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Macrophages induce an allergen-specific and long-term suppression in a mouse asthma model

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ABSTRACT: Increasing evidence suggests that macrophages (Mϕ) play a crucial downregulatory role in the initiation and progression of allergic asthma. Recently, the current authors demonstrated that ovalbumin (OVA)-loaded Mϕ (OVA-Mϕ) suppress subsequent OVA-induced airway manifestations of asthma and that this effect could be potentiated upon selective activation. In the present study, the authors further delineated the underlying pathway by which Mϕ exert this immunosuppressive effect.

To examine the migration of OVA-Mϕ, cells were labelled with 5′chloromethylfluorescein diacetate (CMFDA) and were administered (*i.v.*) into OVA-sensitised BALB/c mice. After 20 h, the relevant organs were dissected and analysed using fluorescent microscopy. Allergen-specificity was investigated by treating OVA-sensitised mice with keyhole limpet haemocyanin (KLH)-Mϕ activated with immunostimulatory sequence oligodeoxynucleotide (ISS-ODN). By lengthening the period between treatment and challenge to 4 weeks it was examined whether OVA-Mϕ exerted an immunosuppressive memory response.

Strikingly, CMFDA-labelled Mϕ were not trapped in the lungs, but migrated to the spleen. ISS-ODN-stimulated KLH-Mϕ failed to suppress OVA-induced airway manifestations of asthma. Moreover, treatment with ISS-ODN-stimulated OVA-Mϕ was still effective after lengthening the period between treatment and challenge.

These data demonstrate that allergen-loaded macrophages can induce an indirect immunosuppressive response that is allergen-specific and long lasting, which are both hallmarks of a memory lymphocyte response.

KEYWORDS: Allergy, interleukin-10, macrophages, regulatory T-cells, T-helper cell type 2 lymphocytes

Allergic asthma is characterised by reversible airway obstruction, increased levels of allergen-specific immunoglobulin (Ig)-E, chronic airway inflammation and persistent airway hyperreactivity (AHR). Allergic asthma is driven and maintained by the persistence of a subset of chronically activated memory T-cells [1, 2]. The maintenance of immunological homeostasis in the respiratory tract requires fine-tuning of T-cell activation, in order to induce a sufficient inflammatory response against inhaled pathogens, while avoiding excessive responses. Increasing evidence suggests that macrophages (Mϕ) play a pivotal role in both the potentiation and the suppression of inflammatory responses [3].

Mϕ trigger the immune responses against microbial pathogens by secreting pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α [4, 5]. Moreover, by releasing IL-12 they can specifically skew immune responses towards T-helper cell type 1 (Th1) responses [6–8]. Though Mϕ favour the induction of Th1 responses [9, 10], it has also been demonstrated that Mϕ can induce differentiation towards Th2 lymphocytes [11, 12]. To exert immunosuppressive responses Mϕ secrete anti-inflammatory mediators, such as prostaglandin (PG)E_2, transforming growth factor (TGF)-β, IL-10 and nitric oxide [3, 5, 13].

Alveolar Mϕ represent the most predominant immune effector cells in the alveolar spaces and conducting airways [3]. By secreting pro-inflammatory cytokines and chemokines alveolar Mϕ direct the recruitment and activation of inflammatory cells, while they also play a key role in dampening immune responses against nonpathogenic antigens [5, 14]. Alveolar Mϕ have

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been shown to suppress T-lymphocyte proliferation *in vitro* [15, 16] and antigen presentation functions of dentritic cells *in vitro* and *in vivo* [17]. Although depletion of alveolar Mϕ potentiated IgE antibody production in response to inhaled allergen [18] and adoptive transfer of alveolar Mϕ suppresses AHR [19], the underlying cellular and molecular mechanisms remain to be elucidated [3].

Previously, the current authors developed a mouse model in which allergen-loaded Mϕ suppressed allergen-induced airway manifestations [20]. The anti-inflammatory effects of Mϕ could be potentiated by activating the Mϕ with the selective toll-like receptor (TLR)-9 or -4 ligands immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) or lipopolysaccharide (LPS), respectively, *ex vivo* [21]. Interestingly, IL-10 is upregulated in these activated Mϕ and plays a crucial role in the anti-inflammatory effects of Mϕ *in vivo* [21]. In this study, the mechanisms underlying these anti-inflammatory effects were investigated in more detail. Strikingly, after administration (*i.v.*), allergen-loaded Mϕ migrated to the spleen. As a consequence, the authors investigated whether Mϕ induce, in the spleen, an indirect allergen-specific and long-term suppressive response.

