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Abbreviations

AHR	Airway hyper responsiveness
AF	Alexa Fluor
Ag	Antigen
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CD	Cluster of differentiation
DC	Dendritic cell
cDC	Conventional dendritic cell
°C	Degree Celsius
DAPI	4',6 Diamidino-2-phenylindoldihydrochloride
DC	Dendritic cell
DNase	Deoxyribonuclease
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
kb	Kilo base(s)
LN	Lymph nodes
mAb	Monoclonal antibody
Mac	Macrophages
mDC	myeloid Dendritic cell
MHC	Major histocompatibility complex

min	Minutes
ml/ μ l	Milliliter(s)/Micro liter(s)
mg/ μ g	Milligram(s)/Microgram(s)
mLN	mediastinal lymph nodes
nm	Nanometer
OVA	Ovalbumin, chicken egg
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
P/S	Penicillin / Streptomycin
RBC	Red blood cell
rpm	Rotation per minute
RT	Room temperature
s	Second(s)
Th	T helper
RT	Room temperature

1. Introduction

1.1 Allergic Asthma

Asthma is a progressively common inflammatory disease caused by an immune response against allergen (1-3). The symptoms are coughing, wheezing, shortness of breath, and chest tightness upon exposure to allergenic Antigen (Ag). Common Ags are proteins, which are derived from inhaled environmental factors such as house dust mite. An example for the mite is *Dermatophagoides Pteronyssinus* (4) present in every home, where it feeds on exfoliated human skin. It grows especially well in moist environments and their faecal pellets contain numerous Ags which have enzyme activity. Other common Ags are well pollen, grass, dogs, cats hair and Proteins present in food for example seafood, peanuts, eggs or milk (5). Recent studies indicate that traffic pollution containing polycyclic aromatic hydrocarbon core of diesel exhaust particles (DEP) (6, 7) can also trigger asthma symptoms and exacerbations.

Each person reacts differently to Ags by either inducing immune tolerance or immune response. It is still ambiguous how the immune system individually works, whether to ignore or to respond to Ags (8). Ags are recognized by Ag presenting cells (APC), which stimulate T cells. They take up Ags, internalize and proteolyse into small peptide fragments and migrate from lung to the draining lymph nodes where they prime Ag-specific T cells. Activation of Th2 response is characterized by increases production of IL-4, IL-5 and IL-13 (9, 10). IL-4 is required to drive production of allergen-specific immunoglobulin E (IgE), whereas IL-5 regulates the eosinophil components of allergy. The IgE production is a significant factor of allergic asthma since IgE binds to allergen and to high-affinity FcεRI receptors on the surface of mast cells which leads to mast cell degranulation and release of vasoactive mediators. The role of eosinophils in asthma disease is observed as an end-stage effector cells. They release granule cationic proteins and generate

paracrine mediators of inflammation (11, 12). IL-13, induce bronchial hyperactivity and mucous metaplasia (9, 13-19) (Figure.1)

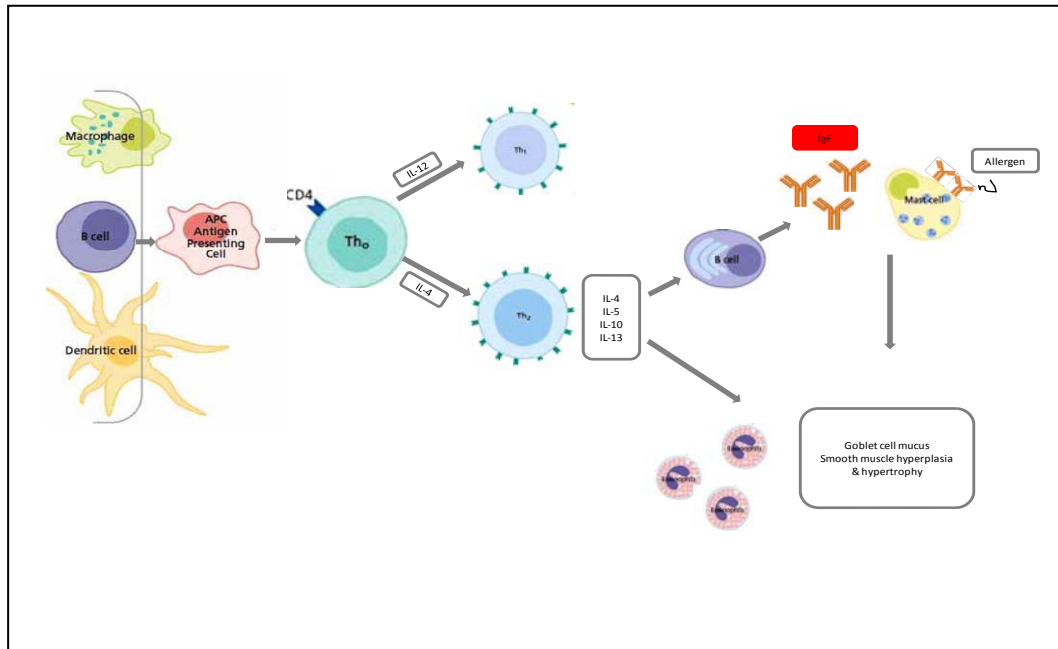


Figure 1. Pathogenesis of allergic asthma

This paradigm represents the pathogenesis of allergic asthma. Briefly, allergen is taken up, internalized, and processed by APCs such as dendritic cells (DCs), B-cells or macrophages (Macs). Intracellular Ags are transported to compartments, where they are proteolyzed into small peptide fragments. MHC class II molecules bind to the peptide and stimulate T cell receptors on naive CD4⁺ T cells. T-cells become activated and differentiate into either Th1 or Th2 cells associated with the production of cytokines. Ags induce Th2 response. Effector cytokines are secreted and induce differentiation of eosinophils and facilitate B-cells to produce IgE. IgE specifically binds to Ag and cross-links Fcε receptors on mast cells. All these mediators cause inflammatory symptoms like mucus production by goblet cells, smooth muscle contraction, edema and eventually, airway remodeling and lung destruction.

1.2 Antigen Presenting Cells

APCs are the first cells to sample inhaled Ags. The major role of APCs constituted of DC, Macs or B cells is to stimulate the T cell response in lymph nodes (LN) (20-22). APCs are located in non-lymphoid tissues where they capture Ags and migrate via afferent lymphatic vessels to the paracortex of the LN where naïve T cells reside (23). During the migration, Ags are fragmented in small peptide fragments which bind to MHC class II and present them to T cells. APCs change their shape and promote activation of T cells (24).

Early 70's Macs and DCs were discovered and have been subdivided from peripheral mononuclear phagocytes (25, 26). Macs and DCs have several subpopulations which are distinguished by their distinct anatomic location and phenotypes. Macs can be found in the serosa, lamina propria, lung, brain (microglia), bone (osteoclasts), and liver (Kupffer cells).

1.3 Dendritic Cells

DCs derived from several lineages have to pass through number of stages during cell development; activation and maturation until they are able to induce immunity or tolerance (27). DCs own the capability to differentiate between pathogens and nonpathogenic proteins to either activate the immune system or induce immune tolerance. But the question, how the immune system distinguishes between response and tolerance, it is still not well known.

There are two important roles of DCs. First, as mentioned before, DCs can recognize Ags and migrate to the draining LNs to present Ags to naïve T cells (28, 29). Second, DCs are able to induce T cells proliferation and activation. Mature DCs express in addition to high levels of MHC molecules CD40, CD80, CD83 and CD86 on their surface (30) and also secrete high levels of cytokines to control T cell polarization, as IL-12, a type I IFNs which promote Th1 responses. Additionally, IL-10, IL-4 and IL-5 are released which implicate Th2 (31).

DCs which present Ag can exist in two states, immature or mature conditions. But only mature DCs have the ability to activate T cells for clonal expansion and prime immune response. This indicates that immature DCs which are located in peripheral tissues capture and process Ags, migrate to the secondary lymphoid tissues, where they become mature DCs with two typical aspects – first their function of Ag uptake is down regulated and second, they are extremely powerful at priming T cells by increasing of surface expression of MHC class II and co-stimulatory molecules. *In vitro*, progenitor of DCs can be converted to mature DCs by specific signals like inflammatory cytokines such as Tumor-necrosis factor (TNF), CD40 ligand but also microbial products as lipopolysaccharide (LPS) (30, 32). LPS is omnipresent in the environment for example in household dusts. It is a cell wall component of Gram-negative bacteria and activates cells through TLR4 with the accessory proteins CD14 and LPS binding protein (33-36). The signal goes through a common adaptor protein MyD88. The role of endotoxin exposure in asthma development in children has been shown controversial affects. It has either a protective role through Th1 induction or an exacerbating effect on asthma severity.

There are several ways how DCs can engulf Ag. First, through fluid-phase endocytosis, this is also used by Macs but not by B cells. Receptor-mediated uptake to internalize Ag is another pathway which is effective in both, Macs and B cells (32). In both pathways, Ags are going through an intracellular processing whereas peptides bind to the MHC class II molecules. MHC class II plays an important role in presenting Ags to T cells. Immature DCs express a large fraction of MHC class II molecules but they are only present in endosomal/lysosomal compartments. Whereas on mature DCs, MHC class II molecules are released from li and are able to bind peptide complexes from Ag. This Ag fragment is transported to the surface to present to T cells.

DCs at mucosal surface are adapted for Ag uptake but have a poor capacity to present Ag to T cells (37). DCs express several specialized receptors which can take up Ag like mannose receptor, Langerin, DC-SIGN -, DEC-205 - and Fc receptors (FcR). FcRs trigger many biological functions including phagocytosis, degranulation,

and cytolysis. Furthermore, they mediate the uptake of immune complexes, degradation and peptide Ag presentation on DCs (38). There are two significant classes of FcγR which are important for modulation of DC functions (39, 40); first the activating receptors FcγRI, FcγRIIA and FcγRIIIA and second the inhibitory receptor FcγRIIB. The activating receptors are characterized by cytoplasmic ITAM which initiates maturation of DCs, whereas inhibitor receptors are distinguished by the presence of ITIM. ITIM inhibits ITAM-mediated activation signals through the recruitment of the inositol-phosphatase SHIP (39, 41).

After digesting Ags, airway DCs transport Ag fragments to the T cell zones of regional LNs (42-44). But interestingly, a significant proportion of Ag-loaded DCs remains in the lung where they can activate T cells locally long after Ag exposure. The migration of DCs into LN is driven by chemokine signals acting on the CCR7 receptor which is recognized by CCL9 and CCL21 (45-47). The relocation of DCs into LNs is controlled by lipid mediators such as leukotrienes and prostaglandins. Prostaglandin D₂ which binds to D prostanoid receptor 1 (DP1) and DP2 receptor has a key role in development of pulmonary inflammatory diseases. According to Lambrecht and his colleague, prostaglandin D₂ is able to suppress the migration of lung DCs into LNs (48).

DCs are capable to control Th1 and Th2 differentiation, but the regulation is incompletely understood. DCs express co-stimulatory and adhesion molecules on their surface and probably this differential expression of surface molecules can influences Th1 or Th2 switching. Molecules like CD86 and CD80 are crucial for Th2 response whereas CD86 seems to be more important compared to CD80 (49). But on the other hand, another group has demonstrated that CD80 is more essential in initiating Th2 responses and airway eosinophilia in mice (50).

Another important factor for regulation either to Th1 or Th2 response is IL-12, a cytokine which initiates and maintains INF-γ producing of Th1 cells. IL-12 cytokines are synthesized when DCs react to components of the bacterial cell wall like LPS or bacterial DNA (CpG oligonucleotides). However, IL-4 cytokine production which is induced by IL-6 is important for Th2 polarization and Th1 inhibition (27).

Airway DCs are important for the induction of pulmonary immunity or tolerance. They build up a dense network in the lung parenchyma, on alveolar surfaces and in the vascular sections, but also within the epithelium and submucosa of conduction airways. They take up Ags and migrate to draining LNs to stimulate naïve T cells (51). Their role is not only in initiating specific Th2 cell immune responses which lead to experimental asthma, but moreover, they are able to restimulate effector cells during an ongoing airway inflammation (52-54). This indicates that DCs are also important for the reactivation of primed Th2 cells after Ag challenge (54).

1.3.1. Division of DCs

Different subsets of DCs can induce different T cell responses (4, 55). In immune system, there are many DC subtypes, which differ in location, migratory pathways, immunological function and dependence on infections or inflammatory stimuli for their generation. The lifespan of DCs is short once they enter the lymphoid tissues to interact with T cells. Therefore, it is important that DCs can continually renew.

1.3.1.1 Conventional- and Precursor DCs

There are two DC forms under steady-state – which means in the absence of maturation signals – conditions or conventional DCs (cDCs) and precursor DCs (pre-DCs) (55-57). CDCs already have a dendritic appearance and exhibit function whereas pre-DCs have to be further developed to gain the dendritic form and the full DC functions. Pre-DCs are the last precursor stage to turn into DCs, and only a little or no cell division is involved in their development. Different pre-DCs produce different types of DC subsets whereas they require microbial or inflammatory stimuli.

Subset of DCs, present in the circulation and tissues are plasmatooid DCs (pDCs) and myeloid DCs (mDCs) derived from bone marrow progenitors (58) and are originate either from common lymphoid or myeloid progenitors. In immature form,

they migrate to the peripheral tissues where they express low levels of MHC class II and B7 family co-stimulatory molecules like CD80 and CD86. To capture Ags, they are using pattern recognition receptors, pinocytosis or phagocytosis. When cDCs take up Ags, they become active and undergo some modifications. First, pattern recognition receptors get down regulated. Secondly, it causes to a change in homing receptors and an up-regulation of co-stimulators. Supplementary, an expression of CCR7 occurs which allows DCs to recognize CCL21 signal to migrate to the draining LNs (59).

1.3.1.2 Myeloid DCs

Myeloid DCs are the predominant type of cDCs in the mouse lung. The mDCs have a lineage relationship with monocytes and Macs which indicates that they need for their survival granulocyte Mac colony stimulating factors (GM-CSF) (37, 59). MDCs as sentinels in peripheral tissues, alert the immune system of the presence of pathogens and initiate rapid immune responses (60). Infections with respiratory viruses (such as Influenza A), bacteria (*Francisella tularensis*) (61) or fungi (*Aspergillus fumigates*) induce in the airways of rodents a migration of mucosal DCs to the draining LNs. These become mature cells with an increased co-stimulatory expression of CD40, CD80, CD86 and MHCII. MDCs are characterized by the expression of β_2 integrin CD11b, CD11c, CD33 and CD13. Mature mDCs induce a Th2 response but under certain conditions they are able to activate a Th1 immune response and protect against asthma (61).

1.3.1.3 Lymphoid DCs

Lymphoid DCs are found in thymus, spleen and LNs (27). Within the thymus, lymphoid DCs mediate negative selections and subsequent destructions of self-reactive T cells (62). These cells have additionally the capacity to activate T cells, but they induce a restricted T cell cytokine profile and apoptosis. Lymphoid DCs are

characterized by the expression of CD8 α , CD11c and CD205 but negative for CD11b, NK1.1 and B220.

1.3.1.4 Plasmacytoid DCs

Plasmacytoid DCs (pDCs), sometimes also referred to as lymphoid DCs have a round shape form, resemble to plasma cells and are present in the circulation and in tissues (37, 55). They are trace populations within the circulation and are relatively long-lived DCs which express B220 and mPDCA-1. The early progenitors of pDCs express Ly6-C and CD3. CD123 and IL-3 receptor α chain can be found as well on the cells. For their growth and survival, they are dependent on IL-3. During viral infection or stimulated with bacterial CpG, pDCs are the main source of IFN- α and IFN- β at the site of viral replication (59, 60). In this case they could play a critical role in the innate immune response to viral infections. PDCs differ from myeloid DCs by high level of CD45RA, 120G8, and 440c, and by low levels of CD11c and MHC class II. An *in vitro* study exhibits that freshly isolated pDCs have a low expression of CD8 α , whereas activated pDCs instead have a high expression of CD8 α (37).

Several studies were indicated that lung pDCs have an essential role in preventing asthmatic reactions to harmless inhaled Ag (52). In a paper published in 2004 by Lambrecht and Co. (52) found out that pDCs in the lung were able to take up harmless Ags and migrate to the draining LNs to induce tolerance through the induction of regulatory T cells. This observation was indicated by depletion of pDCs in the lung and mLNs during the first exposure to OVA Ag given by i.t. injection in mice. Three weeks later, after aerosol challenge with OVA, they observed that pDCs depleted mice developed symptoms of asthma whereas control mice did not. Taken together, pDCs in the lung and in mLNs are immature and express low levels of MHC class II and co-stimulatory molecules. However they express high levels of PD-L1 inhibitory molecules which interact with PD-1 and are able to lead to a suppression of T cell effectors.

1.3.1.5 Langerhans Cells

Langerhans cells (LC) are another subpopulation of DCs (37, 55), and are present in the epithelial of the intestinal, respiratory and reproductive tracts which were mostly characterized in the epidermis of the skin. LCs belong to the classical model for migratory DCs by collecting the Ag in the skin, then migrate to the LNs to pass the antigenic information to the T cell system. This subset of DCs expresses molecules like E-cadherin, Langerin (CD207) and Birbeck granules which help LCs to track among other migratory cells into LNs (63).

1.3.2. DCs in lung and upper airway

DCs are dispersed throughout the whole lung. They can be found in conducting airways, lung parenchyma, alveolar space, visceral pleura and the pulmonary vascular bed (60). However, different subsets of lung DCs are differentially distributed. Mice contain in their conducting airways DCs which express CD11c, MHC class II and CD11b, and low to intermediate levels of DEC205. But they are negative for CD8 α , a marker which is present on a subset of lymphoid organ DCs, and are also negative for Gr-1, a pDC marker. The interstitial DCs are found in lung interstitium, a space in between alveolar epithelial cells and alveolar capillaries. These cells are predominantly CD11c⁺ myeloid. They have a low expression of co-stimulatory molecules CD40, CD80 and CD86, but a high expression of inflammatory chemokines and endocytic receptors which indicates that these cells are immature DCs. In the airway compartment, DCs are positive for F4/80, CD11c and CD11b, and they are able to capture airborne Ags. They maintain the capacity to activate specific T cells long after Ag exposure. Vermaelen and colleagues found another subtype of pDCs in lung (60) which express low levels of CD11c, but positive for Gr-1, B220 and 120G8.

According to a paper published in 2004 by the same group, DCs were distinguished in the lung by means of high CD11c expression (64). Their study point out that cells with morphological, functional and immunophenotypical features of

DCs and Macs can be examined by flow cytometry. They indicate that pulmonary DCs are sentinel cells with a fast turnover. They transport inhaled Ag to the T cell areas of LNs and initiate a primary immune response, whereas pulmonary Macs appear as poorly migratory cells with a slow turnover and inefficient in stimulating naïve T cell proliferation. Moreover, they also display immunosuppressive properties. Another study revealed that lung Macs can suppress APC functions of lung DCs *in situ* through the secretion of soluble mediators such as nitric oxide (65), prostaglandin, transforming growth factor-beta or IL-10 (64, 66, 67). The authors assumed that CD11c⁺ cells with low autofluorescence were myeloid DCs and/or immediate precursors; whereas CD11c⁺ cells with high autofluorescence represent pulmonary Macs.

1.3.3. DCs in Lymph nodes

After Ag uptake, cells migrate to the LN to activate naïve T cells. Currently it is known that there are several cell types within the murine LNs which express MHC class II molecules and have the potential to activate CD4 T cells (68). These are Macs, B cells and DCs. Macs are located in non-lymphoid tissues, in the red pulp of the spleen and in the subcapsular and medullary sinuses of LNs, but not in the T cell areas which indicate that these cells don't play a role as initial APCs (69). Also B cells are not initiated in activating of T cells. Currently, Germain (70) indicated that B cells in the LNs produce lysozyme peptide MHC class II complexes after injection of lysozyme. They are very inefficient at taking up Ags and activating T cells. Therefore probably DCs are the essential APCs for activating naïve T cells. Several DC subsets are found in LNs which have been described previously. To summarize them, there are myeloid DCs which express CD11b, CD4 but not CD8α and CD205 integrin (23, 66, 69, 70). The second type of DC subset is negative for CD11b but express CD8α and CD205. These DCs are referred to as the lymphoid DCs (71). Another known subset - plasmacytoid DCs are also exist in LNs which are CD11c⁺ and B220⁺ but CD11b⁻.

There are another three subset (69) found in LNs. One of them consists of MHC class II^{high}, CD11c⁺, CD11b⁺ and CD8α⁻. Another population is described as MHC class II^{high}, CD205^{high}, CD11b^{low}, CD8α^{int} and expressed Langerhans cell specific protein Langerin. These cells are seldom found in non-skin-draining LNs or in the spleen. The third subset is CD11c^{low} DCs derived from monocytes.

1.4 Macrophages

Macs exhibit immense phenotypic variation engaged in many antagonistic processes as immunogenic and immunosuppressive functions. These cells are participated in tissue destructive but also in reparative processes (72).

Macs are derived from monocytes. They share the same precursors with mDCs until late stages of differentiation (58, 73). The determination of DCs versus Macs differentiation, it is still unclear. In the paper of Gutierrez, they explained that transcription factors Gata1 play a role in the final DCs and Macs separation (58). Additionally, determining cytokines such as Mac colony-stimulating factor (M-CSF) and granulocyte-Mac colony-stimulating factor (GM-CSF) are important factors for growth and differentiation of Macs. Also Interleukin-3 (IL-3), tumor-necrosis factor (TNF)-family proteins, TNF-receptor-related molecules and transcription factors such as PU.1 are important for Mac determination in bone marrow (74).

A progenitor gives rise to Macs which distribute through the blood stream and enter to all tissues compartments of the body. There are resident Mac populations in different organs like Kupffer cells in liver, alveolar Macs in lung or microglia found in central nervous system. Another Mac population is recruited which can elicit non-specific Ags, activate alternative Ag or classical Ag (74). Classical activation which is induced by IFN-γ and LPS, and leads to a high microbicidal activity (75). Macs cultured *in vitro* with IL-4 or IL-13 increase the endocytic activity, the expression of mannose receptor, but also the cell growth, tissue repair and parasite killing which indicate as alternative activation. Innate activation is mediated in culture by Toll-like-receptor (TLR) (34, 76) and is associated with microbicidal activity and cytokine production (77, 78). Another role of Macs is the

deactivation by IL-10 or TGF- β which reduced the expression of MHC class II molecules (77).

