trigger Th2

responses [242]. Also, E-cigarettes have been shown to alter cytokine production by various cell types (see section 1.2), but no studies have assessed the impact on lymphocytes so far. Lymphocytes are crucial in mounting adaptive immune responses which provide specific and long-lasting protection against pathogens, but are also associated with the pathogenesis of inflammatory and autoimmune diseases. Examining the effects on ECVE on lymphocytes may help us to gain deeper understanding of the effects of E-cigarettes on adaptive immunity.

The present study did not investigate the effects of ECVE treatment of Poly I:C-matured DCs on their ability to stimulate T cells. This would be an area of interest for further investigation, particularly given that there was significant global suppression of signalling molecules in the Poly I:C-maturing DCs treated with 1% ECVE for 30 minutes. Thus, it is possible that ECVE treatment of poly-I:C matured DCs may suppress their ability to stimulate T cells, rather than enhance their ability to stimulate T cells, as found for ECVE-treated LPS-matured DCs (Chapter 7). In this context, it is interesting to note that Poly I:C mimics the stimulation of TLR3 by double-stranded RNA in the context of viral infections, and it has very recently been reported that E-cigarette smokers have reduced innate and adaptive immunity to influenza infection [311]

Thousands of flavoured E-liquids are available and some flavourings have been shown to induce alterations in various cell types, including affecting their viability, proliferation, phagocytic ability and cytokine production (see section 1.2.5). Since the E-liquid used in the current study did not contain flavouring and there are no other studies assessing the effects of flavourings on DCs, it would be interesting to use

flavoured E-liquid to generate ECVE and evaluate how they affect the phenotype and functionality of DCs. Finally, MoDCs were generated using blood of healthy never-smoker donors in the current study: DCs could also be generated from current and previous E-cigarette users in the future to investigate their biological behaviour, and the results could be compared with in vitro model used in the present study.

Unlike the generation of CSE, there is no standard protocol for ECVE generation. In addition, a broad variety of brands and flavourings of E-liquid are available. It may, therefore, be difficult to compare results of E-cigarette studies using different brands and formulations of E-liquid. An internationally agreed standard protocol for ECVE generation is required.

9.2 General conclusion

This study established an in-vitro model to investigate the effects of ECVE on human monocyte-derived DCs. Several biological aspects of DCs have been examined including signalling pathways, surface markers, cytokine production and T cell activation, using a variety of culture conditions: i.e. types of maturation stimulation (LPS or Poly I:C) and ECVE concentrations. Apart from causing reduced expression of certain surface markers, ECVE generally had stimulatory effects on DCs, demonstrated by increased levels of signalling molecules, the pro-inflammatory cytokine IL-6 and T cell activation, suggesting its potential role in promoting inflammation. A clinical case of hypersensitivity pneumonitis supported the

inflammatory effects of ECVE in humans. Therefore, E-cigarettes are not completely innocuous to human health and this thesis supports concerns about E-cigarette use.

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