METHODS

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific pathogen-free (according to the Federation of European Laboratory Animal Science Associations [22]) male BALB/c mice (6-weeks old) were purchased from Charles River (Maastricht, The Netherlands). The mice were housed in macrolon cages in a laminar flow cabinet. All mice were provided with food and water *ad libitum*.

Materials

OVA (grade V), purified LPS from *Escherichia coli* 0111:B4, and methacholine (acetyl-β-methylcholine chloride) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Keyhole limpet haemocyanin (KLH) was obtained from Calbiochem (San Diego, CA, USA). Fluorescent dye 5′chloromethylfluorescein diacetate (CMFDA) was acquired from Molecular Probes Europe BV (Leiden, The Netherlands). CpG-containing phosphorothioate ISS-ODN was synthesised by Isogen Bioscience BV (Maarsen, The Netherlands). The ISS-ODN used had the sequence 5′-TGACTGTGAACGTTCGAGATGA-3′ [23].

Loading and stimulation of macrophages

Peritoneal Mϕ were isolated from naïve BALB/c mice as described previously [20]. Subsequently, 1×10^7 Mϕ·mL^{-1} were loaded with 2 mg·mL^{-1} OVA or 2 mg·mL^{-1} KLH and were stimulated with 10 μg·mL^{-1} LPS or 3 μg·mL^{-1} ISS-ODN [21]. After incubation for 3 h at 37°C and 5% CO_2, the Mϕ were extensively washed to remove all residual soluble OVA, LPS, and ISS-ODN.

Sensitisation, treatment and challenge

Mice were sensitised to OVA by active sensitisation with 7 *i.p.* injections of 10 μg OVA in 0.5 mL pyrogen-free saline on alternate days [24]. Treatment was performed 17 days after the last sensitisation by administration (*i.v.*) of 3×10^5 Mϕ (OVA-loaded or KLH-loaded) in 50 μl saline. As an additional control group, mice were *i.v.* injected with 50 μl saline. After 1–4 weeks post treatment, mice were exposed to OVA (2 mg·mL^{-1} saline) aerosol challenges for 5 min on eight consecutive days.

Fluorescent labelling and localisation of macrophages

A total of 1×10^7 Mϕ·mL^{-1} were loaded with OVA (2 mg·mL^{-1}) and stimulated with ISS-ODN (3 μg·mL^{-1}) for 3 h at 37°C and 5% CO_2. At the last 30 min, 10 μM CMFDA was added to the Mϕ, the Mϕ were then extensively washed. To test the labelling efficiency, 1×10^5 CMFDA-labelled Mϕ·mL^{-1} were cultured in 96-well round-bottomed plates (Greiner Bio-One GmbH, Kremsmuenster, Austria) in Roswell Park Memorial Institute medium-1,640 enriched with 2% foetal calf serum, penicillin/streptomycin (all GIBCO BAL division of Invitrogen, Breda, The Netherlands) and 50 μM β-mercaptoethanol (Sigma-Aldrich). After 20 h, the Mϕ were harvested and cytospins of these Mϕ were analysed using fluorescent microscopy. Subsequently, OVA-sensitised mice were treated (*i.v.*) with 3×10^5 Mϕ. After 20 h, blood, lungs, spleen, lymph nodes (axillary, brachial, thoracic), liver and kidneys were collected. Several 6-μm thick cryosections of the tissues were analysed using fluorescent microscopy.

Measurement of airway responsiveness in vivo

Airway responsiveness was measured in conscious, unrestrained mice using barometric whole-body plethysmography by recording respiratory pressure curves (Buxco corp., EMKA Technologies, Paris, France) in response to inhaled methacholine (ranging from 0–50 mg·mL^{-1} methacholine). Airway responsiveness was expressed in enhanced pause (Penh), as described previously [25].

Determination of ovalbumin-specific immunoglobulin-E levels in serum

After measurement of *in vivo* airway responsiveness, mice were sacrificed by injection of 1 mL 10% urethane (*i.p.*, Sigma-Aldrich). Mice were bled by cardiac puncture. Subsequently, serum was collected and stored at -70°C until analysis. Serum OVA-specific IgE was measured as described previously [26]. A reference standard was obtained by *i.p.* immunisation of mice with OVA and arbitrarily assigned a value of 1,000 experimental units·mL^{-1} (EU·mL^{-1}). The detection level of the IgE ELISA was 0.5 EU·mL^{-1} for IgE.

Analysis of the cellular composition in the bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with 1 mL saline (37°C) containing 2 μg·mL^{-1} aprotinine (Roche Diagnostics, Almere, The Netherlands) and 5% bovine serum albumin (Sigma-Aldrich). Cytokines in the supernatant of the first mL of the BAL fluid (BALF) were determined by ELISA. Subsequently, mice were lavaged a total of four times with 1 mL saline (37°C). The cells in the BALF were pooled in cold phosphate-buffered saline (including those from the first mL). Subsequently, cells in the BALF were differentiated into mononuclear cells (monocytes, Mϕ and lymphocytes), eosinophils, and neutrophils as described previously [26].