Alveolar Macs in the lung have a distinctive phenotype. They express high levels of pattern-recognition receptors (PPRs) such as mannose receptor, scavenger receptor-A (SR-A) and the β -glucan receptor. The target for these receptors is called pathogen-associated molecular patterns (PAMPs) (79). Phagocytic receptors are important to discriminate between infectious agents and self-antigens. Pulmonary Macs also secrete large amount of lysozyme (74). They have a high expression of pro-inflammatory cytokines and secrete various immunosuppressive products; therefore they are important for innate defense of the airways and modulate lung reactivity in acquired immunity. In the gut, there are the richest sources of Macs in the body whereas in the Lamina propria, there are the unique Mac phenotypes which have high phagocytic and bactericidal activity but weak production of pro-inflammatory cytokines (77, 80).

As known, microbial and cytokine milieu lead to a polarized functional properties of Macs which can diverse these cells in two classes. There are class M1 which is activated by IFN- γ , GM-CSF, TNF and microbial products like LPS; and is characterized by IL-12^{high}, IL-23^{high} and IL-10^{low} phenotype (81). M1 produces large amount of nitric oxide which inhibits cell replication and pro-inflammatory cytokines. Macs M1 are both inducer and effector in polarization of Th1 immune response and also occur in mediation of resistance against intracellular parasites and tumors (82). The second class of Macs is called M2 which is actually a name for various forms of Mac activations. These cells express IL-4, IL-13, IL-10 and glucocorticoid hormones; have an IL-12^{low}, IL-23^{low} and IL-10^{high} phenotype (78, 81, 82). Class M2 is characterized by a high production of scavenger- (83), mannose- and galactose-type receptors and has Th2 immune activity which includes immunoregulation, angiogenesis, tissue remodeling, tumor promotion and parasite encapsulation.

1.5 B cells and eosinophils

Another airway APCs are B cells which expressed high levels of DC marker CD205 and CD11b (66, 84). B cells isolated either from lung parenchyma or main conducting airways present Ags and activate T cells *in vitro*. This suggests that B cells may also be potential contributor to T cell activation within the airway mucosa. As mention before, APCs capture Ags by receptor mediated uptake, but only DCs and Macs are able to take up fluid Ags through endocytosis. This pathway is inefficient in B cells.

Another candidate of APCs in allergic airways is eosinophils. Eosinophils are recruited in allergic disease to inhaled Ags but can also be function as APCs. Weller and colleagues (11, 85) examined the migration of fluorescent labeled eosinophils from within the trachea into regional LNs. They established that eosinophils exposed to aerosolized Ags have the capabilities to migrate from the airway Lumina into regional LNs and like a professional APCs, they are able to stimulate naïve T cells. In summary, they can interact directly with inhaled Ags within the airways (11, 17, 85). These cells have also the capacity to cooperate with particulate Ags like other APCs.

1.6 Mice using as asthma model

Mice are the most widely used species in experimental asthma research because of their availability of immunological reagents and transgenic strains. Mouse models are not able to reproduce all the features of human allergic disease after sensitization and respiratory tract challenges with Ag, but they develop syndromes which have similarities with allergic asthma such as eosinophilic lung inflammation, AHR, increased IgE, mucus hypersecretion and airway remodeling (86). BALB/c and C57BL/6 are simple mice model which is used in the laboratory (4, 86).

1.7 Aims of the study

In this study, we were interested in characterization of APCs at early time points after i.n. instillation with soluble and particulate Ags. As soluble Ags we used OVA conjugated with fluorochrome, whereas for particulate Ags we took India ink particles.

First, we were interested to determine the phenotype and morphology of APCs by analyzing with surface markers and May-Grünwald Giemsa. In addition, we investigated the ability of cells taken up both soluble and particulate Ags. Secondly, we wanted to separate Macs and DCs by using their adhesion properties. Finally, our study was concentrated on the pathway of cells taking up inhaled Ags in the airway.

2. Methods

2.1 Animals

BALB/c and C57Bl/6 female mice at the age of 6 - 8 weeks were used in the experiments. They were obtained from Charles River Laboratories (Germany). Mice were provided ovalbumin (OVA)-free food and water ad libitum. All experiments complied with the requirements of the Animal Care Committee of the Austrian Ministry of Science.

2.2 Intranasal administration of soluble OVA conjugated to fluorochrome

The animals were anesthetized with 100 µl mixture of 2 ml 2% Rompun solution (Bayer AG, Leverkusen) and 10 ml Ketanest (BayerAG, Leverkusen; Pfizer) dissolved in 18 ml distilled water. When mice became paralyzed, they were instilled intranasal (i.n.) either with 50 µl 1 x PBS (as control) or with 50 µl OVA–AlexaFluor594 (10 µg/ 50 µl). Chicken egg OVA–AF594 was purchased from Molecular Probes (USA). After either 1 h or 4 h of i.n. instillation, mice were administered a lethal dose of 500 - 1000 µl of Rompun-Ketanest (Figure 1).

2.3 Preparation of bronchoalveolar lavage fluid cells

According to the experimental procedure bronchoalveolar lavage (BAL) fluid was performed after i.n. instillation at either 1 h or 4 h by flushing lungs with 500 µl of 1 x PBS 4 times using a pediatric i.v. catheter. BAL fluid was collected and lysed with 500 µl FACS Lysing Solution (1:10) to lyse red blood cells (RBC). After 10 min centrifugation at 1200 rpm, supernatant was removed and pellet was resuspended with 1% BSA (Albumin, Bovine, Fraction V purchased from Sigma) mixed with 1 x PBS. Pellet was washed 3 times with 1% BSA in 1 x PBS. BAL fluid cells were stained

with Trypan blue (1:1 dilution) to exclude death cells to determine the total cell count with a hemacytometer - Neubauer chamber under light microscope (Nikon Y-IDP). A total cell number either of either 1×10^4 or 1×10^5 cells were resuspended in 100 μ l 1% BSA in 1 x PBS and centrifuged for 5 min at 8000 rpm with Cytospin II (Thermo Shandon) machine. Then the slides were kept in dark at RT. These prepared cells were used for the next experimental steps as immunofluorescence - or May-Grünwald Giemsa staining.

2.4 I.n. administration of India ink

To observe the difference of the uptake between soluble and particulate Ags, India ink which was purchased from Windsor & Newton, was used as particle. A dilution of 5%, 10% or 20% India ink particles attenuated in 1 x PBS mixed with OVA-AF594 (10 μ g/ 50 μ l) were delivered i.n. to anesthetized mice. After either 1 h or 4 h of i.n. instillation, mice were administered a lethal dose of 500 - 1000 μ l of Rompun-Ketanest (Figure 2).

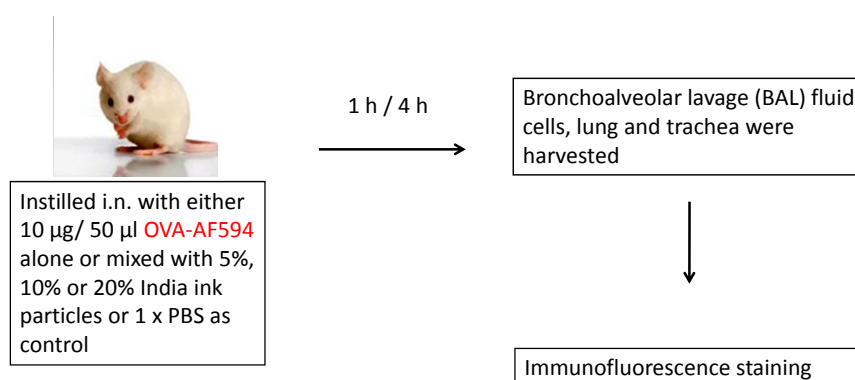


Figure 2. Represent the schematic procedure of i.n. administration of soluble and particulate Ags.

2.5 Harvesting of trachea and lung tissues

After performing BAL, lungs were perfused with O.C.T / PBS (1:1) through the trachea to increase the size of the lungs for a better observation of the inner phase under microscope. To embed lung and trachea tissues, they were transferred into plastic cassettes which were filled with Tissue-Tek O.C.T, a substance which is similar to wax, and stored in liquid nitrogen. Tracheas were cut twice horizontal before freezing.

2.6 Immunofluorescence microscopy

To investigate the phenotype of APCs in BAL fluid and in tissues, we prepared BAL cytopsin slides and frozen tissue sections (5 μ m) which were kept at RT for 10 min and fixed with acetone for another 10 min at 4°C. The slides were washed with 1 x PBS. Primary monoclonal antibodies (mAbs) were added and incubated at 37°C for 1 h. After 1 h incubation, slides were washed with 1 x PBS and secondary mAbs were added for another 35 min in dark at RT. Nuclei were then stained with 50 μ l DAPI (1:500) diluted in 2% BSA in 1 x PBS for 10 min. Slides were evaluated by a Nikon optical fluorescence microscope. The following mAbs were purchased from Serotec: A3-1 (rat anti-mouse F4/80), N418 (anti-mouse CD11c, hamster IgG), MR5D3 (rat anti mouse CD206); from BD Pharmingen: RM4-5 (purified rat anti-mouse CD4), 1A8 (anti-mouse Ly6G, rat IgG2a), 2.4G2 (anti-mouse CD16/CD32, rat IgG2b), RA3-6B2 (rat anti-mouse B220), M1/70 (anti-mouse CD11b, rat IgG2b), 2G9 (rat anti-mouse I-A/I-E); from ACRIS: MOMA-2 (anti-mouse MOMA-2, rat IgG2b), FA-11 (anti-mouse CD68, rat IgG2a), NLDC 145 (rat anti-mouse CD205), rat anti mouse CD8 α . Monoclonal Abs were primarily conjugated with FITC, biotin, or unconjugated. Unconjugated mAbs were visualized using secondary mAbs, goat anti-rat AF-488 (Molecular Probes) or goat anti-hamster AF-488 (Molecular Probes). All mAbs were diluted in 2% BSA in 1 x PBS.

2.7 May-Grünwald Giemsa staining

Prepared BAL cytopsin slides kept at RT were fixed with methanol (Merck) for 5 min. Then, slides were incubated in around 3 - 4 ml May-Grünwald (Merck) for 15 min. Slides were washed carefully with deionised water. In the next step, cells were stained with 10% Giemsa (Merck) for another 20 min, and then washed with deionised water. Slides were air dried and allowed us to observe the cells under light microscope. This staining solutions which contained methylene blue, azure and eosin dye nuclei, granules of basophile and eosinophil and also red blood cells. Therefore, it gives us an idea with which cells we were confronted with.

2.8 Adoptive cell transfer

Using a similar experimental approach as described in 2.1, recipient mice were instilled i.n. with a 1:10 dilution of OVA-AF488 (50 µl) on day 1. After 24 h, recipient mice were divided in two groups. One group received i.n. BAL fluid cells from donor mice, which were instilled with OVA-AF647. These mice were sacrificed 1 h after instillation. Lung and mediastinal LNs (mLN) were harvested for FACS. The second group of the recipients was instilled i.n. with BAL fluid cells from donor mice, which received OVA-AF594. After 24 h, BAL fluid cells, lung and mLNs were taken for further studies (Figure 3).

In the second experimental approach of adoptive cell transfer study, donor C57Bl/6 mice were instilled i.n. with 10 µg/ 50µl OVA-AF594. After 1 h, BAL fluid cells were harvested, counted and instilled i.n. (8×10^4 cells/ 50µl) into recipient mice. After 23 h, recipients received another dose of OVA-AF488 (10 µg/ 50µl) i.n. and we waited for another 1 h before harvesting BAL fluid cells, lung, trachea and LNs (Figure 4).

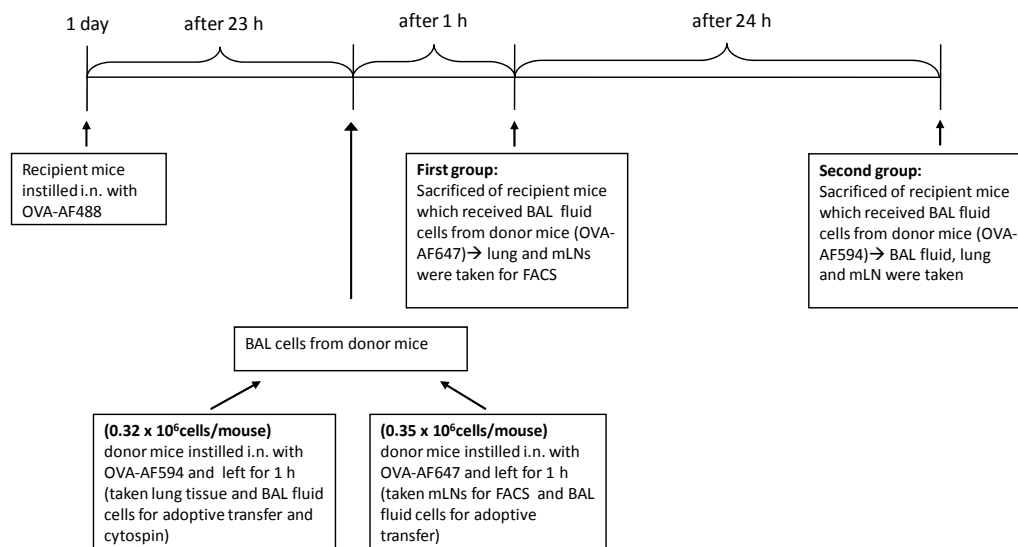


Figure 3. Represent the schematic experimental approach of adoptive cell transfer study

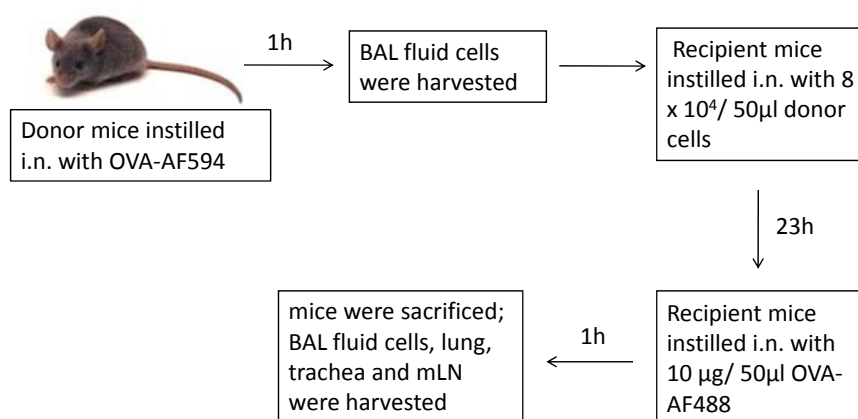


Figure 4. Represent the schematic experimental approach of the second adoptive cell transfer study.

2.9 Analysis of mLN by flow cytometry

MLNs were isolated from recipient mice and placed in petri dishes filled with RPMI (1640 Medium 25 mM Hepes + L-Glutamine; GIBCO) + 5% FCS. MLNs were transferred to a cell strainer filter (70 μ m) and smashed with the bottom of a syringe. Smashed MLNs were rinsed through the filter into a 50 ml falcon which was filled with RPMI + 5% FCS. Cells were diluted with Trypan blue and counted with hemacytometer - Neubauer chamber under light microscope. MLN cell suspensions (100 μ l) were transferred in 96-well plates (7×10^6 cells in each well). Plates were centrifuged for 2 min at 2000 rpm. The pellet was mixed with 5% mouse serum and left for 15 min at RT. After 15 min, 150 μ l of 1 x PBS + 2% FCS were supplemented and centrifuged at 2000 rpm for 2 min. After centrifugation, cells were given 100 μ l of primary mAb and incubated for 30 min on ice in dark. Then, cells were washed with 100 μ l of 1 x PBS + 2% FCS and centrifuged at 2000 rpm for 2 min. Supernatants were discarded before adding the second mAb and incubated for another 20 min. Cells were washed with 1 x PBS + 2% FCS. At last, the pellet was resuspended with 200 μ l 1 x PBS + 2% FCS and transferred to FACS tube for measurement.

2.10 Preparation of lung suspension cells

Isolated lungs were flushed 3 times with 5 ml 1 x PBS + 2% FCS to eliminate red blood cells until the lung slowly turned white. Then, tissues were minced with a scissor in as small as possible pieces before transferring into a falcon filled up with RPMI containing 5% FCS, 2% DNase (important for the cleavage of phosphodiester linkage in the DNA backbone) and 4% Collagenase (to destroy connective tissues), and were incubated in water bath at 37°C for 1 h. After incubation, lung tissues were sucked up with 50 ml pipette und homogenized gently with a glass homogenizer. Suspensions were passed through a 70 μ m strainer into another falcon tube and centrifuged at 1200 rpm for 10 min. The pellets were resuspended in RPMI + 5% FCS and filtered through a 40 μ m strainer. To achieve a density

separation, 13 ml of Lympholyte M (a density separation medium) was added with 10 ml pipette very slowly at the bottom of the cell suspension. The lympholyte gradient was centrifuged without brakes for 20 min at 1500 rpm. The interphase was collected with a 10 ml pipette, resuspended in 30 ml of RPMI + 5% FCS and centrifuged at 1200 rpm for 10 min. After centrifugation, pellets were lysed with 1 ml lysis buffer for 2 min on ice by continuous pipetting. The lysis reaction was stopped with the addition of RPMI + 5% FCS, and then centrifuged at 1200 rpm for 10 min. Pellets were resuspended again in RPMI + 5% FCS. The number of cells was counted with hemacytometer - Neubauer chamber under light microscope. To exclude dead cells, cell suspensions were mixed with Trypan blue with a 1:1 dilution. These isolated lung suspension cells were either used for adhesion experiment or for cytospin.

2.11 Preparation of glass cover slips for BAL fluid - and lung suspension cells

Several cover slips were incubated in sterile cell culture dishes filled with prepared medium which contained RPMI, 5% FCS and 1% Penicillin-Streptomycin (P/S) at 37°C with 5% CO₂ overnight. Cells were transferred onto glass cover slips in which parafilm slices were plated to prevent an overflowing of the medium containing the cells.

2.12 Cell transfer to glass cover slips

According to the experimental procedure explained in 2.1., 200 µl of our stock cell solution (0.1×10^6 cells / cover slip) was transferred to the prepared cover slips and was incubated for either 2 h or 24 h at 37°C with 5% CO₂ (Figure 3). After incubation, the cover slips were washed twice with 500 µl RPMI mixed with 5% FCS and 1% P/S. During the washing process non adherent cells were collected and transferred to another 50 ml falcon filled with RPMI, 5% FCS and 1% P/S. Cover slips were placed in a 6-well plates containing 2 ml 1 x PBS and stored at 4°C in dark.

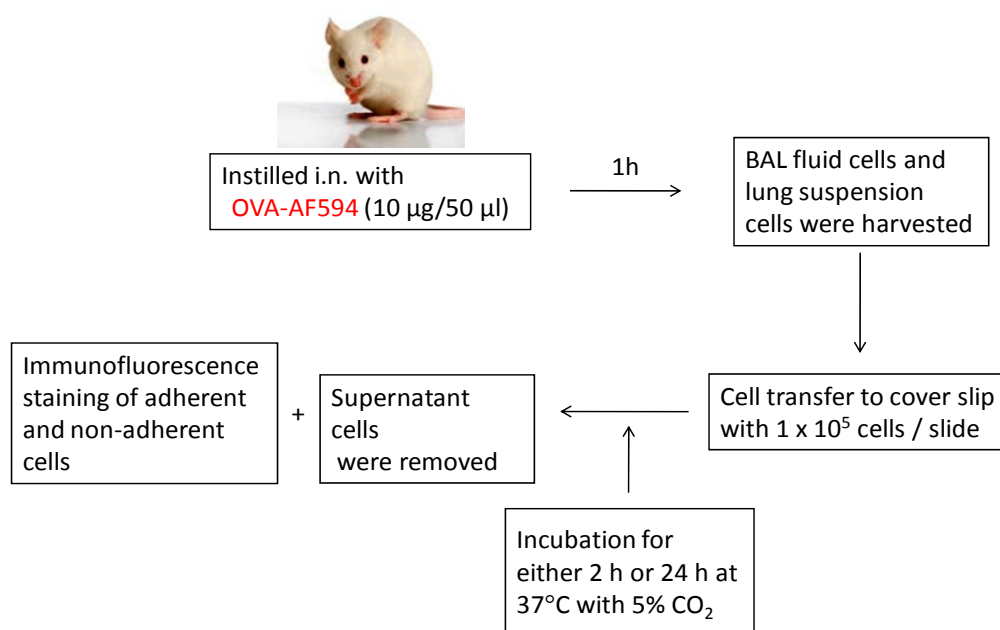


Figure 5. Represent the schematic approach of adhesion experiment

2.13 Fixation of adherent cells

Formaldehyde (Accustain R Formalin solution; SIGMA, 1 ml x 3.7%) was pipetted into each well where cover slips resided and were incubated for 10 min. After removing formaldehyde, cover slips were washed twice with 2 ml 1 x PBS for 2 min and twice with 1 ml deionised water for 2 min. Then, slides were kept in 1 x PBS, packed with aluminium foil and stored at 4°C for further use.

2.14 Preparation of adherent cells for fluorescence microscopy

Cells adhered on cover slips were kept in 6-well plates and fixed with 2 ml acetone for 10 min at 4°C. Then, cells were washed with 2 ml 1 x PBS for 5 min in the dark. Cells on cover slips were stained with DAPI (300µl) and incubated for 10 min. After incubation, cells were washed first with 1 x PBS for 1 min then twice with 2 ml deionised water for 1 min. Cover slips were transferred upside down onto a slide with one drop of FA mounting fluid (DIFCO) and fixed with fixogum.

2.15 Preparation of non-adherent cells for fluorescence microscopy

Non-adherent cells were obtained during the washing process of cover slips incubated either for 2 h or 24 h. These cells were pooled and prepared for cytopsin, as described previously in 2.3. Briefly, non-adherent cells were centrifuged for 10 min at 1200 rpm. Then, pellets were resuspended with RPMI and 5%FCS. Cells were stained with Trypan blue and counted with hemacytometer- Neubauer chamber to receive a total cell number. Next, 1×10^4 cells were resuspended in 100 µl RPMI + 5% FCS and centrifuged for 5 min at 8000 rpm with Cytospin II. Slides were kept in dark at RT to dry. Later, we used Rot®Liquid Barrier Marker (Carl Roth) to mark the cells on the dried slides and fixed with acetone for 10 min at 4°C, then washed with 1 x PBS for 2 min in dark. Cells were stained with 50 µl DAPI for 10 min. After incubation, cells were washed first with 1 x PBS for 1 min and then with deionised water also for 1 min. Finally we put a drop of Fluorprep on cells, covered with a 10 x 23 mm cover slip and fixed with fixogum.