Cytokine ELISAs

IL-5 ELISA (BD PharMingen, Alphen aan den Rijn, The Netherlands) was performed according to the manufacturer's instructions. The detection limit of the IL-5 ELISA was 10 $pg \cdot mL^{-1}$.

Statistical analysis

All data are expressed as mean±SEM. The airway dose-response curves to methacholine were statistically analysed by a general linear model of repeated measurements followed by *post hoc* comparison between groups. Data were log transformed before analysis to equalise variances in all groups. Statistical analysis on BALF cell counts was performed using the nonparametric Mann-Whitney U-test (2-tailed). For ELISA, results were analysed statistically using a t-test (2-tailed, homoscedastic). Results were considered statistically significant at the $p<0.05$ level.

RESULTS

Allergen-loaded macrophages migrate to the spleen

Allergen-loaded Mϕ suppress anti-inflammatory responses in a mouse model of allergic asthma [20, 21]. To gain more insight in the underlying mechanism, the localisation of OVA-Mϕ after administration (*i.v.*) was examined. Peritoneal Mϕ were loaded with OVA (OVA-Mϕ) and stimulated with ISS-ODN. Subsequently these Mϕ were labelled with the fluorescent dye CMFDA.

As a control, the CMFDA-labelled OVA-Mϕ were cultured for 20 h at 37°C and 5% CO_2 and the labelling efficiency was analysed. As shown in figures 1 a) and b), 20 h afters labelling >95% of the Mϕ were still fluorescent.

Additionally, OVA-sensitised mice were treated (*i.v.*) with 3×10^5 CMFDA-labelled OVA-Mϕ. After 20 h, the blood, lungs, spleen, lymph nodes (axillary, brachial, thoracic), liver and kidney were dissected and analysed for the presence of CMFDA-labelled Mϕ. Analysing the different tissues, no CMFDA-labelled OVA-Mϕ was found throughout the lungs (fig. 1c), whereas labelled Mϕ were clearly detectable in the spleen (fig. 1d–f). Staining with haematoxylin (not shown) revealed that most (~90%) CMFDA-labelled Mϕ were localised in the marginal zones of the spleen. No CMFDA-labelled Mϕ were detectable in the blood or any other analysed tissue, including the main clearance sites; liver and kidney (data not shown).

Since OVA-Mϕ migrated to the spleen, it was hypothesised that these *M*ϕ can accomplish an indirect suppressive effect, most probably mediated by the induction of allergen-specific memory lymphocytes.

Allergen-loaded macrophages induce an allergen-specific suppression

Allergen-specificity was examined by treating OVA-sensitised mice with ISS-ODN-stimulated and KLH-loaded Mϕ. In sham-treated mice, the airway responsiveness to increased concentrations of methacholine (1.6–50 $mg \cdot mL^{-1}$) was significantly ($p<0.05$) increased upon OVA inhalation challenge. As an example the airway hyperresponsiveness to 50 $mg \cdot mL^{-1}$ methacholine is depicted in figure 2a. As compared with sham-treated mice, the increase in AHR was significantly ($p<0.05$; up to 40%) suppressed upon treatment with ISS-ODN-stimulated OVA-Mϕ (fig. 2a). In contrast, ISS-ODN-stimulated KLH-Mϕ failed to suppress the AHR to methacholine (fig. 2a).

Additionally, the analysis of serum OVA-specific IgE levels demonstrated that the OVA-specific IgE levels dramatically increased upon challenge in sham-treated mice (fig 2b). ISS-ODN-stimulated KLH-Mϕ were not capable of suppressing the upregulation of OVA-specific IgE (fig. 2b). As a control, ISS-ODN-stimulated OVA-Mϕ suppressed the upregulation of OVA-specific IgE for 68% ($p<0.01$), as compared with sham-treated mice (fig. 2b).

In agreement with these results, ISS-ODN-stimulated KLH-Mϕ failed to suppress both the airway eosinophilia and IL-5 levels in the BALF (fig. 2c and d), whereas ISS-ODN-stimulated OVA-Mϕ significantly ($p<0.05$) suppressed these asthma manifestations (89% and 83% compared with sham-treated mice, respectively).