3. Results

3.1 Ag uptake *in situ* lung and airway at early time points after i.n. instillation with OVA-AF549

Our first approach was to determine cells engulfed in uptake of Ag and migrated into respiratory tract at early time points. Therefore BALB/c mice were instilled i.n. with 10 µg/ 50 µl of OVA (OVA is commonly used to stimulate an allergic reaction) conjugated with fluorochrome. After either 1 h or 4 h, mice were sacrificed; BAL fluid cells and lungs were harvested. Bronchoalveolar lavage is a technique which is often used experimentally to sample the cellular or biochemical components of the conducting airways and lung alveoli. It is carried out by putting a tube in the trachea and rinsing the downstream compartment of saline. Lung frozen sections and BAL cytopsin slides were analyzed by fluorescence microscope to demonstrate the locations of Ags.

We found an accumulation of OVA⁺ cells *in situ* lung and in BAL in large quantities (Figure 6) at 1 h and remained present after 4 h. Additionally, to determine the quantity of OVA⁺ cells in BAL after 1 h and 4 h of i.n. instillation, BAL cytopsin slides were prepared and counterstained nuclei with DAPI. OVA-AF594⁺ cells were counted and the percentage was calculated. The calculation process was performed by counting the cells on pictures taking by fluorescence microscope and the average was examined. We observed that the percentage of OVA⁺ cells in BAL did not change in large quantities within the first 4 hours (Figure 7). Out of total DAPI⁺ cells, there are 40~45% BAL cells which took up OVA-AF594 soluble antigen.

Figure 6.

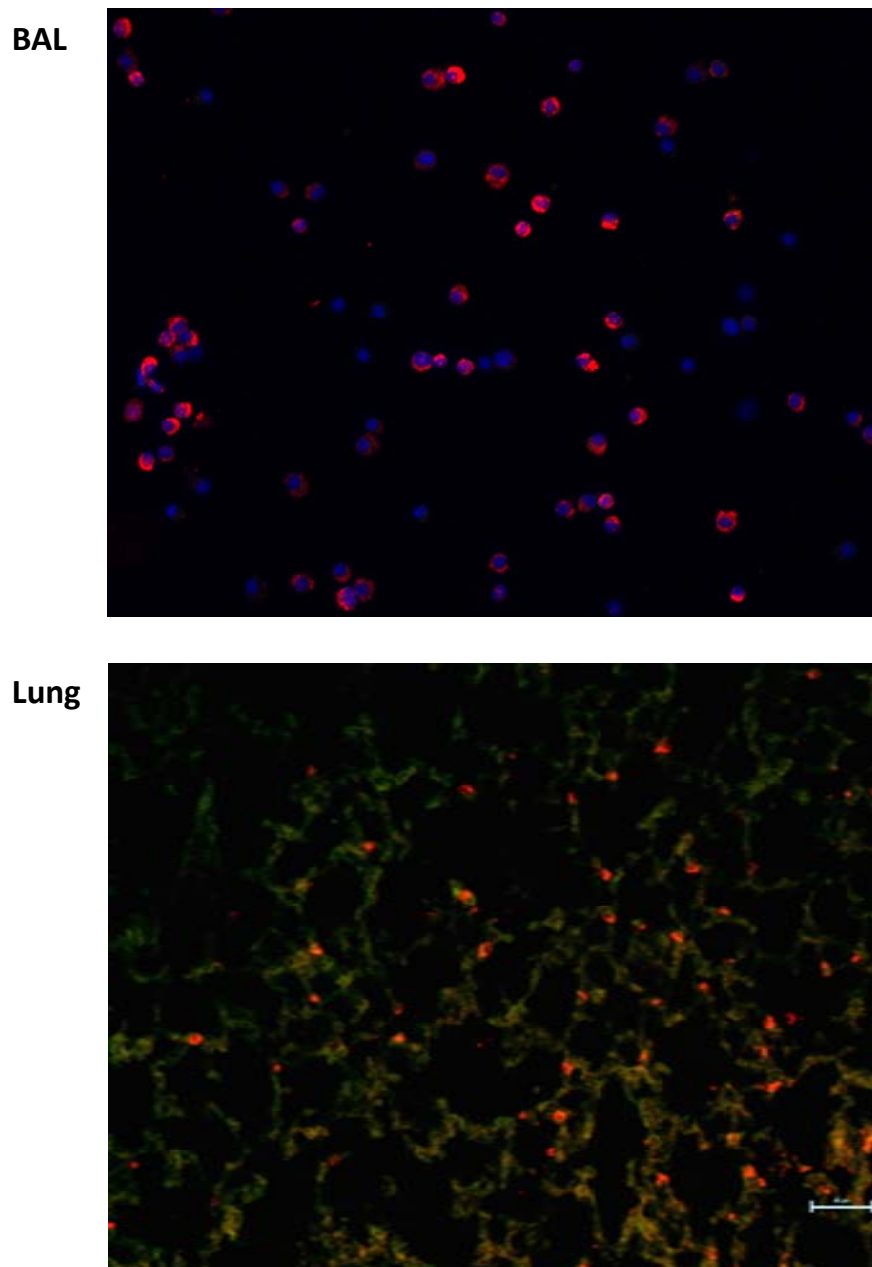


Figure 6. OVA^+ cells are present in BAL and in situ lung. BALB/c mice were instilled *i.n.* with 10 μ g/ 50 μ l of OVA-AF594. After 1 h, BAL was performed with 4 x 1ml of 1 x PBS. Lung tissues were flushed with O.C.T. and were frozen in nitrogen. Cells in BAL were stained with DAPI and lung frozen sections (5 μ m thick) were stayed unstained. Pictures were taken either with 10x or 20x objective of the fluorescence microscope.

Figure 7.

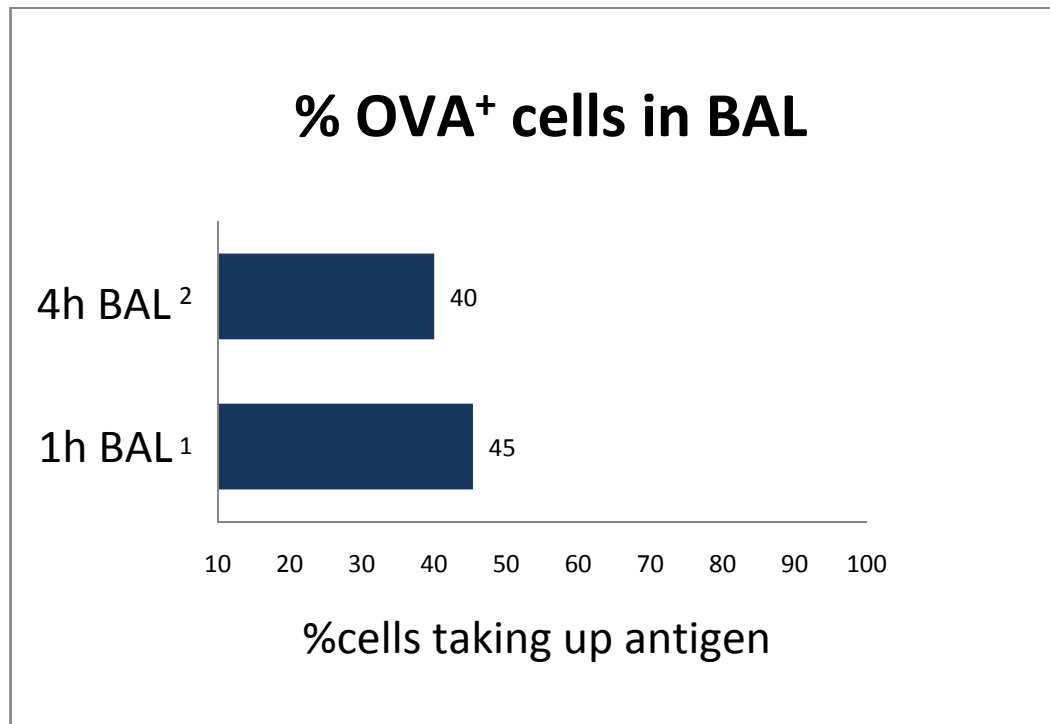


Figure 7. Percentage of OVA⁺ /DAPI⁺ cells. Lightly anesthetized mice were administered i.n. 10 µg/ 50µl OVA-AF594. After either 1 h or 4 h, BAL cells were harvested by flushing 4 x with 0.5ml 1 x PBS. Collected BAL cells were pooled and cytopspin was performed - each slide contained 1 x 10⁴ cells. Nuclei were stained with DAPI. Pictures were taken by fluorescence microscope with a magnification of 10x. OVA⁺ cells were counted versus DAPI positive cells. One representative experiment of three is shown.

3.2 Morphology of BAL cells following fluorescent-labeled OVA inhalation

To determine the morphological features of BAL cells, mice were instilled i.n. with 10 µg/ 50 µl of OVA-AF594. After 1 h, BAL fluid cells were collected by flushing 4 x with 0.5 ml of 1 x PBS. Cytospin slides with 1×10^5 cells were prepared to perform May-Grünwald Giemsa staining (Figure 8).

We observed that majority of cells have morphology of Macs which contain a big nucleus with large cytoplasm.

Figure 8.

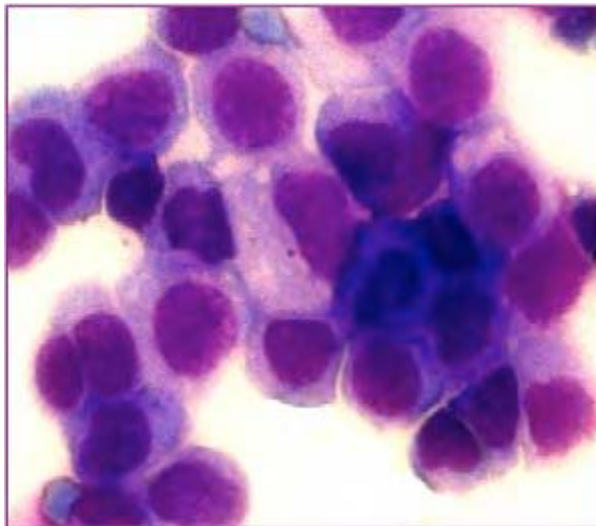


Figure 8. Morphology of BAL cells exhibit Macs structure. Lightly anesthetized mice were instilled i.n. with 10 µg/ 50 µl of OVA-AF594. After 1 h of i.n. instillation, BAL cells were collected by canulating 4 x with 0.5 ml of 1 x PBS. Cells were cytospun on slides, stained with May-Grünwald Giemsa and were analyzed by light microscope at 40x magnification.

3.3 Characterization of APCs after 1 h of i.n. instillation in BAL

To identify the phenotype of APCs which are engulfed in uptake of Ag in the respiratory tract at early time point we used common markers as CD11b which consists α -chain of the $\alpha_M\beta_2$ or CD11b/CD18 integrin also known as Mac-1 or complement receptor 3, is generally considered as a typical myelomonocytic marker (64, 87). But it is also expressed on Macs and eosinophils. Other surface markers are MHC class II (expressed on all APCs), Gr-1 (marker for granulocytes and pDCs) and B220 (found on B cells and pDCs). We further used CD11c (a DC and Mac marker), F4/80 (a 7-transmembrane protein expressed by some Mac subsets and Langerhans cells) or CD205, which is a multilectin receptor involved in glycoprotein uptake and known as a marker for DCs (64). CD68 recognized by FA-11 express on Macs and Langerhans cells (88) and CD206, a Mac marker also provided for the characterization of APCs (Table 1).

At 1 h and 4 h after instillation of Ag, we examined that OVA⁺ cells from BAL were negative for CD11b, MHC class II, Gr-1 and B220 (Figure 9.A and 9.B). Referring to this observation, we could exclude Granulocytes, B cells and probably pDCs from the subpopulation of early APCs in the respiratory tract. In turn, we observed that OVA⁺ cells were positive for CD11c, CD205 (Figure 10.A), F4/80 and MOMA-2 (Figure 10.B). MOMA-2 mAbs express in Macs and monocytes but low in DCs. To continue characterization, APCs were stained with surface markers CD68, CD206 (Figure 10.C) and FcγRII/III which expressed on all APCs (Figure 10.D) revealed that a majority of cells were double positive –OVA⁺ and positive for certain markers.

Table 1.

Surface marker	Cell population
I-A/ I-E (MHC class II)	Macs, DCs, Eosinophils
CD11c	Macs, mDCs
CD11b	Macs, mDCs, Eosinophils
F4/80	Macs, Langerhans cells
B220	Macs, pDCs, B-cells
MOMA-2	Macs, DCs ^{low} ,
DEC205 (CD205)	DCs
CD206	Macs
FcRγIII/II(CD16/CD32)	Macs, DCs
Gr-1	Granulocytes, pDCs
CD68	Macs, Langerhans cells ^{low}

Table 1. Surface markers used in characterization of APCs. To identify the phenotype of APCs, surface markers conjugated with fluorochrome were used to analyze cells by fluorescence microscope.

Figure 9.A

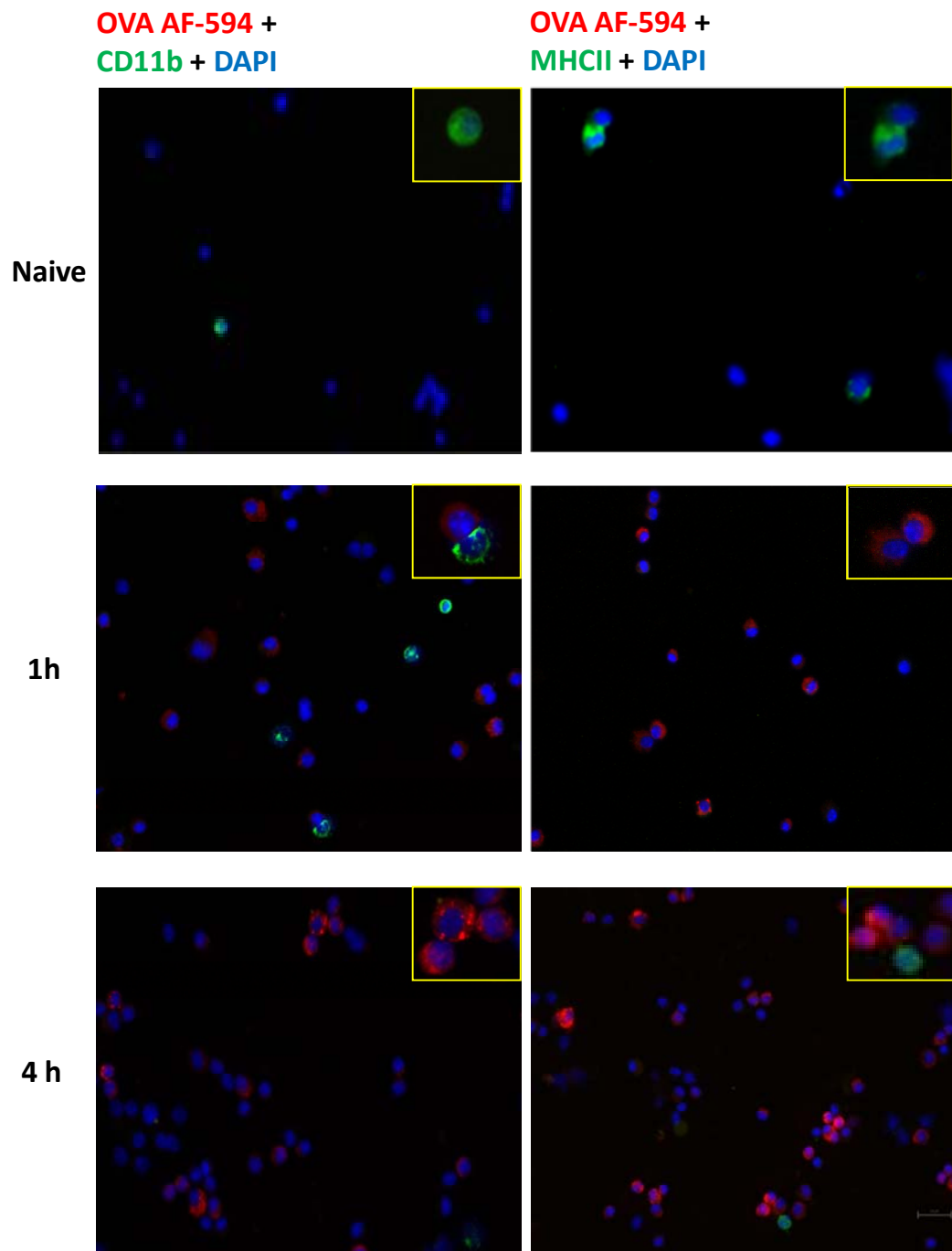


Figure 9.A. Detection of single positive cells in BAL at 1 h and 4 h time points (CD11b and MHC class II). Mice were instilled i.n. with 10 μ g/ 50 μ l of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytopsin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs CD11b and MHC class II. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 9.B

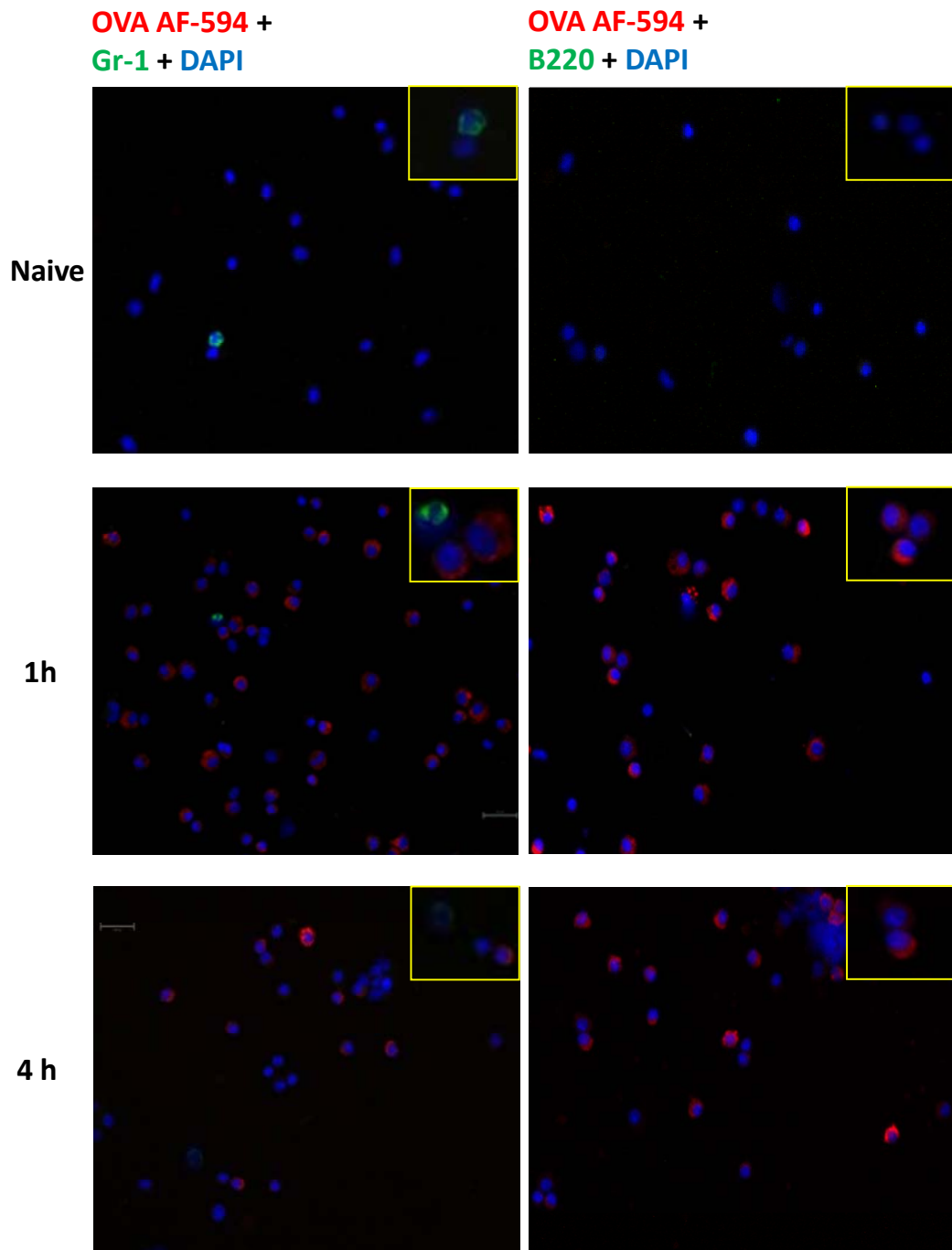


Figure 9.B. Detection of single positive cells in BAL at 1 h and 4 h time points (Gr-1 and B220). Mice were instilled i.n. with 10 μ g/ 50 μ l of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytopspin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs Gr-1 and B220. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 10.A

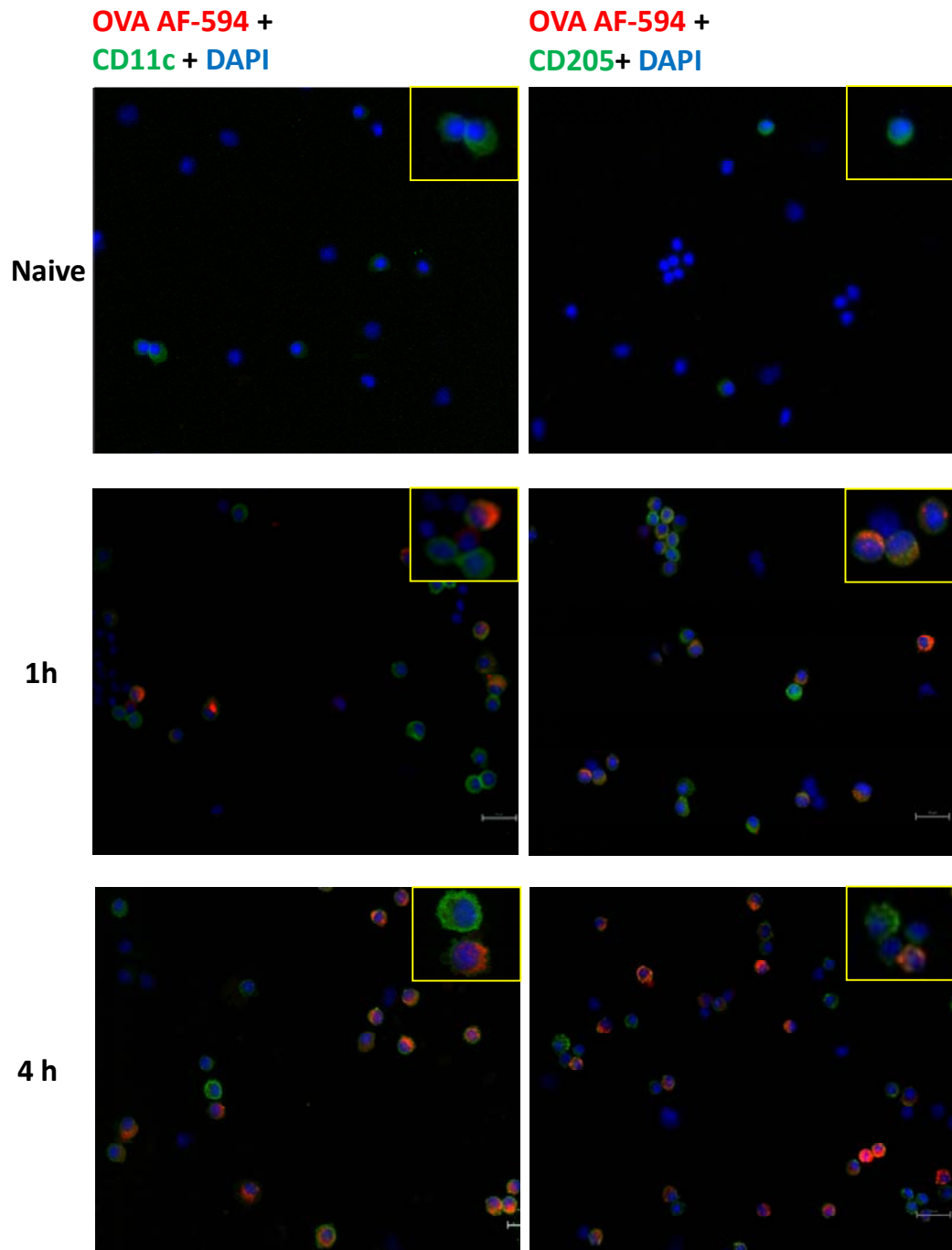


Figure 10.A. Detection of double positive cells in BAL at 1 h and 4 h time points (CD11c and CD205). Mice were instilled i.n. with 10 μ g/ 50 μ l of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytopsin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs CD11c and CD205. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 10.B

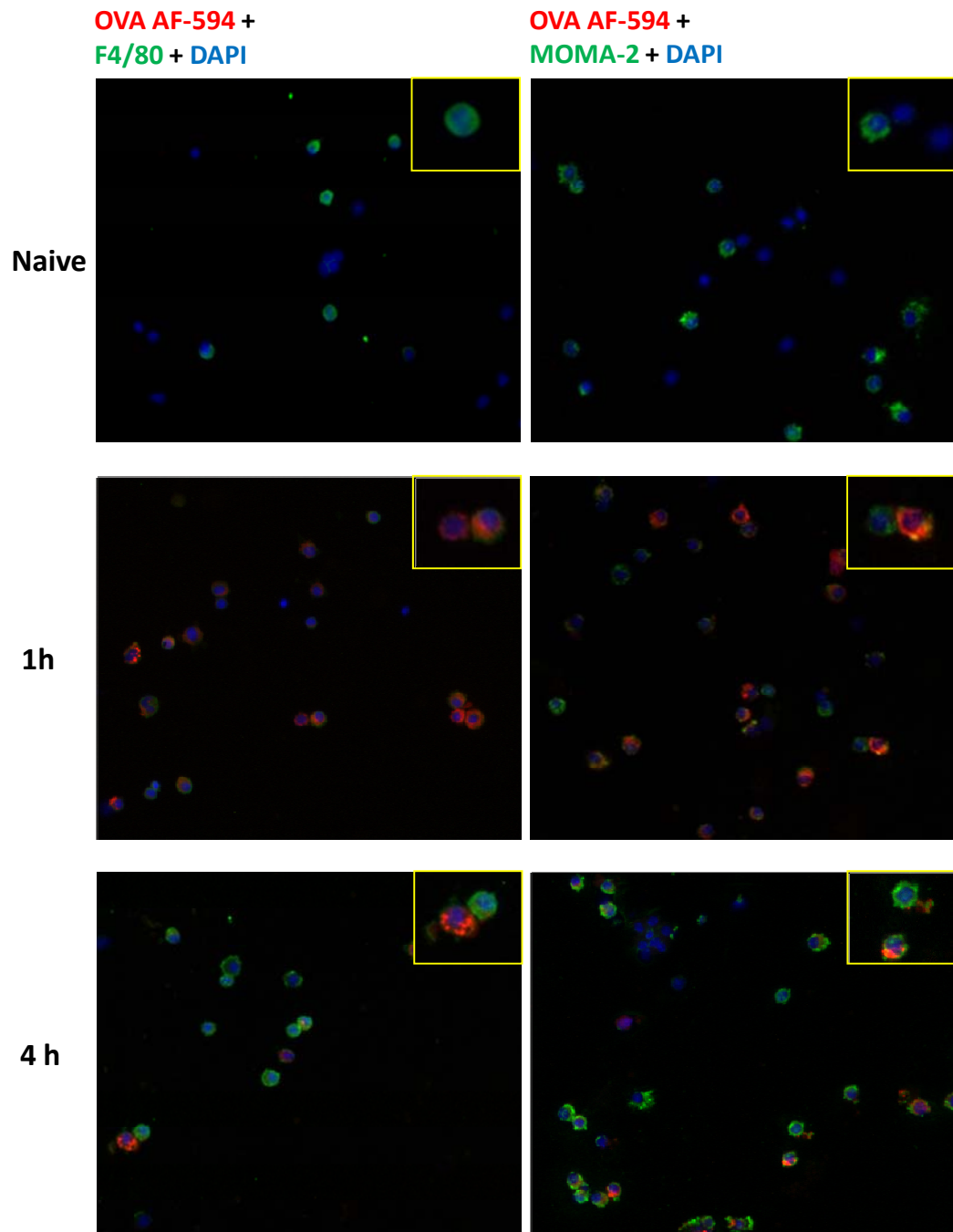


Figure 10.B. Detection of double positive cells in BAL at 1 h and 4 h time points (F4/80 and MOMA-2). Mice were instilled i.n. with 10 μ g/ 50 μ l of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytopsin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs F4/80 and MOMA-2. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 10.C

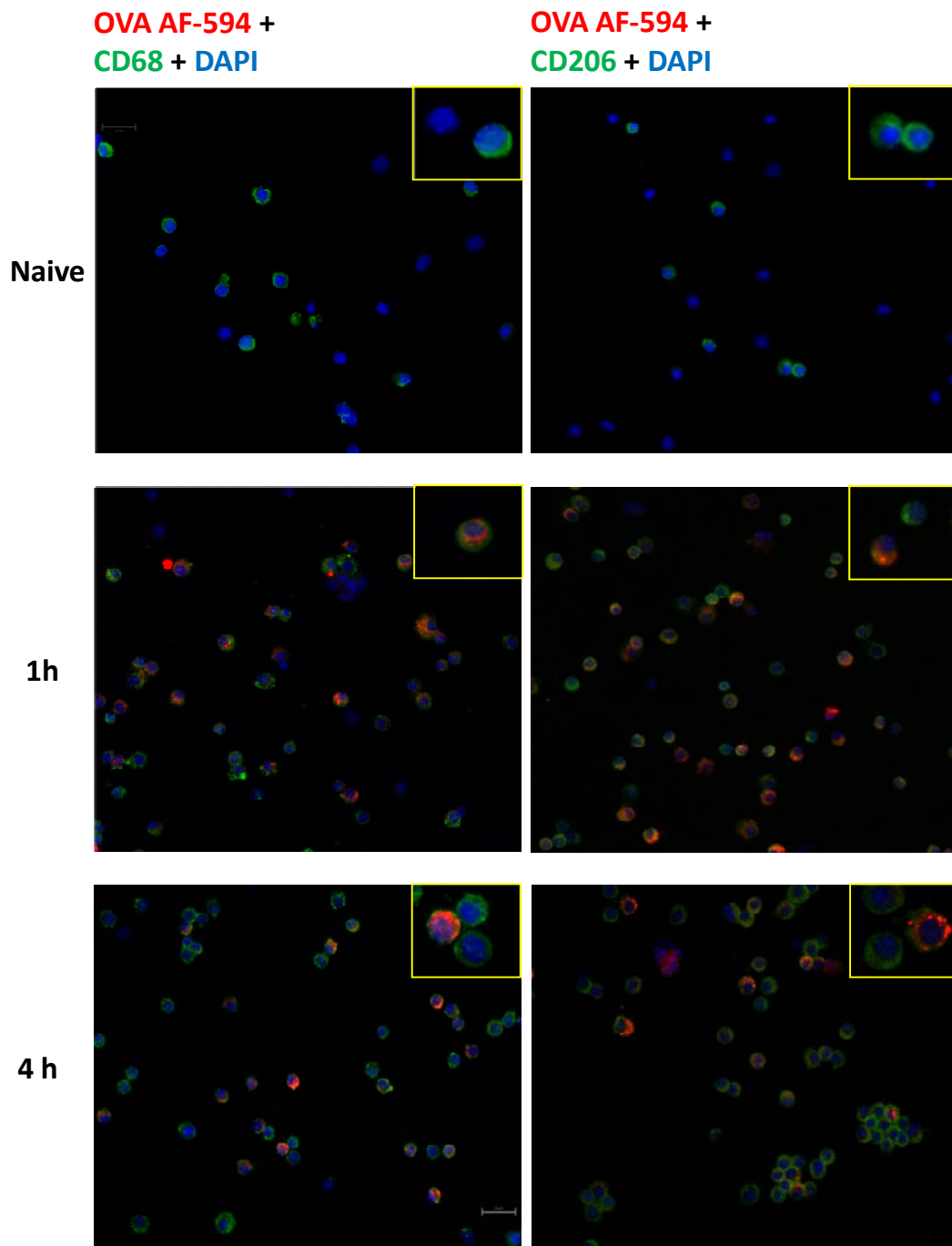


Figure 10.C. Detection of double positive cells in BAL at 1 h and 4 h time points (CD68 and CD206). Mice were instilled i.n. with 10 μ g/ 50 μ l of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytopspin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs CD68 and CD206. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 10.D

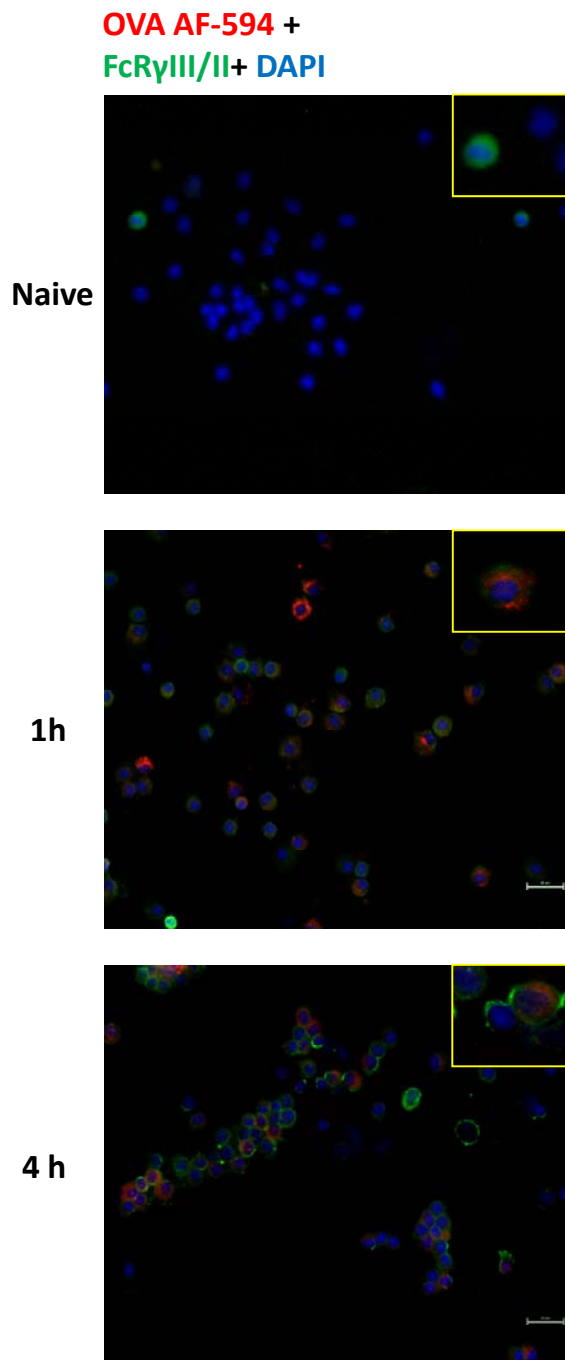


Figure 10.D. Detection of double positive cells in BAL at 1 h and 4 h time points (FcγRII/III). Mice were instilled i.n. with 10 µg/ 50µl of OVA-AF594. After either 1 h or 4 h, BAL was performed and cytospin slides with a total amount of 1×10^4 BAL per slide were prepared and stained with mAbs FcγRII/III. As control, we instilled naïve mice with 50 µl 1 x PBS.

3.4 Characterization of APCs after 1 h of i.n. instillation *in situ* lung

In BAL, there were no surface markers which could characterize the exact phenotype of APCs. Therefore we investigated in lung tissues. To determine the phenotype of APCs localized in lung after either 1 h or 4 h of i.n. instillation with soluble OVA protein, lung frozen sections were stained with the same surface molecules. As in BAL, CD11b, MHC class II (Figure 11.A), Gr-1 and B220 (Figure 11.B) did not express OVA⁺ cells in lung sections. But surface molecules CD11c, CD205 (Figure 12.A), F4/80, MOMA-2 (Figure 12.B), CD68, CD205 (Figure 12.C) and FcγRII/III were detected on OVA⁺ cells (Figure 12.D).

Taken together, APCs in respiratory tract may be either Macs or DCs since OVA positive cells *in situ* and in BAL do express Macs and DCs markers like CD11c and FcR. Markers which are more characteristic for Macs than DCs as F4/80, CD68 or MOMA-2 or typical DC marker CD205 express on OVA⁺ cells. But they are negative for MHC class II and CD11b. These APCs also do not bind to mAbs Gr-1 and B220 (Table 2). Typical Mac and DC markers do not distinguish the cells in the respiratory tract.

3.5 Accumulation of APCs in trachea

In addition, we were interested if we can find OVA⁺ cells in trachea. Therefore trachea was harvested, cut twice horizontal and frozen in nitrogen. 5 μm thick sections of trachea tissue were either unstained or stained with the same mAbs as explained previous and observed by fluorescence microscope.

We observed that cells in trachea could take up Ags (Figure 13) but do not express any APCs markers (data not shown). Cells were located in the inner part of the cell wall.

Figure 11.A

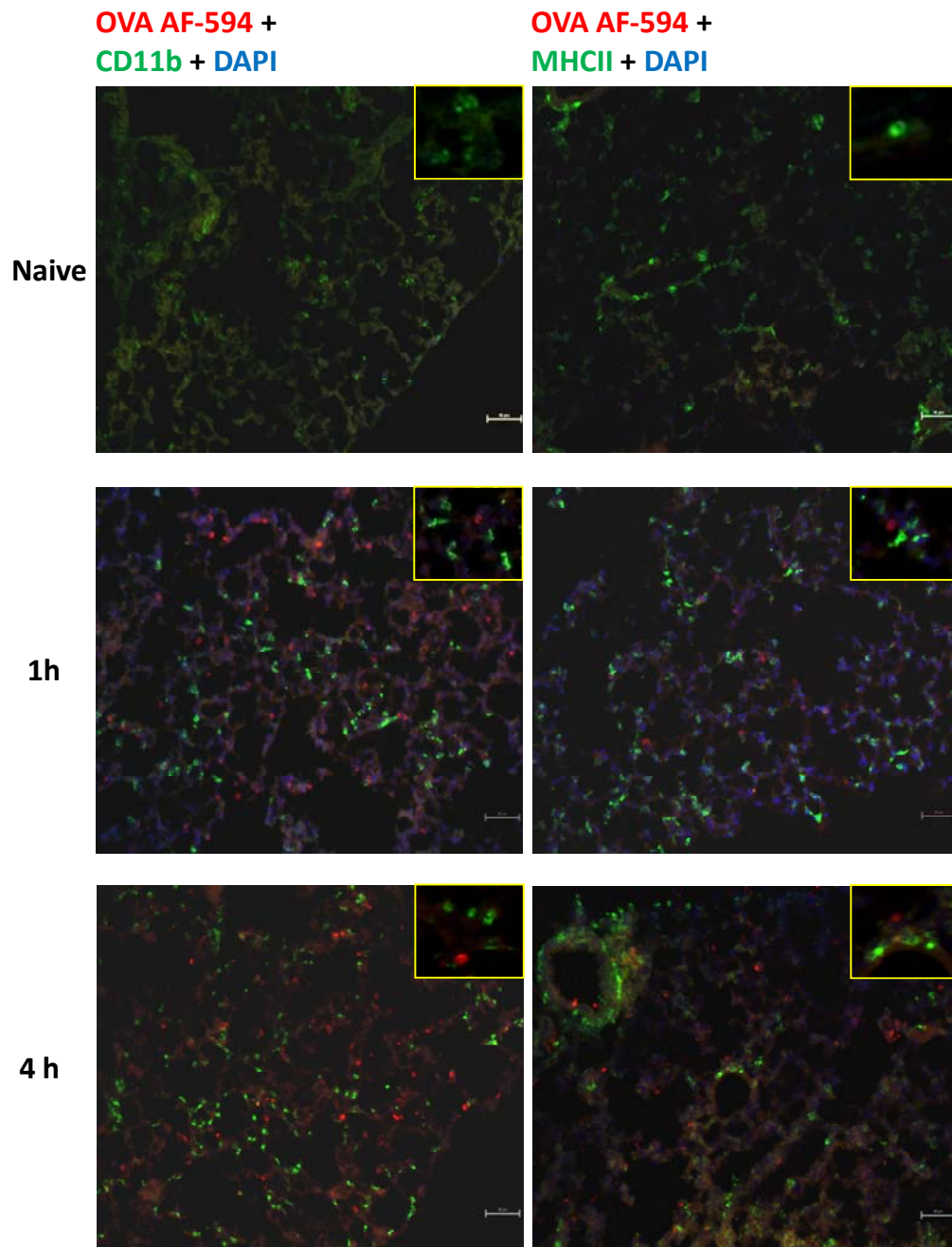


Figure 11.A. Detection of single positive cells in lung at 1 h and 4 h time points (CD11b and MHC class II). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Lungs were perfused with O.C.T to increase the size within the lungs for a better observation and analysis of the cells. Frozen lung tissues were cut 5 μ m thick, stained with DAPI and with mAbs CD11b and MHC class II. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 11.B

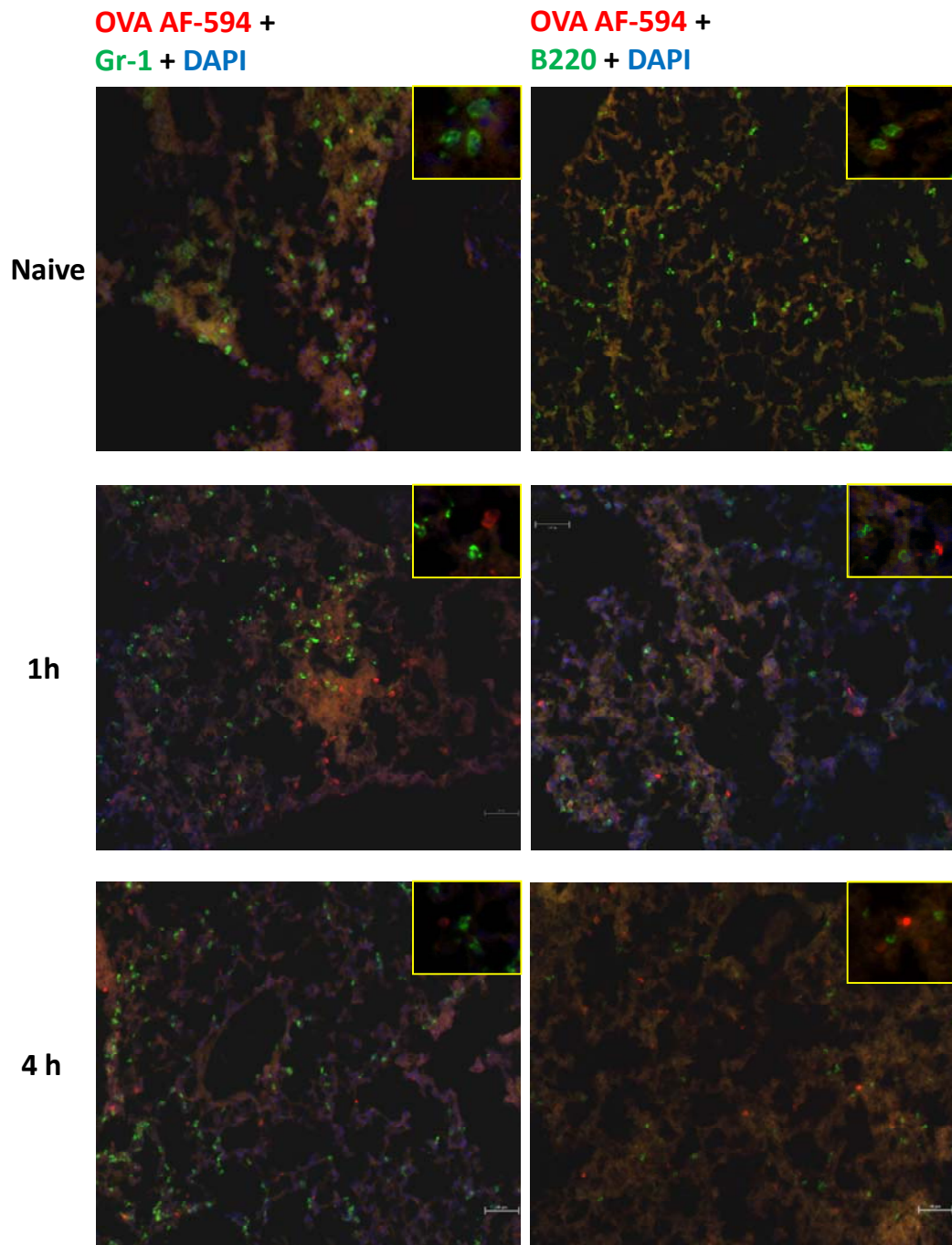


Figure 11.B. Detection of single positive cells in lung at 1 h and 4 h time points (Gr-1 and B220). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Frozen lung tissues were cut 5 μ m thick and stained with DAPI and with mAbs Gr-1 and MHC B220. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 12.A

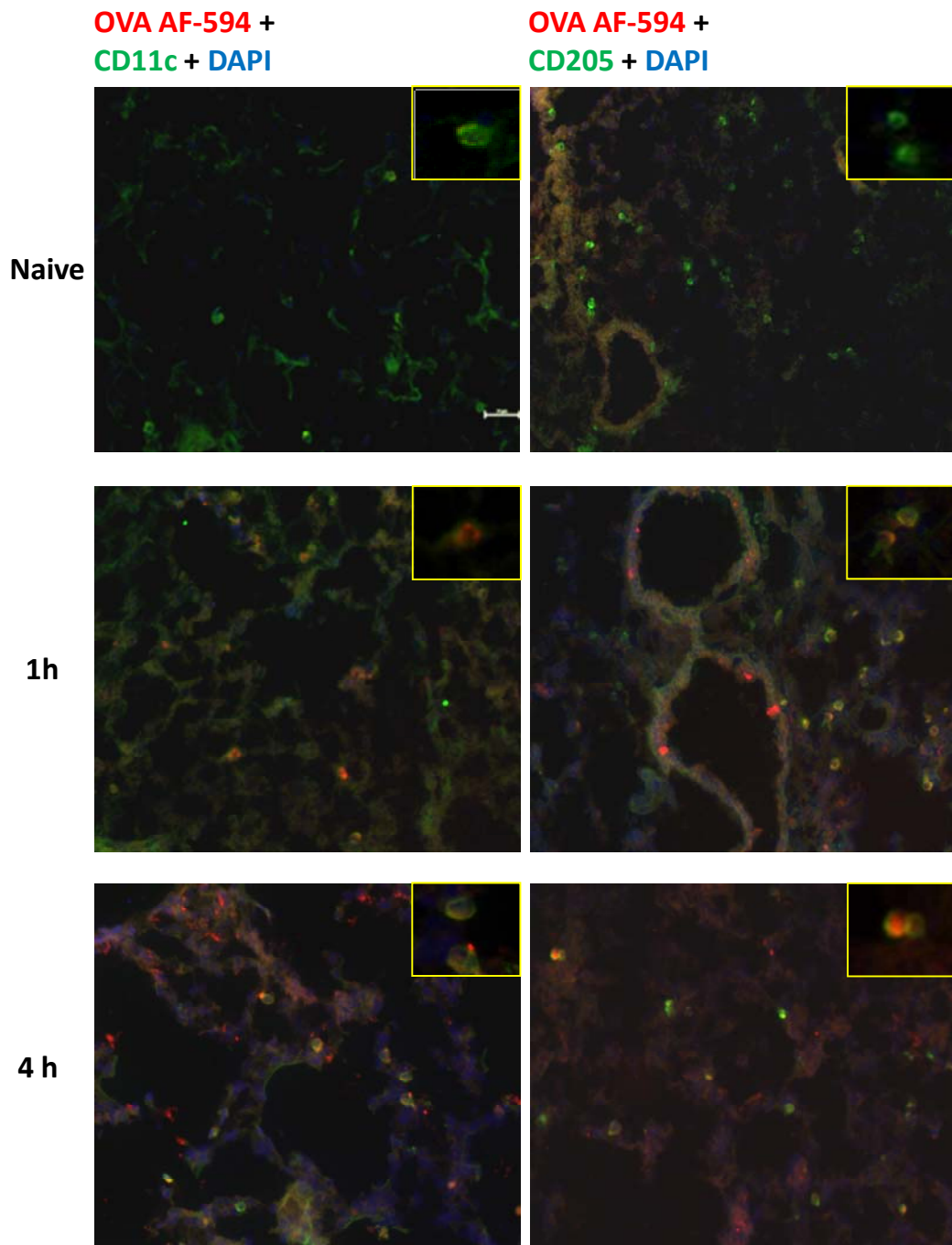


Figure 12.A. Detection of double positive cells in lung at 1 h and 4 h time points (CD11c and CD205). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Lungs were perfused with O.C.T to increase the size within the lungs for a better observation and analysis of the cells. Frozen lung tissues were cut 5 μ m thick, stained with DAPI and with mAbs CD11c and CD205. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 12.B

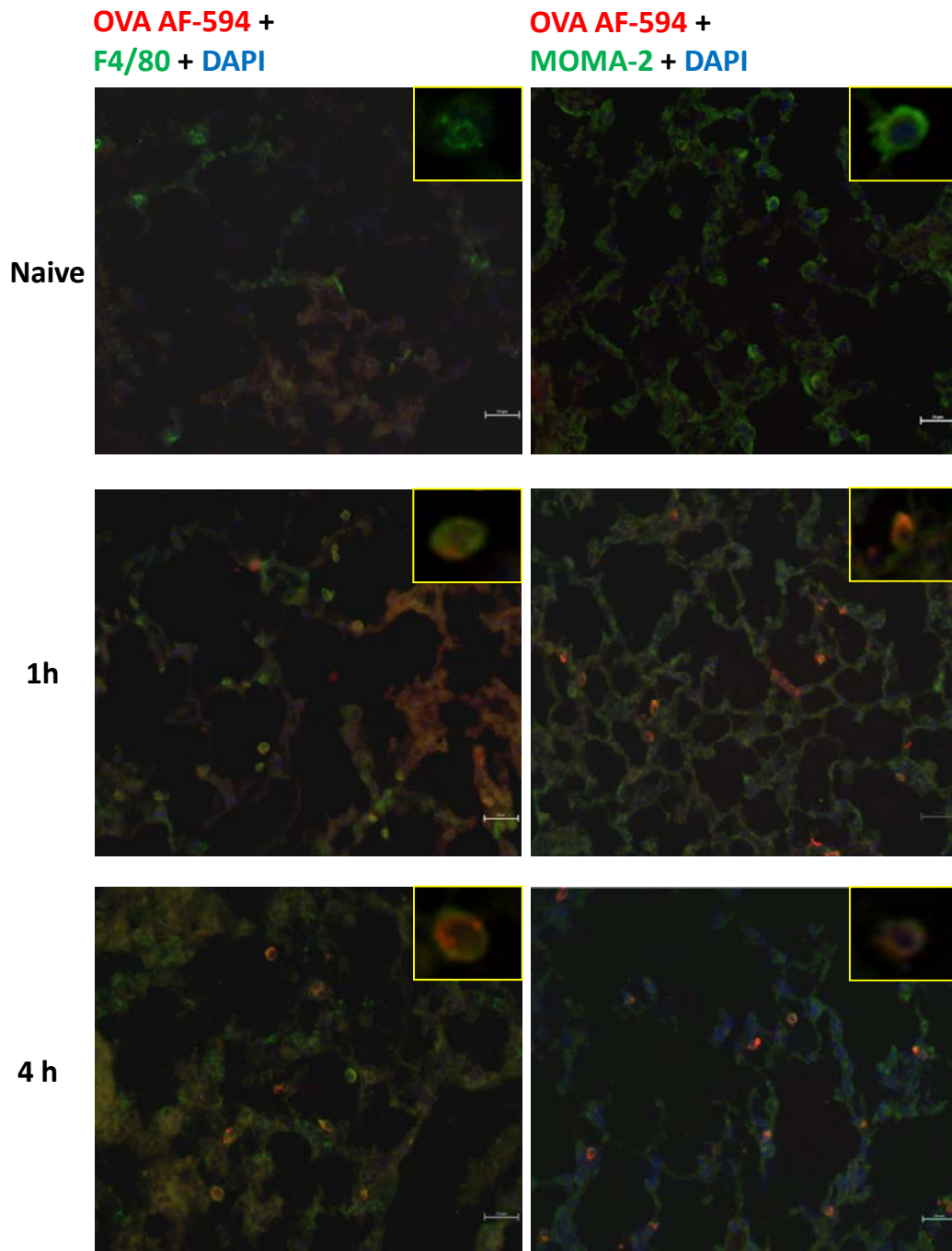


Figure 12.B. Detection of double positive cells in lung at 1 h and 4 h time points (F4/80 and MOMA-2). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Frozen lung tissues were cut 5 μ m thick and stained with DAPI and with mAbs F4/80 and MOMA-2. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 12.C

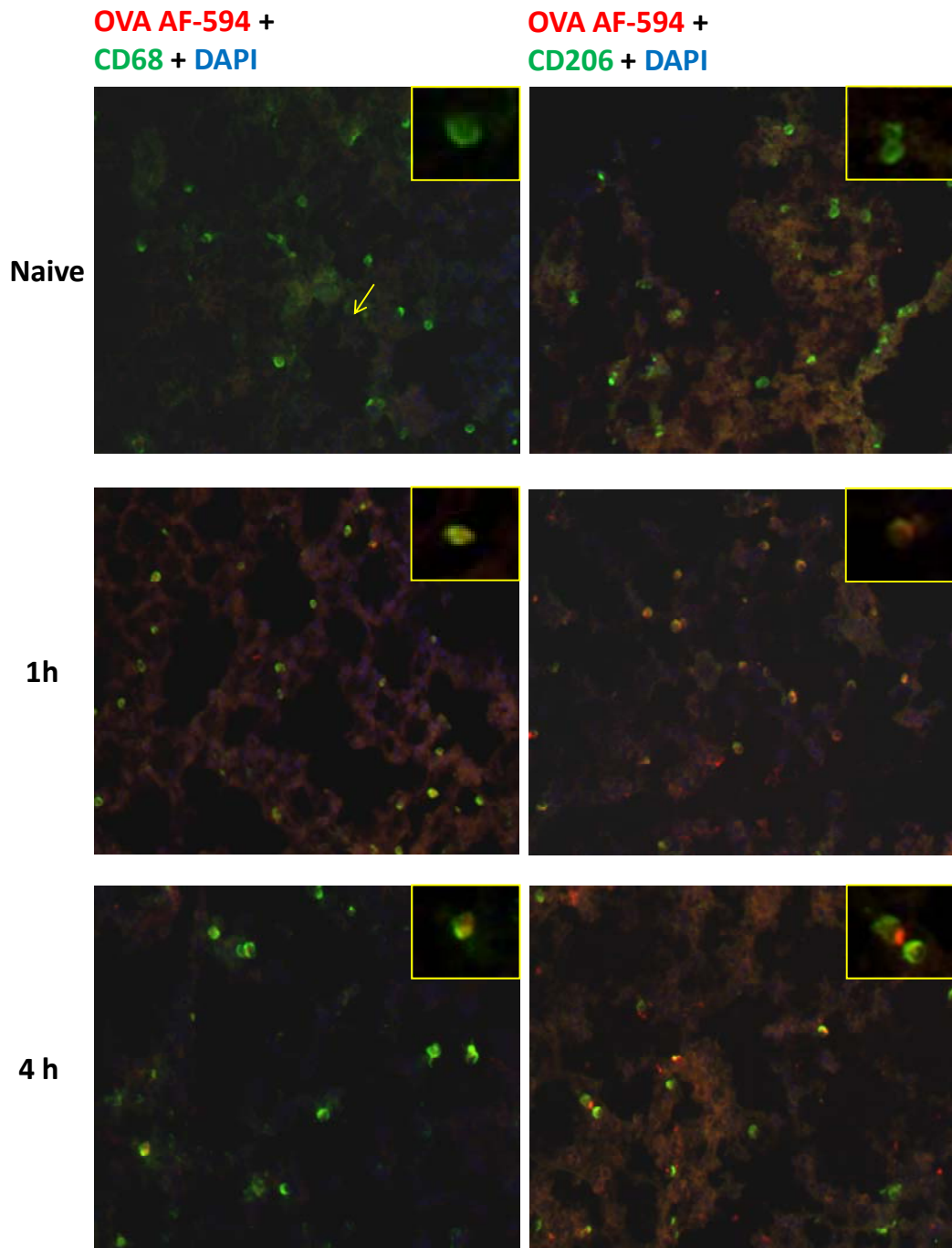


Figure 12.C. Detection of double positive cells in lung at 1 h and 4 h time points (CD68 and CD206). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Frozen lung tissues were cut 5 μ m thick and stained with DAPI and with mAbs CD68 and CD206. As control, we instilled naïve mice with 50 μ l 1 x PBS.

Figure 12.D

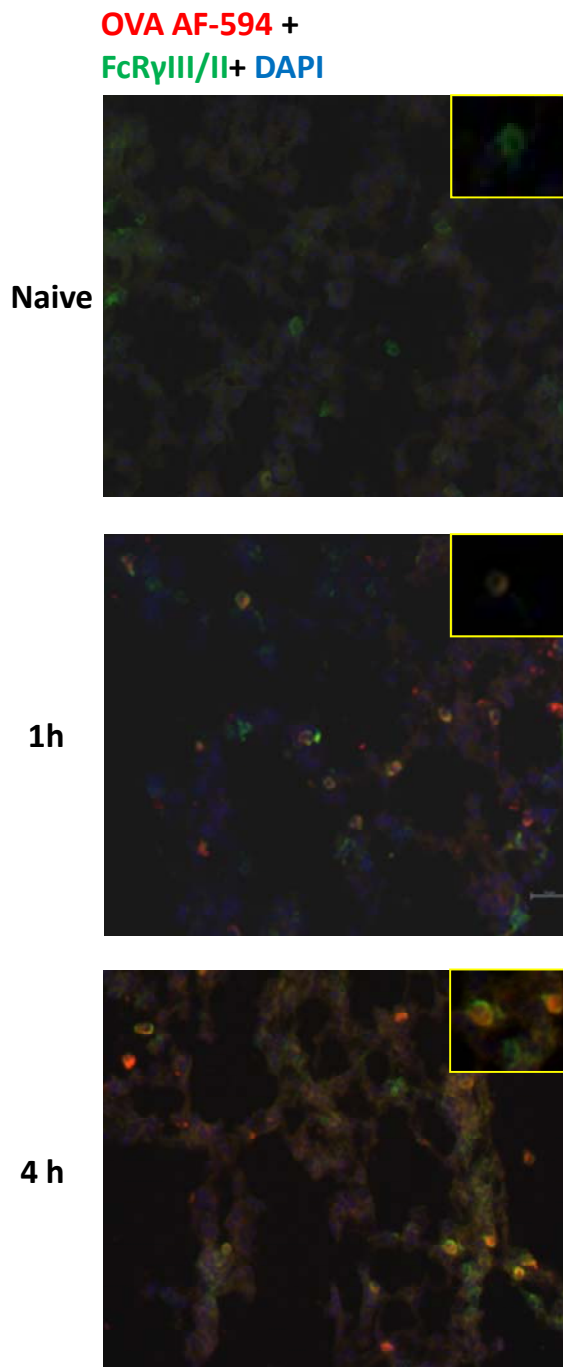


Figure 12.D. Detection of double positive cells in lung at 1 h and 4 h time points (Fc γ RII/III). Mice were instilled i.n. with 10 μ g/ 50 μ l OVA-AF594. After either 1 h or 4 h, lungs were harvested. Frozen lung tissues were cut 5 μ m thick and stained with DAPI and with mAbs Fc γ RII/III. As control we instilled naïve mice with 50 μ l 1 x PBS.

Table 2

macs = DC	macs > DC	macs = DC	DC
↑	↑	↑	↑
CD11c ⁺	F4/80 ⁺ CD68 ⁺ MOMA-2 ⁺	FcR ⁺	CD205 ⁺
MHC class II ⁻ CD11b ⁻ Gr-1 ⁻ B220 ⁻			
↓	↓	↓	
macs = iDC	not mDC macs ? DC	not pDC	

Table 2. Summarize of important surface markers which either do or do not express on APCs. Cells harvested from BAL and lung frozen sections are positive for CD11c, F4/80, CD68, MOMA-2, FcR and CD205 but negative for MHC class II, CD11b, Gr-1 and B220.

Figure 13.

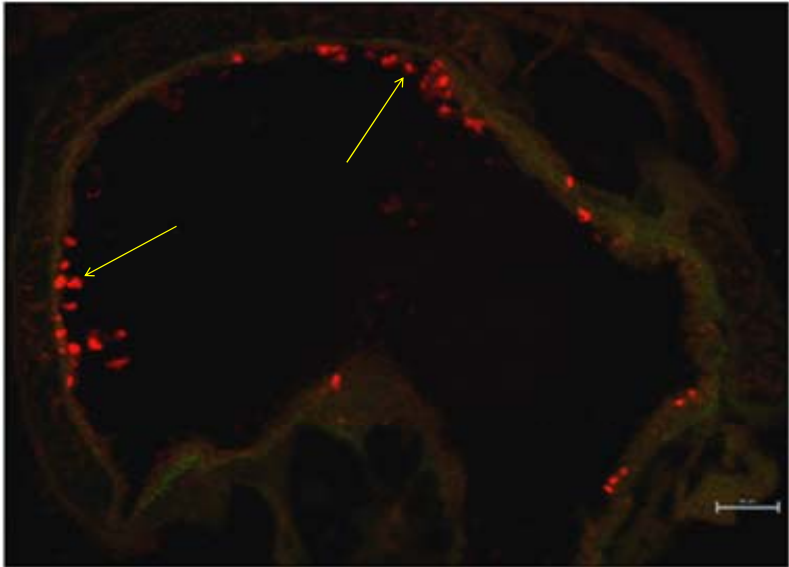


Figure 13. Co-localization of OVA⁺ cells in trachea. After 1 h, trachea was harvested and cut twice horizontal before freezing. Unstained frozen trachea sections (5 μ m thick) were analyzed by fluorescence microscope with a magnification of 20x. Arrow indicated OVA⁺ cells in trachea.

3.6 Characterization of APCs in BAL and lung after i.n. instillation of soluble and particulate Ags

Our study suggested that typical DC and Mac markers do not distinguish the subtypes of cells, which take up soluble Ags in respiratory tract. From this observation, we were interested if particulate and soluble Ags were picked up by the same cells and probably given us a define phenotype. Therefore, we used India ink particles. From literatures published 1959 (89, 90), it was found that India ink particles migrated into the lung 20 min after first i.n. instillation. Therefore, we instilled i.n. mice with 10% India ink mixed with OVA-AF594 (10 μ g/ 50 μ l) and stained BAL cytospin cells and lung frozen sections with surface markers. We found that OVA and India ink co-localized in airway cells 1 h after inhalation (Figure 14). However, not all OVA⁺ positive cells were India ink positive in lung and BAL (Figure 15.A and 15.B). We instilled mice as well with 5% and 20% India Ink but there was no significant differences (data not shown) therefore we used the mean value 10%.

To determine the phenotype of OVA⁺ India Ink⁺ cells in respiratory tract, BAL cells and lung frozen sections were stained with the surface markers. We observed that OVA⁺ India Ink⁺ cells did not express the same surface markers like CD11b, MHC class II (Figure 16.A and 16.B), Gr-1 and B220 but did express F4/80, MOMA-2, CD68, CD206 and CD205 (Figure 17.A, 17.B, 17.C and 17.D), also CD11c and Fc γ RII/III (data not shown) compared with APCs which only took up soluble Ags.

In summary, our studies demonstrated that APCs took up either soluble or particulate Ag share the same phenotype (Table 3) and that all India ink positive cells were OVA⁺ but not all OVA⁺ cells were India Ink⁺.

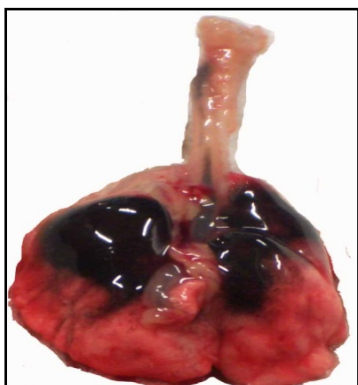


Figure 14. India ink particles within the respiratory tract at 1 h. Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, lung was harvested and analyzed.

Figure 15.A

OVA AF-594 + DAPI + India Ink

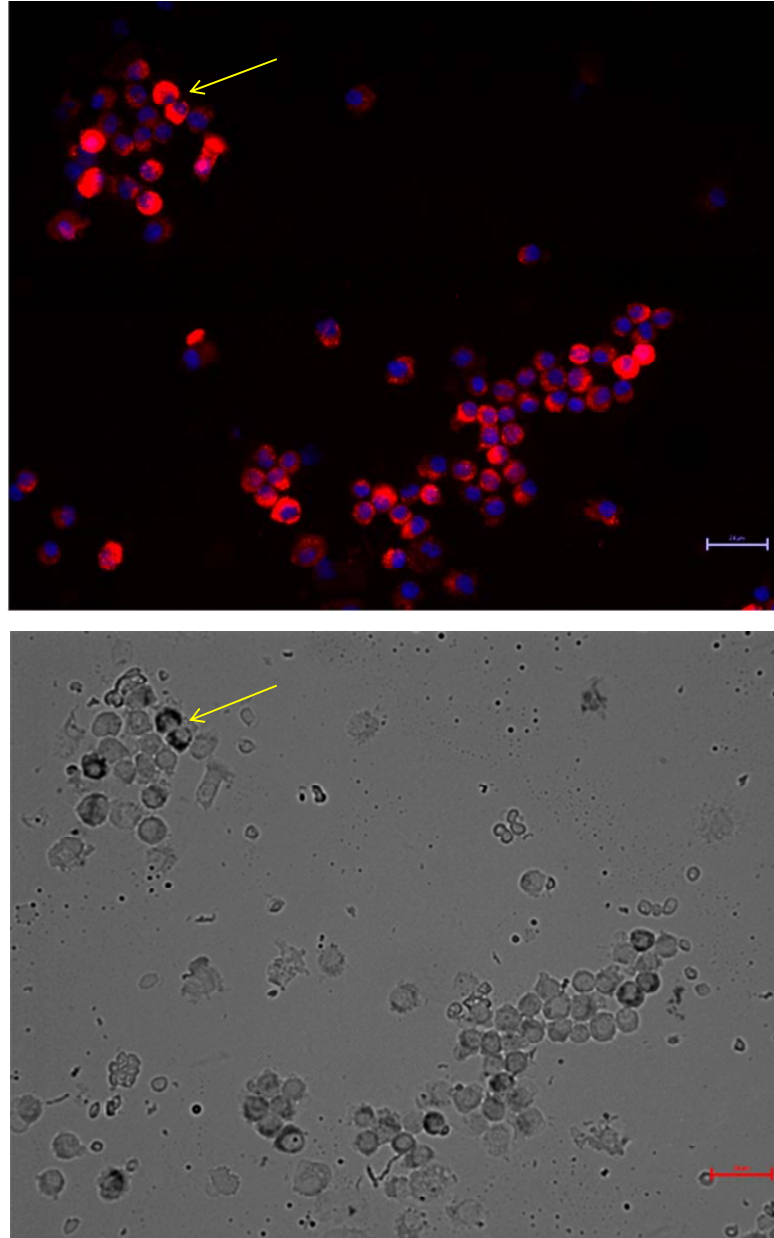


Figure 15.A. OVA and India ink co-localize in BAL. Mice were instilled i.n. with 10 μg / 50 μl OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. As explained before, cytopsin slides with an amount of 1×10^4 cells were prepared for staining. Cells were stained with DAPI and observed by fluorescence and light microscope with a magnification of 20x. Arrows indicated the engulfment of soluble and particulate Ags in the same cell.

Figure 15.B

OVA AF-594 + India Ink

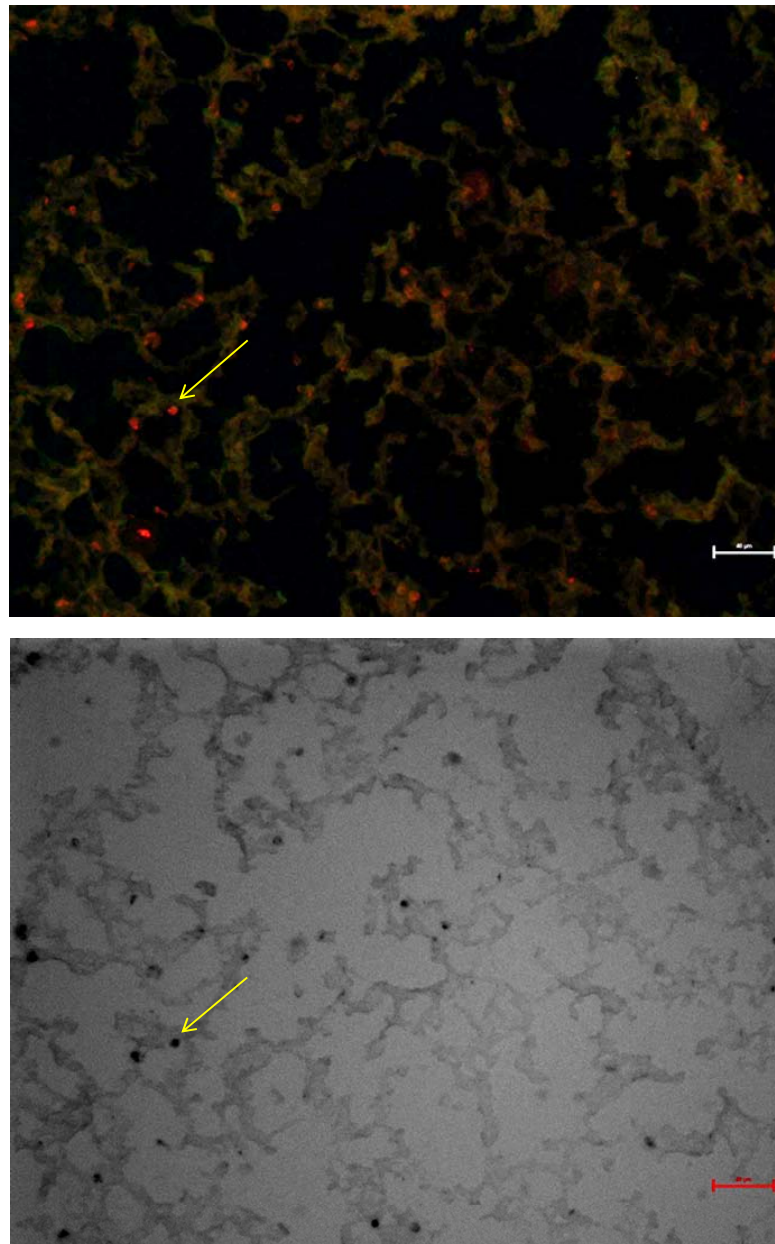


Figure 15.B. OVA and India ink co-localize in situ lung. Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, lung tissues were harvested. Unstained frozen lung sections were observed by fluorescence and light microscope with a magnification of 10x. Arrows indicated the engulfment of soluble and particulate Ags in the same cell.

Figure 16.A

OVA AF-594 + CD11b + DAPI + India Ink

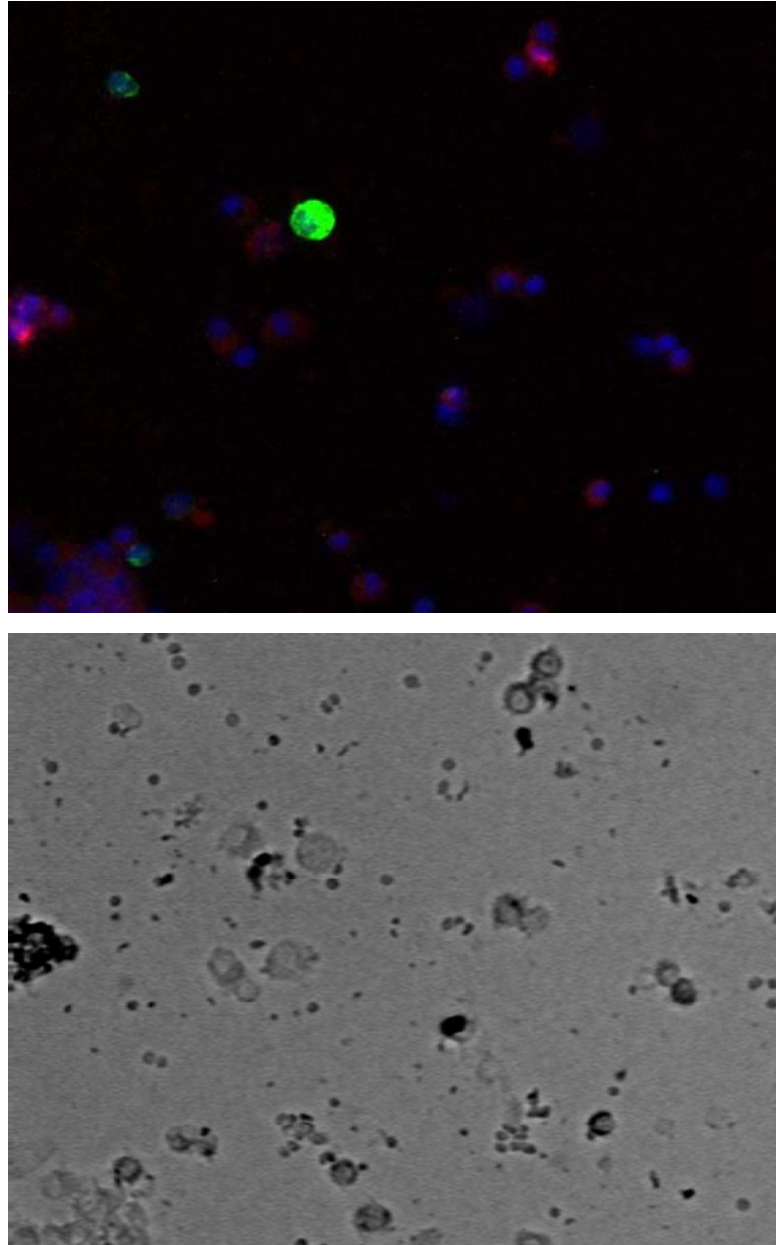


Figure 16.A. *OVA⁺ India Ink⁺ cells do not express CD11b.* Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb CD11b and analyzed by fluorescence and light microscope with a magnification of 20x.

Figure 16.B

OVA AF-594 + MHC class II + DAPI + India Ink

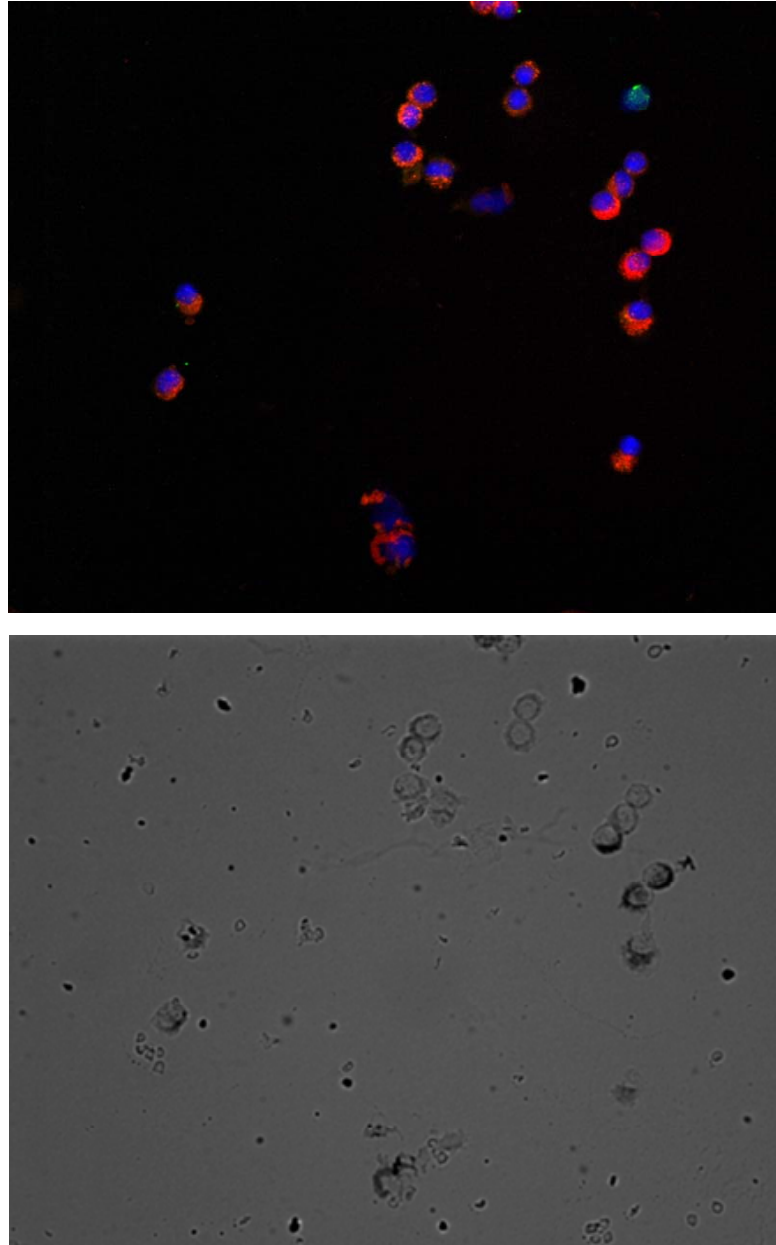


Figure 16.B. *OVA⁺ India Ink⁺ cells do not express MHC class II. Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb MHC class II and analyzed by fluorescence and light microscope with a magnification of 20x.*

Figure 17.A

OVA AF-594 + MOMA-2 + DAPI + India Ink

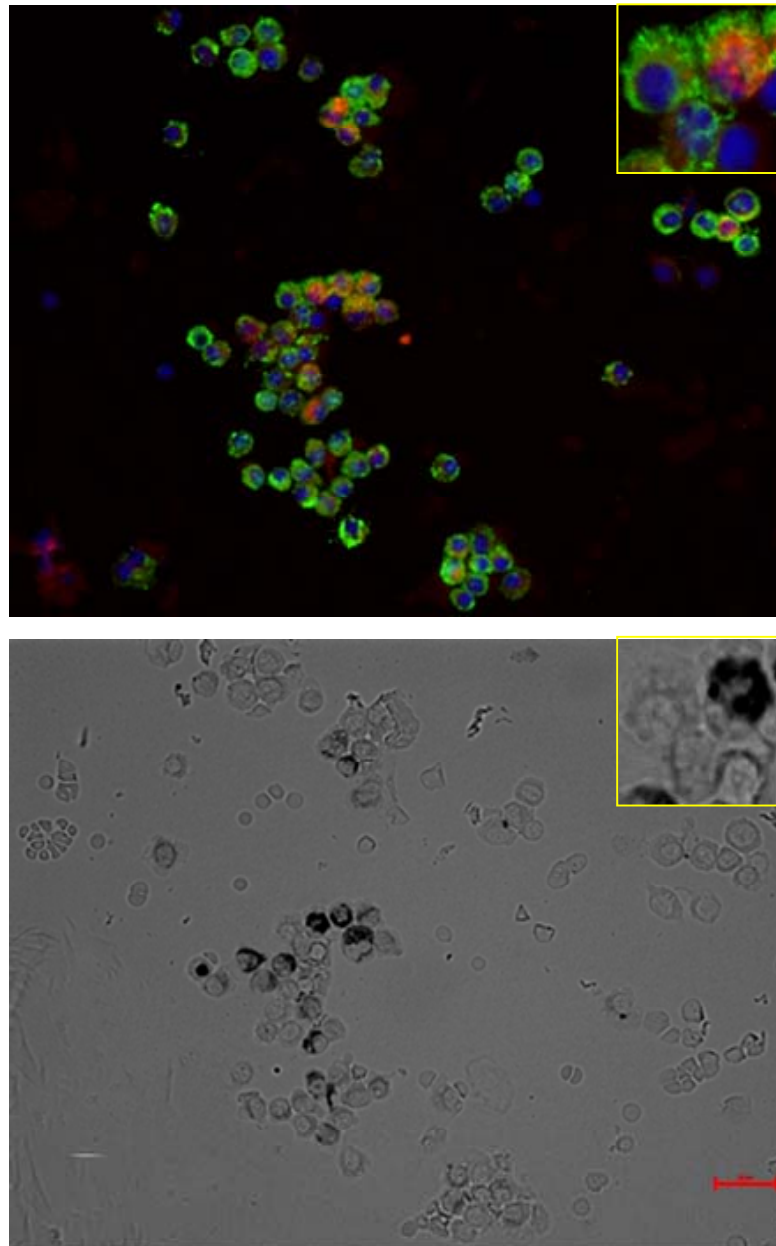


Figure 17.A. *OVA⁺ India Ink⁺ cells do express MOMA-2.* Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb MOMA-2 and observed by fluorescence and light microscope with a magnification of 20x.

Figure 17.B

OVA AF-594 + F4/80 + DAPI + India Ink

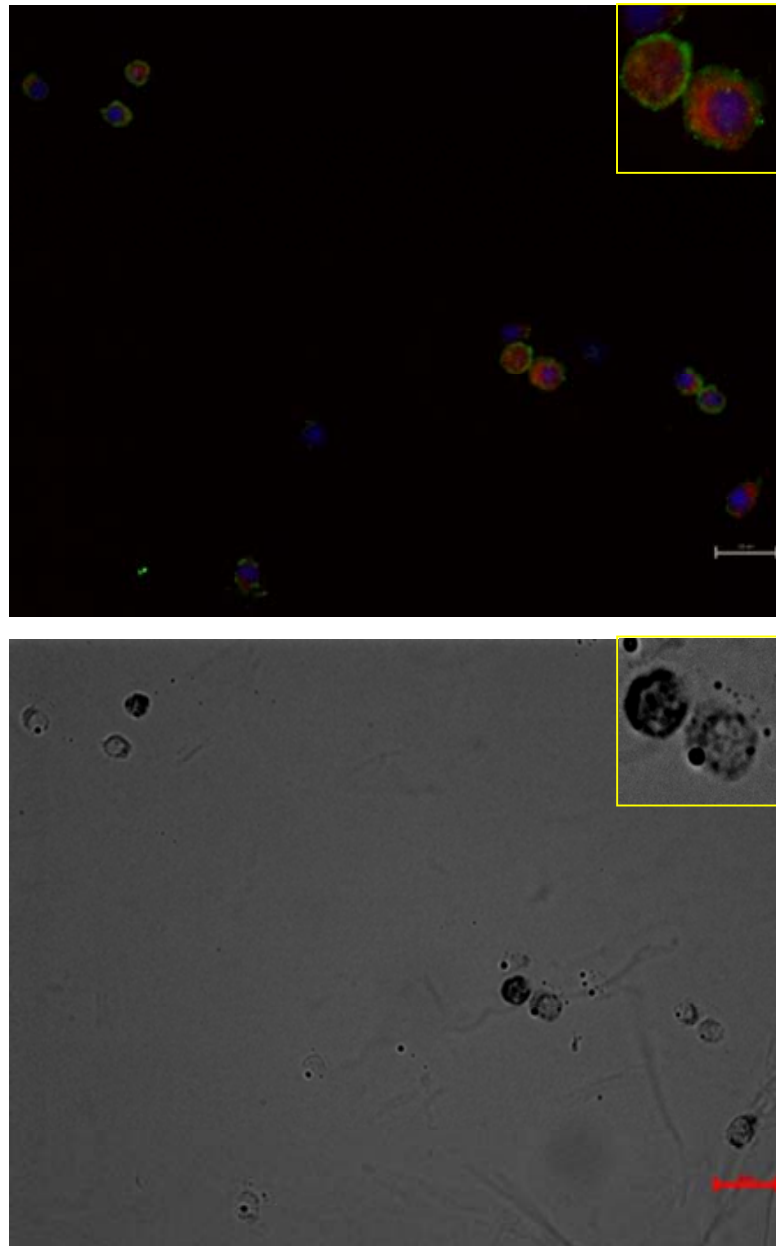


Figure 17.B. *OVA⁺ India Ink⁺ cells do express F4/80.* Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb F4/80 and observed by fluorescence and light microscope with a magnification of 20x.

Figure 17.C

OVA AF-594 + CD68 + DAPI + India Ink

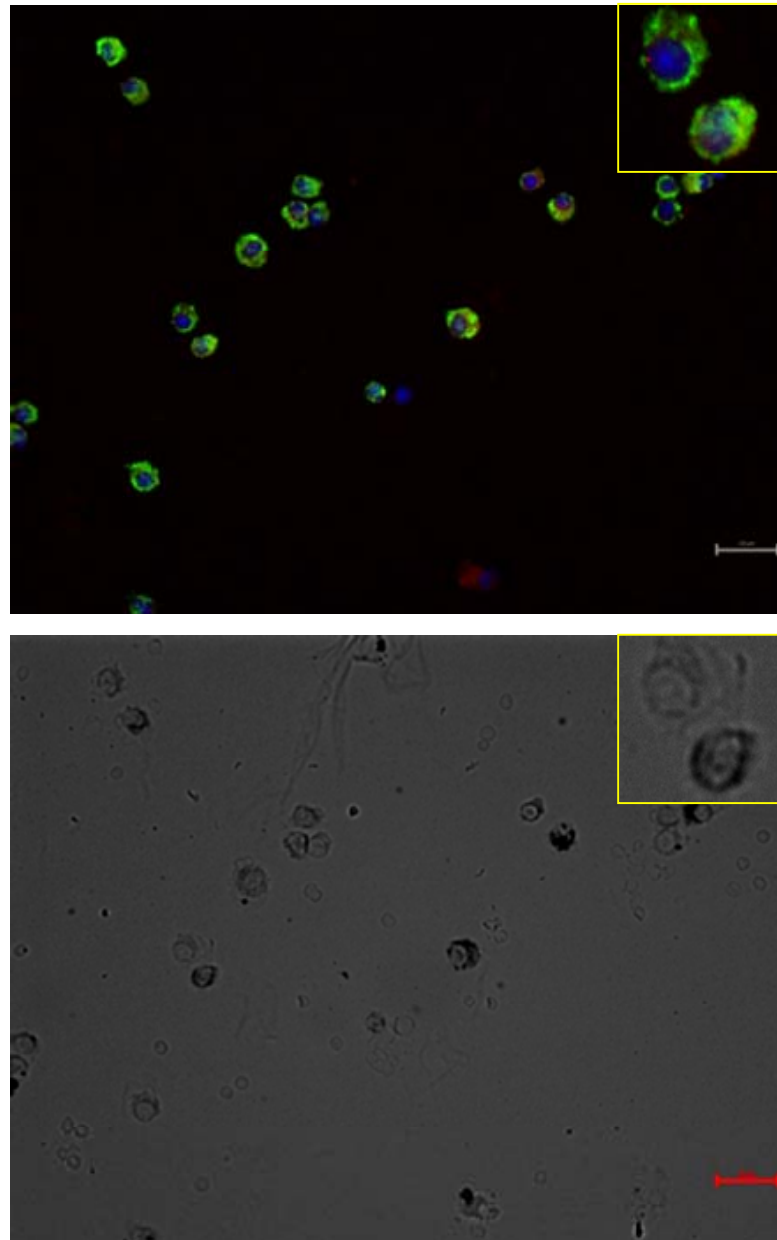


Figure 17.C. *OVA⁺ India Ink⁺ cells do express CD68.* Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb CD68 and observed by fluorescence and light microscope with a magnification of 20x.

Figure 17.D

OVA AF-594 + CD206 + DAPI + India Ink

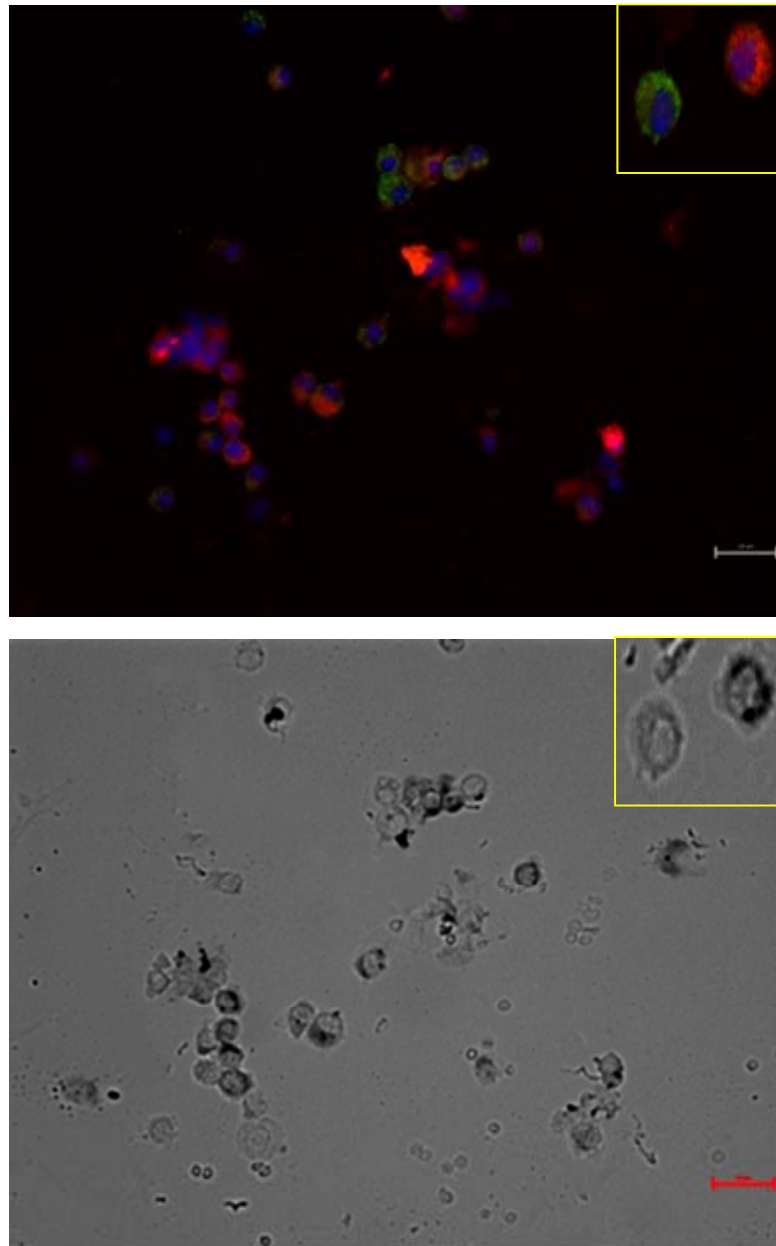


Figure 17.C. *OVA⁺ India Ink⁺ cells do express CD206.* Mice were instilled i.n. with 10 μ g/ 50 μ l OVA mixed with 10% India ink. After 1 h, BAL cells were harvested. Cells were stained with DAPI and mAb CD206 and observed by fluorescence and light microscope with a magnification of 20x.

Table 3.

	Surface marker		Surface marker
	CD11c ⁺		CD11b ⁻
	CD205 ⁺	OVA ⁺ India Ink ⁺	Gr-1 ⁻
	CD206 ⁺		MHCII ⁻
OVA ⁺ India Ink ⁺	MOMA-2 ⁺		B220 ⁻
	CD68 ⁺		
	F4/80 ⁺		
	FcRγIII/II ⁺		

Table 3. Summarize of surface markers which do and do not express on cells after uptake of soluble and particulate Ags. This experiment was reproduced three times.

3.7 Differentiation of DCs and Macs in BAL

To differentiate the phenotype of APCs, we separated subtypes of cells by adhesion experiment. Mice were instilled i.n. with OVA-AF594 (10 µg/ 50 µl) mixed with 5% India ink. After 1 h, BAL cells were collected, transferred to glass cover slips and incubated at 37°C with 5% CO₂ either for 2 h or 24 h. After incubation, supernatant (non-adherent cells) was collected. Adherent cells and non-adherent cells were stained with DAPI and evaluated by fluorescence and light microscopy (Figure 18). We observed that there was no difference between 2 h and 24 h incubation. As we have seen previously, OVA⁺ cells were also India ink⁺ found in adherent and non-adherent cells.

To examine the partition we counted the adherent and the non-adherent cells after 2 h and 24 h incubation (Table 4). At 2 h incubation, we received 99% OVA⁺/DAPI⁺ and 53% India Ink⁺/DAPI⁺ cells compared with 24 h incubation, we received 100% OVA⁺/DAPI⁺ and 67% India Ink⁺/DAPI cells. From these data, we suggested that after 24 h incubation more Macs were attached to glass cover slips than after 2 h incubation.

Figure 18.

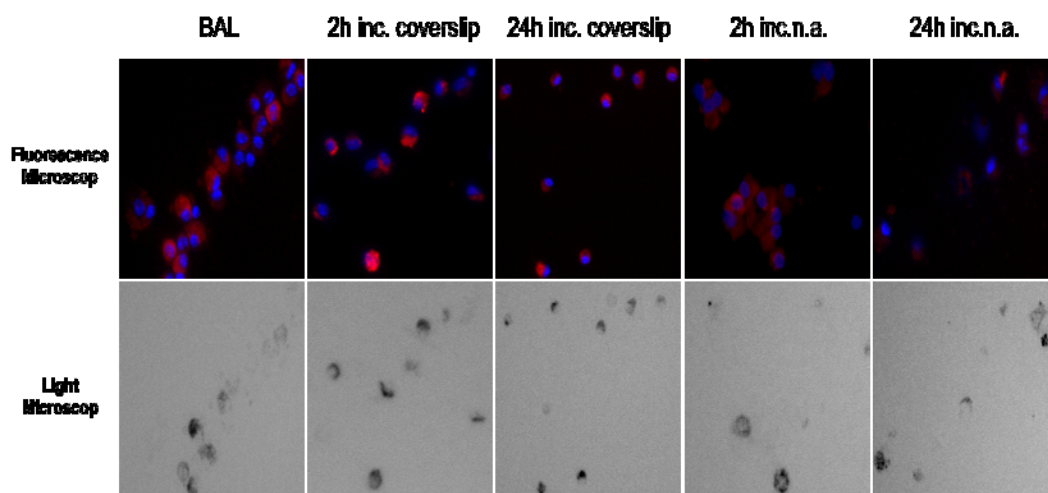


Figure 18. Detection of OVA⁺ India ink⁺ cells of adherent and non-adherent cells after 2 h and 24 h incubation. Mice were instilled i.n. with 10µg/50µl OVA-AF594 mixed with 5% India ink. After 1 h BAL cells were collected and transferred to glass cover slips for 2 h and 24 h incubation. Supernatants were collected. Adherent cells and non-adherent cells were stained with DAPI.

Figure 19.

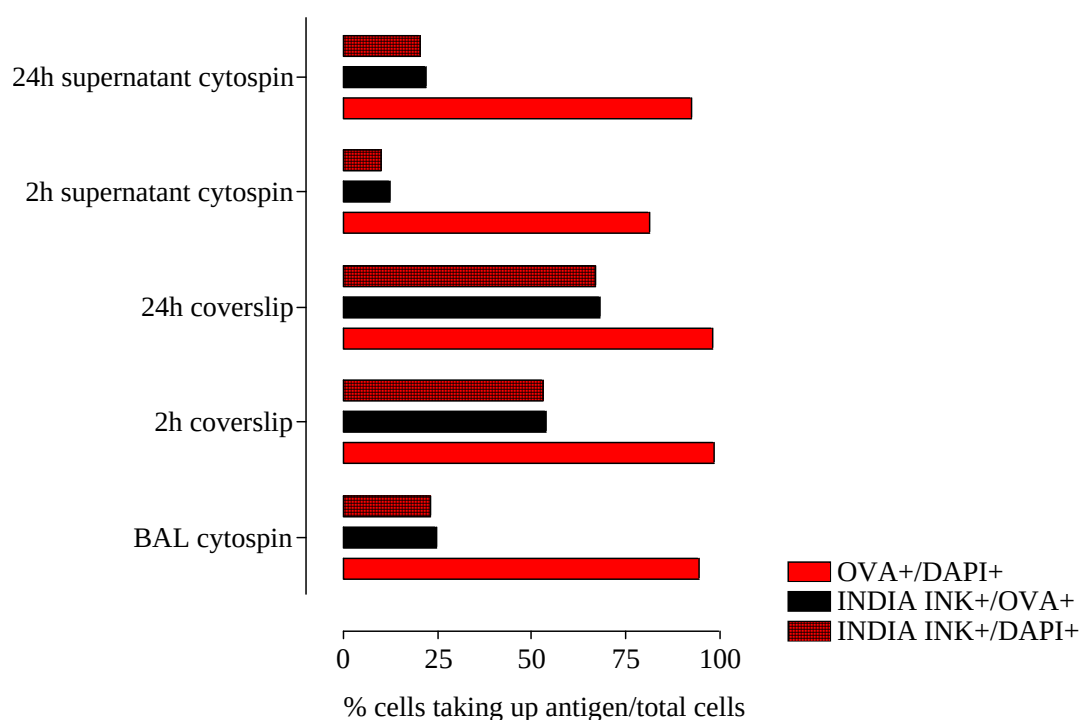


Figure 19. Percentage of single and double positive cells detected either on cover slips or in supernatant. Mice were instilled i.n. with 10 µg/ 50 µl OVA-AF594 mixed with 5% India ink particles. After 1 h, BAL cells were collected in RPMI, 5% FCS and 1% P/S. After several washing processes, cells were transferred to glass cover slips and incubated either for 2 h or 24 h. After incubation, supernatant contained non-adherent cells were collected. Adherent cells on cover slips and non-adherent cells were stained with DAPI and were valued by fluorescence microscope.

3.8 Adherent and non-adherent cells in respiratory tract determined by adhesion studies

To continue characterization of APCs at early time point after uptake of Ag, we separated subtypes of cells obtained from BAL and lung suspension by adhesion experiments. This technical method gives us the ability to distinguish DCs from Macs. From previous study (91, 92), we know that Macs adhere on cover slips within 30 min to 60 min and that DCs are loosely adherent cells (93). Therefore lightly anesthetized mice were instilled i.n. with OVA-AF594 (10 µg/ 50 µl). After 1 h, cells from lung and BAL were harvested and put in RPMI mixed with 5% FCS and 1% P/S. After several washing processes, cells with an amount of 1×10^5 in 200µl were transferred on each glass cover slips and incubated at 37° C with 5% CO₂ for either 2 h or 24 h. After incubation, supernatants which are non-adherent cells were collected. Additional, we washed three times the cover slips to get rid of all non-adherent cells. Supernatants were collected and pooled as after 2 h also after 24 h incubation. Non-adherent cells were washed and centrifuged with Cytospin II. On the other hand, adherent cells on glass cover slips were fixed with 3.7% Formaldehyde. Both, adherent- and non-adherent cells were stained with DAPI and analyzed by fluorescence microscope.

Our study indicated that there was no difference in number of adherent and non-adherent cells after 2 h and 24 h in BAL (Figure 20). However, in lung more non-adherent cells were determined after 24 h incubation than after 2 h (Figure 21). We suggest that there are probably more DCs in lung than in BAL (Table 4).

Figure 20.

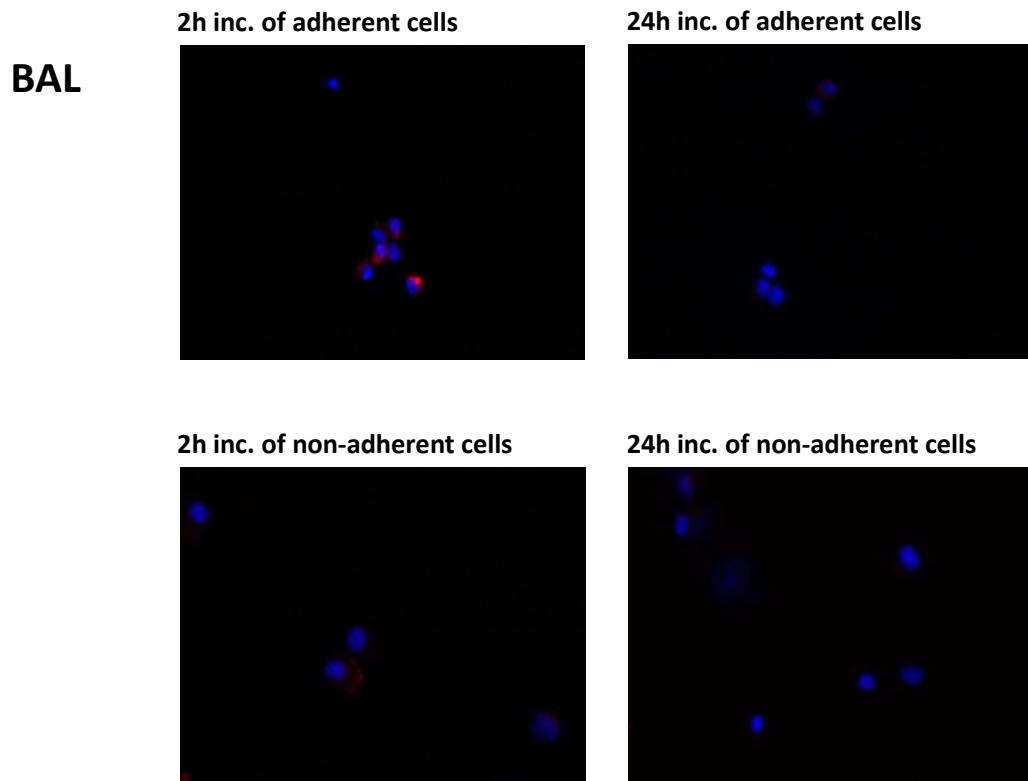


Figure 20. Adherent and non-adherent BAL cells after 2 h and 24 h incubation. BALB/c mice were instilled i.n. with OVA-AF594 (10 μ g/ 50 μ l). Animals were rested for 1 h. Then, BAL fluid cells were harvested by flushing 4 x times with 0.5ml 1 x PBS. Cells were put in RPMI, 5% FCS and 1% P/S, washed 2 times and lysed with lyses buffer to get rid of RBCs. 1×10^5 cells in 200 μ l were transferred to each glass cover slips and incubated either for 2 h or 24 h. After incubation, non-adherent cells were collected and pooled at each time point and stained with DAPI. Adherent cells on cover slips were fixed with 3.7% Formaldehyde and stained as well with DAPI. Both adherent- and non-adherent cells were analyzed by fluorescence microscope.

Figure 21.

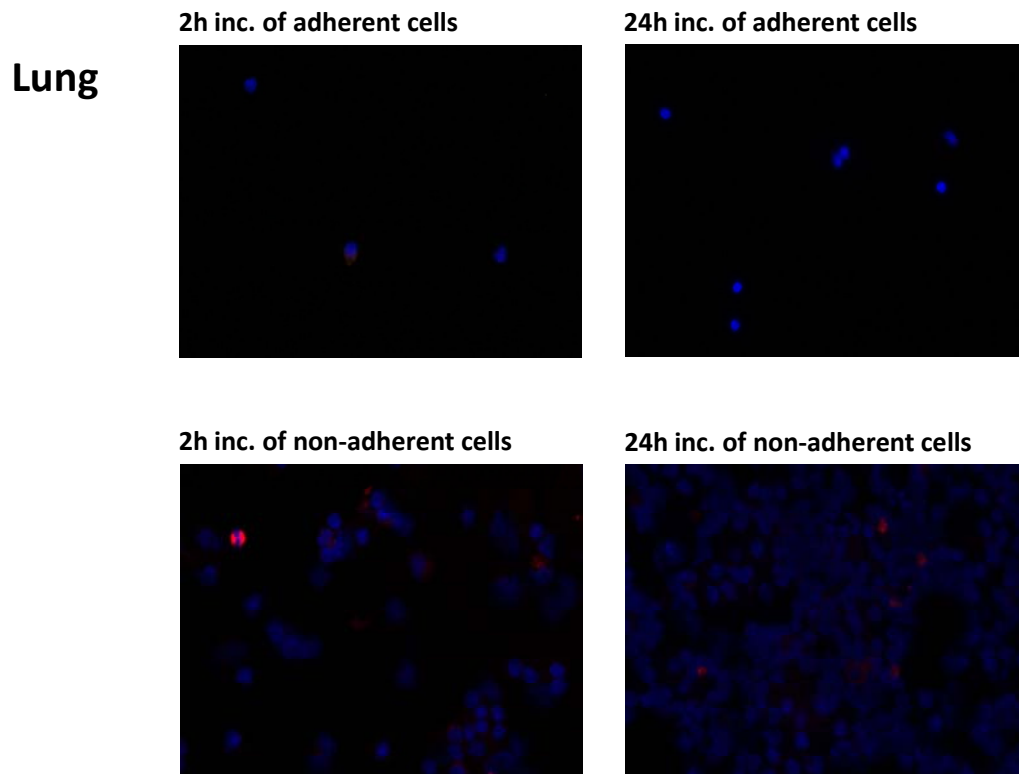


Figure 21. Adherent and non-adherent lung suspension cells after 2 h and 24 h incubation. BALB/c mice were instilled i.n. with OVA-AF594 (10 μ g/ 50 μ l). Animals were rested for 1 h. Then, lungs were perfused with O.C.T/ 1 x PBS (1:1). Lung suspension cells were prepared and put in RPMI, 5% FCS and 1% P/S, washed twice before 1×10^5 cells in 200 μ l were transferred to glass cover slips and incubated either for 2 h or 24 h. After incubation, non-adherent cells were collected and pooled at each time point and stained with DAPI. Adherent cells on cover slips were fixed with 3.7% Formaldehyde and stained as well with DAPI. Both adherent- and non-adherent cells were analyzed by fluorescence microscope.

Table 4.

	Lung suspension	Adherent	Non-adherent
0h	1x10 ⁵ /200μl/cover slip		
2h		0,7 x 10 ⁵	0,3 x 10 ⁵
24h		0,36 x 10 ⁵	0,64 x 10 ⁵

	BAL	Adherent	Non-adherent
0h	1x10 ⁵ /200μl/cover slip		
2h		0,52 x 10 ⁵	0,52 x 10 ⁵
24h		0,48 x 10 ⁵	0,48 x 10 ⁵

Table 4. Summarize of adherent- and non-adherent BAL and lung suspension cells after 2 h and 24 h incubation. After taking pictures with a magnification of 10x, cell numbers on cover slips were counted and substrated from total cell number to get an idea of the cell number of non-adherent cells. One representative experiment of three is shown.

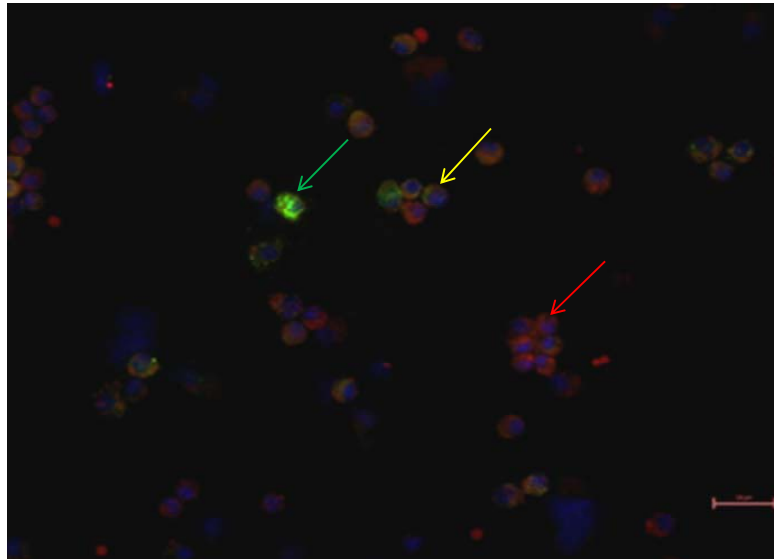
3.9 OVA⁺ cells remain in respiratory tract within 24 h after inhalation

To a different approach, we were interested in migrating of cells taking up inhaled Ag in respiratory tract. To examine the trafficking process, we used in our experimental model OVA-AF594 and OVA-AF488 (Figure 4). First, B6 donor mice were instilled with OVA-AF594 (10 µg/ 50 µl) after 1 h, BAL fluid cells were harvested and transfer to B6 recipient mice by i.n. instillation of 8×10^4 cells/ 50µl. Mice were rested for 23 h before given an another dose of OVA-AF488 (10 µg/ 50 µl) by i.n. instillation. After 1 h, BAL cells were harvested and centrifuged by Cytospin II whereas lung tissues were frozen and cut in 5 µm sections. Both were stained with DAPI and analyzed by fluorescence microscope.

In this adoptive transfer experiment, we observed that OVA-AF594⁺ cells were found in airway and lung parenchyma 24 h after inhalation. Furthermore OVA-AF594 and 488 co-localize in the same cell suggesting that either the same cell is able to pick up both Ags (Figure 22) or it can exchange Ags among each other. In addition, we detected OVA⁺ cells localized in mLN of recipient mice. This observation suggests that OVA⁺ cells migrate to draining LN within 24 h (Figure 23).

Figure 22.

OVA-AF594 + OVA-AF488 + DAPI



OVA-AF594 + OVA-AF488 + DAPI

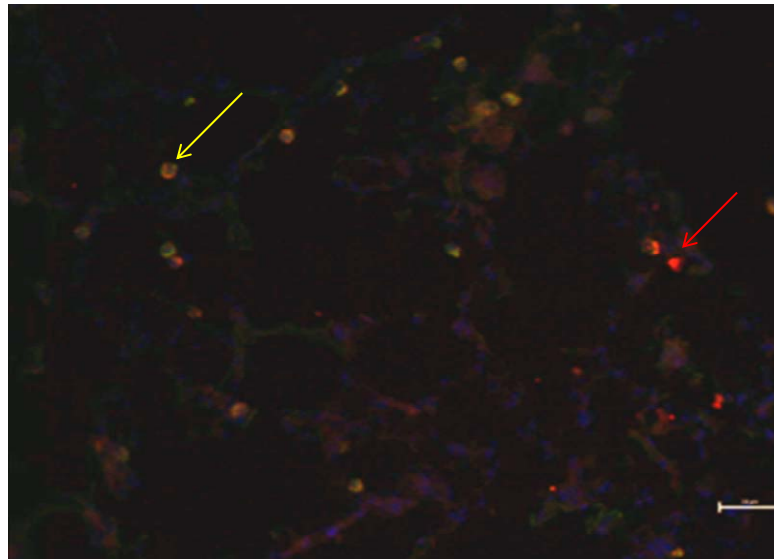


Figure 22. OVA⁺ cells remain in respiratory tract within 24 h after inhalation. B6 donor mice were instilled i.n. with OVA-AF594 (10 µg/ 50 µl). After 1 h, mice were sacrificed and BAL cells were harvested by flushing 4 x with 0.5 ml 1 x PBS. Collected BAL cells were transferred to B6 recipient mice by i.n. instillation (8×10^4 cells/ 50µl). After 23 h, animals were given another dose of OVA-AF488 which is green

fluorescent (10 µg/ 50 µl). Mice were rested for another 1 h before BAL and lung tissues were harvested. BAL cells were collected, washed and centrifuged with Cytospin II whereas lung frozen tissues were cut 5 µm thick before stained with DAPI. Slides were observed by fluorescence microscope with 10x and 20x magnification. Red arrow indicates cells which took up OVA-AF594, green for uptake of OVA-AF488 and yellow arrow centers on cells where a co-localization of OVA-AF594 and OVA-AF488 Ags was detected.

Figure 23.

OVA-AF594 + DAPI

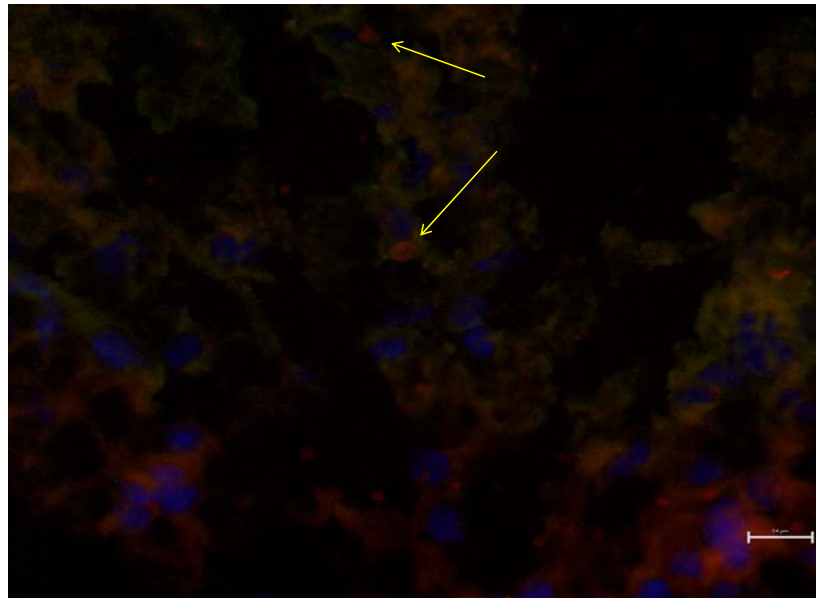


Figure 23. OVA⁺ cells migrate to draining LN within 24 h after inhalation. B6 donor mice were instilled i.n. with OVA-AF594 (10 µg/ 50 µl). After 1 h, mice were sacrificed and BAL cells were harvested by flushing 4 x with 0.5 ml 1 x PBS. Collected BAL cells were transferred to B6 recipient mice by i.n. instillation (8×10^4 cells/ 50µl). After 23 h, animals were given another dose of OVA-AF488 which is green fluorescent (10 µg/ 50 µl). Mice were rested for another 1 h before BAL and lung tissues were harvested. Frozen mLNs were cut 5 µm thick before stained with DAPI.

Tissues were analyzed by fluorescence microscopy with a magnification of 20x. Arrow indicates cells which took up OVA-AF594 and migrate within 24 h from respiratory tract to mLN.

4.0 OVA-AF488 detected in mLNs within 24 h by FACS

Because Ags were detected in draining LNs by fluorescence microscopy, we suggest that APCs migrate into mLNs within 24 h. To prove this observation, we performed a different model of experimental adoptive cell transfer. Therefore we administered i.n. B6 mice with OVA-AF488 (10 µg/ 50µl). After 23 h, mice received BAL cells from donor mice. Donor mice were instilled i.n. with either OVA-AF594 (10 µg/ 50µl) or OVA-AF647 (10 µg/ 50µl) 1 h before they were sacrificed (Figure 3). OVA-AF647 is a fluid ovalbumin conjugated with a fluorochrome which can be detected by FACS. Mice were rested for 1 h before mLNs were harvested and analyzed by FACS. Recipient mice which received OVA-AF594 were rested for another 24 h before collecting BAL cells by flushing 4 x with 0.5ml 1 x PBS. Lung and draining LNs were harvested as well.

As demonstrated by fluorescence microscopy, APCs which have taken up Ag could be found in mLNs within 24 h (Figure 24). There are 4.38% OVA-AF488⁺ cells found in mLNs but there were no cells which indicated a co-localization of OVA-AF647 and OVA-AF488 Ag. Whereas after 48 h, neither OVA-AF488 nor OVA-AF594 positive cells could be detected in the respiratory tract (data known shown).

Figure 24.

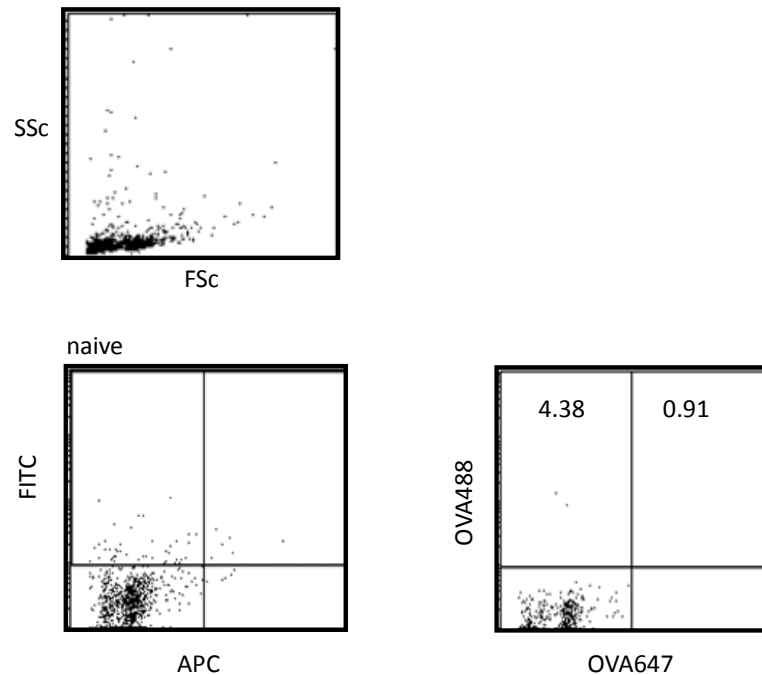


Figure 24. Percentage of OVA-AF488 in mLNs after 24 h. B6 mice were instilled i.n. with OVA-AF488 (10 μ g/ 50 μ l). After 23 h, they received from donor mice BAL cells. These donor mice were administered i.n. with OVA-AF647 (10 μ g/ 50 μ l), rested for 1 h before mice were sacrificed. After 1 h of the last i.n. instillation, mice were sacrificed and mLNs were harvested. Smashed LNs were centrifuged and resuspended with RPMI + 5% FCS. Received LN suspension cells were measured by FACS. As control, naïve LN suspension cells were used.

4. Discussion

In this study, we examined the phenotype of APCs at early time points after giving i.n. OVA conjugated with Alexa-Fluor 594. The results imply that OVA-AF594 was taken up by APCs after 1 h and 4 h of instillation. We found OVA⁺ cells in BAL and lung, and there was no difference in number of OVA⁺ cells between 1h and 4 h after inhalation. We have investigated at shorter time points – after 15 min and 30 min – as well, but we couldn't detect any differences compare to 1 h time points. Parallel, we were interested in the morphology of BAL cells; therefore we stained the cells with May-Grünwald Giemsa. BAL cells have the morphological feature of Macs, with a big nucleus and large cytoplasm. This result gives us an idea what kind of cells we are confronted with, but to come closer to our main question of this study – which subtypes are situated in the respiratory tract – more specific experimental methods have to be used.

Therefore to characterize the phenotype of APCs, we applied surface molecules CD11c, CD11b, MHC class II, F4/80, FcRγIII/II, CD205, CD206, CD68, MOMA-2, B220 and CD207. As we expected, there were no OVA⁺ cells, which expressed CD207 (data not shown), therefore Langerhans cells, a population of DC can be excluded from the subsets of APCs in the respiratory tract. As known, Langerhans cells which are immature DCs are present in the epidermis and in mucosal epithelium (94, 95). Respectively, these cells are not in the airways. However, our results indicated that OVA⁺ cells were CD11c⁺ F4/80⁺ but Gr-1⁻, B220⁻ and MHC class II⁻, in BAL and lung. Lambrecht and Co. (52) demonstrated that lung mDCs and pDCs were able to take up fluorescent OVA but might have a significant difference in Ag processing and presentation efficiency. They established that mDCs were CD11c⁺ Gr-1⁻ B220⁻ cells whereas pDCs were CD11c^{int} Gr-1⁺ B220⁺ in lung of naïve mice. Both subtypes were able to take up FITC-labeled OVA. Therefore, if we compared our results with that of Lambrecht, we could exclude pDCs. However,

whether these cells are DCs and/or Mac lineage is not obvious as the monoclonal antibody F4/80 recognizes both cell types.

Others proclaimed that the majority of cells present in the airways of normal mice was classified as alveolar Macs, however alveolar Macs have also been reported to express some surface markers which were often associated with DCs including expression of CD11c. Steven W. Dow and Co. (96) used *Yersinia pestis* to track APCs in the mouse lung and LNs. They focused on investigation of early immune responses in pneumonic plague, in particular in identifying the early target cells in the lung and BAL. They established that after i.t. or i.n. instillation, the majority of *Y. pestis* organisms were taken up by a distinctive population of CD11c⁺ cells which co-expressed DEC-205 but did not express CD11b. In our study after 1h of i.n. instillation, OVA⁺ cells were positive for CD11c, DEC205 but CD11b⁻ in BAL and lung frozen sections. Considering this observation, we hypothesized that the same phenotype of APCs able to capture *Y. pestis* (96) had the ability to pick up OVA-AF594 as well. To distinguish the phenotype of APCs with surface markers was not able, since OVA⁺ cells expressed both DC and Mac markers.

Another impact was the critical role of DCs in the lungs and airways (61). Recently, it has been published that in lungs, DCs are organized in a structural arrangement which indicated a DC subset distribution within the airways and lung parenchyma (97). It has been shown that in lung parenchyma, there are several different CD11c⁺ DC populations that express CD11b, CD8 or B220. In contrast, airway DCs represent a homogeneous population, maybe because of their immaturity. Airway DCs are indicated to be more responsible for sampling inhaled Ags after i.n. delivery (4, 97). In our study, we were concentrated at the early time points after inhalation, but these cells can be compared with their airway DCs as our cells were negative for CD11b and B220 too. However, as mention previously, our cells express as well typical Mac markers therefore our question – what subsets of cells we are confronted with- remains open.

A different experimental model was used to define the subtypes of APCs in BAL and lung. We treated mice with particle (India ink) mixed with soluble Ags. India ink is a widely used particle to investigate the migration of cells. In the paper published 1993 represent a study using colloidal carbon (Pelican Ink) (88). In their experimental approach, they injected carbon intravenously (i.v.) into mice and waited either for 2 h or 24 h before phenotype the phagocytic cells in liver and spleen. In early years, literatures published in 1959 (89, 90) when DCs were not known, demonstrated that India ink particles were taken up by Macs 20 min after i.n. instillations, and these cells were increased in size and in amount after phagocytosing the Ag. They were found accumulated in alveolar Macs and inside membrane bound vacuoles or vesicles of the cytoplasm. In our study, we were confronted with two questions. First, if India ink particles were picked up by cells at early time points and second, if the same cell, which picked up OVA taken up India ink particles as well. Our results indicated that the same cells, which picked up OVA, also engulfed India ink particles. It demonstrated that all India ink⁺ cells were OVA⁺, however not all OVA⁺ cells were India ink⁺. BAL cells, which were OVA⁺ India ink⁺ expressed surface markers CD11c, MHC, class II, F4/80, FcRγIII/II, CD205, CD206, MOMA-2 and CD68. Although these APCs were positive for India ink, it is still unclear which cell population they are, DCs or Macs.

Our study suggested that already after 1 h of i.n. instillation, particulate Ags were taken up by APCs in lung, which concurred with the report of Chaplin and colleagues. (42), who used fluorescent latex bead particles as Ag. They demonstrated the appearance of beads in lung APCs as early as 2 h after i.n. instillation and presented until 46 h. Their data demonstrated that at 6 h after instillation of beads, most bead⁺ cells from BAL and lung were positive for CD11c and F4/80, but negative for CD11b. Therefore they suggested that bead⁺ CD11c⁺ F4/80⁺ CD11b⁻ cells represent a unique subset of airway DCs. In our research, we obtained India ink⁺ CD11c⁺ F4/80⁺ CD11b⁻, therefore we hypothesized that the same phenotype of APCs, which captured bead particles, also picked up India ink particles.

To further characterize APCs, we used in our recent study another technique to distinguish DCs and Macs. This method, described by Laurent P. Nicod and colleagues, was utilized in the present study (98). In our research, we instilled i.n. mice with OVA-AF594 mixed with 5% India ink particles. BAL cells were harvested and transferred to glass cover slips for 2 h and overnight incubation. It is known that DCs are loosely adherent cells whereas Macs attach to cover slips within 2h incubation (98). Non-adherent cells appear as lymphocytes, eosinophils and neutrophils. We obtained, after 24 h incubation, only Macs attached to cover slips. After 2 h incubation, almost all OVA⁺ cells were attached to cover slips whereas only 53% of India ink⁺ cells were adhered to. However, after 24 h incubation all OVA⁺ cells were attached and there was an increase of India Ink⁺ cells on cover slips. There was not an obvious difference between 2 h and 24 h incubation of OVA⁺ cells. In contrast, we received 14% difference in India ink uptake. Only 10 - 20% of non-adherent cells picked up India ink. In conclusion, our finding suggested that after 24 h incubation more OVA⁺ India ink⁺ cells attached to the cover slips.

In addition, we were interested in numbers of adherent and non-adherent cells in general. We found that mice administered with only soluble Ag (OVA), lung suspensions cells were less attached to the cover slips after 24 h incubation whereas, there was no difference in the percentage of adherent and non-adherent BAL cells after 2 h and overnight incubation. Our results indicated that there are probably more DCs found in the lung than in BAL. The numbers in our results have to be considered carefully as our technical model causes cell loss during the washing steps of glass cover slip after incubation.

In summary, we observed a cell population in airways of mice that is able to capture OVA-AF594 at early time points after i.n. instillation. The same APCs are able to pick up soluble and particle Ags at the same time and express both DC and Mac surface receptors. The separation of DCs from Mac population is complicated by the fact that neither cell type can be identified by a single antigenic determinant.

In adoptive cell transfer studies, we wanted to determine if the same cells would pick up two different Ags, and furthermore, if these cells would move to mLNs within 24 h or would remain in the lung. In the first experimental approach, we observed that the same cell was able to take up OVA-AF594 and OVA-AF488 soluble proteins. The possibility of co-localization of two Ags in one cell can be explained by either exchange of the Ags – “cell to cell communication”, or already OVA loaded cells take up Ag which are released from dead cells. Furthermore, we could detect double positive cells within 24 h in the mLNs by immunofluorescence microscopy. To proof this observation, we performed another adoptive cell transfer experiment which allowed us to detect APCs by FACS. The result confirmed our previous study, which cells from recipient mice which received i.n. OVA-AF488 in the first day remained in the lung. These cells are able to pick up another Ag which was administered after 23 h. To point out, the second Ag was not given directly but through adoptive cell transfer. Probably, there is a cross-linking between two cells which could be defined as two different subsets of APCs; or there is an exchange of Ag through the same phenotype. Recently, a study group (99, 100) has argued that Macs are able to fuse in multinucleated giant cell, characteristic of granulomatous infections as well as multinucleated osteoclasts. Therefore, there is a possibility that our APCs which could be Macs are fused together to explain the finding of two Ags in the same cell. But another question was appeared during analyzing our data: are these cells which we could detect with FACS and immunofluorescence staining, are from recipient or from donor mice? Further studies would be necessary to answer this question.

In addition, our result indicates that already after 24 h DCs or Macs are in the draining LNs caring both Ags. During this short time point, there isn't a proliferation of the T cells to Ags but we could observe a mobilization of APCs into the mLNs showing by FACS and immunofluorescence staining. In this case, it was not possible to describe with which APCs we were confronted with in mLNs. Interestingly, after 48 h of i.n. instillation APCs still remained in lung as mentioned in Randolph paper (101). But we are not exactly sure with which cell type we were confronted with. However, Randolph and his colleagues demonstrated that the cell

type which takes up particulate Ags (latex beads) and motiles to the mLN after 48 h is Macs. They described that DCs carrying Ags are more inefficient in transporting particulate Ags into mLN than Macs. They proved by enhance of DCs which migrated to the mLN bearing latex beads when Macs were eliminated.

Finally, in BAL and lung single and double positive cells (red- and green fluorescent) could be observed in recipient mice which were instilled OVA-AF594 after 24 h whereas after 48 h only double positive cells could be observed. We suggested that maybe single positive cells might migrate to mLN faster than double positive cells because of their density difference. We were face with many difficulties in these two kind of adoptive cell transfer experiments therefore many questions remained open.

Thus, in this study we provide evidence that APCs at early time points cannot be determined their subtypes by functional surface markers. In addition, APCs are able to take up soluble and particulate Ags and migrate into mLN within 24 h.

5. Summary

Allergic asthma is a complex inflammatory disease of the airways caused by immune response to common aerosol Ags. The main entrance of these allergic Ags is the respiratory tract where they are recognized and taken up by Ag presenting cells (APCs). This interaction is able to lead to either immune allergic response or immune tolerance. In our study, we were interested in the characterization of APCs which might be involved in causing an allergic response. To determine the phenotype of APCs in the respiratory tract, we instilled i.n. mice with fluorescent-labeled soluble protein (OVA) and particulate Ag (India ink). We evaluated BAL cells and frozen lung tissues under fluorescence microscope. Additionally, cells were stained with common surface markers. Our results indicated that APCs had taken up both soluble and particulate Ags at 1 h and 4 h after instillation. However, surface molecules which were indicated either specific for DCs or for Macs are expressed on the cells. The cells were CD11b⁺, MHC class II⁺, Gr-1⁺ and B220⁺ but positive for CD11c, CD205, CD68, CD206, F4/80 and MOMA-2. We couldn't find any significant differences in the cell phenotype as these cells were positive for both DC and Mac markers. In addition, DCs and Macs were separated by adhesion experiment. We found out that after 24 h of incubation cells are non-adherent. These results suggest that probably more DCs as APCs are situated in the lung. In summary, our data provide evidence that there is a unique subset of lung APCs taking up foreign Ags which share the markers of both DCs and Macs. These APCs could play a key role in regulation of the immune response against inhaled foreign Ag which can imply in understanding the mechanisms leading to immune response and may be useful as a therapeutic strategy.

6. Zusammenfassung

Allergisches Asthma ist eine chronisch entzündliche Erkrankung mit dauerhaft bestehender Überempfindlichkeit. Die Krankheitssymptome sind Atemnot, Sekretion von Schleim, Verkrampfung der Bronchialmuskulatur oder Husten. Diese werden meist durch harmlose Fremdkörper, wie beispielsweise Pollen, Schimmelpilze, Tierhaare, Staub oder Nahrungsmittel hervorgerufen. Diese allergischen Antigene wandern nach der Inhalation in den Respirationstrakt, wo sie von Antigen präsentierenden Zellen erkannt und zerkleinert werden, um eine Immunantwort oder Immuntoleranz zu aktivieren. Unsere Studie beschäftigt sich vorwiegend mit jenen Zellen, die kurz nach Aufnahme von Fremdsubstanzen im Atmungsweg bzw. in der Lunge verweilen. Fraglich ist, mit welchem Phänotyp der Antigen präsentierenden Zellen wir konfrontiert sind. Aus diesem Grund, wurde Mäusen intranasal flüssiges Ovalbumin gekoppelt an Fluorochrome verabreicht, und nach einer bzw. vier Stunden BAL und Lungen entnommen und experimentell untersucht. Zusätzlich haben wir diese Zellen mit den in den Literaturen oft beschriebenen Oberflächenmarkern gefärbt, um die Zellen zu charakterisieren. Es stellte sich heraus, dass sich bereits nach einer und vier Stunden, die Zellen sich im Respirationstrakt befinden und negativ für CD11b, MHC class II, Gr-1 und B220 aber positiv für CD11c, CD205, CD68, CD206, F4/80 und MOMA-2 Marker sind. Zusammengefasst zeigt unsere Studie, dass Antigen präsentierende Zellen sowohl Merkmale von dendritischen Zellen als auch von Makrophagen besitzen. Desweiteren wurde Mäusen intranasal flüssiges Protein vermischt mit Partikeln (India Ink) verabreicht, um zu beobachten, ob die gleichen Zellen beide verschiedene Formen von Fremdkörpern aufnehmen können. Wir sind zu der Erkenntnis gelangt, dass sie diese Eigenschaften besitzen. Diese Zellen scheinen eine eigene Untereinheit der Antigen präsentierenden Zellen zu sein und können eine wichtige Rolle in der Immunantwort und in der Anwendung therapeutischen Strategien spielen.

7. References

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Curriculum Vitae

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Education

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09/1987 – 06/1996	Middle School and High School
09/1996 – 06/1997	Nurse School
10/1997 – 06/1998	Technical University in Vienna, Major subject: Architect
09/1998	University of Vienna Major subject: Microbiology/Immunology
11/2006 – 01/2008	Diploma thesis at the Department of Dermatology; Division of Immunology, Allergy and Infectious Diseases; Group Dr. Michelle Epstein

Activities

09/2001 – 05/2002 of	Exchange Student of Erasmus/Socrates Programme the European Union in Manchester, Great Britain (University of Manchester)
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Poster presentation

Poster presentation at ÖGAI Congress (Austrian Society of Allergology and Immunology) in December 2007.

Title of my poster: "Characterization of lung antigen presenting cells taking up soluble and particulate aeroantigens"