Activated ovalbumin-macrophage induce a long-lasting immunosuppressive effect

By lengthening the time period between Mϕ treatment and OVA inhalation challenge from 1 to 4 weeks, it was possible to investigate whether allergen-loaded Mϕ induced memory suppressive effects. OVA-sensitised mice were treated with OVA-Mϕ, LPS-stimulated OVA-Mϕ, or ISS-ODN-stimulated

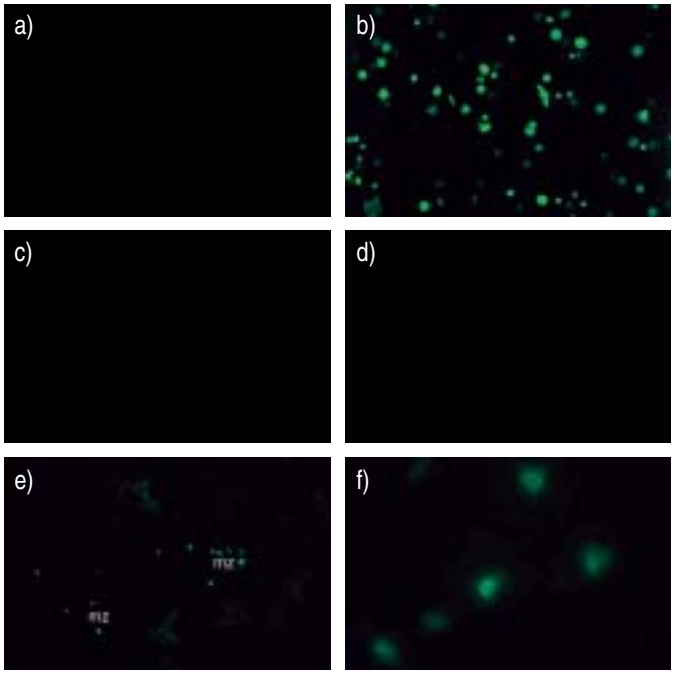

FIGURE 1. Immunostimulatory sequence oligodeoxynucleotide (ISS-ODN)-stimulated ovalbumin (OVA)-macrophages (Mϕ) migrate to the spleen. ISS-ODN-stimulated OVA-Mϕ were labelled with 5'chloromethylfluorescein diacetate (CMFDA). As a control, unlabelled Mϕ and CMFDA-labelled Mϕ were cultured for 20 h at 37°C and 5% CO_2 and subsequently, cytospins of unlabelled Mϕ (a) and CMFDA-labelled Mϕ (b) were analysed for labelling efficiency. OVA-sensitised mice were treated (*i.v.*) with CMFDA-labelled Mϕ (n=6; c, e and f) or with saline (n=3, d). After 20 h, lungs (c) and spleen (d, e and f) were dissected and analysed. mz: marginal zone.

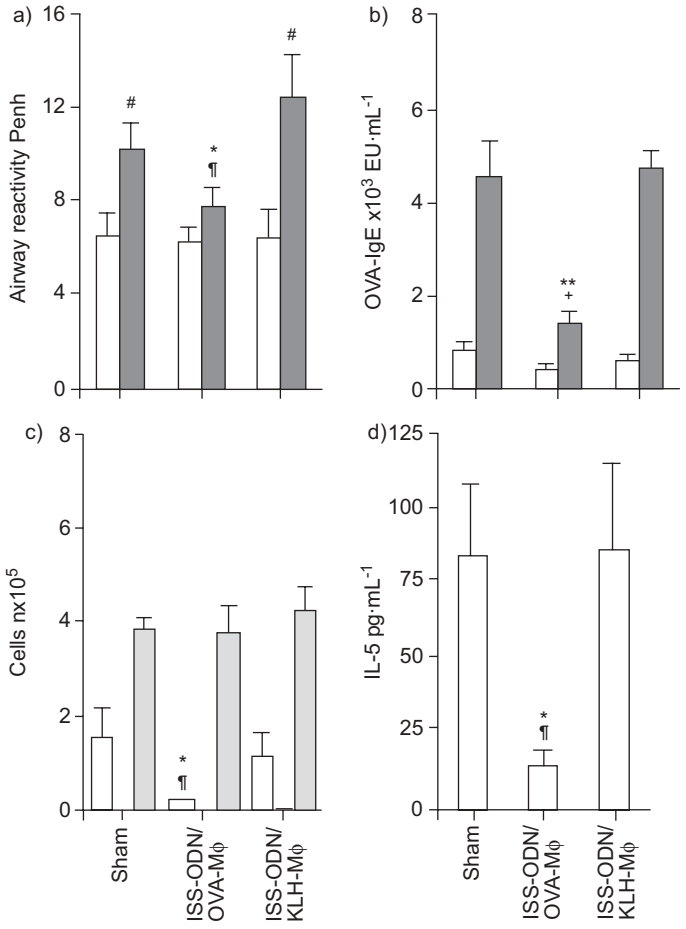

FIGURE 2. Allergen-loaded macrophages (Mϕ) induce an allergen-specific immunosuppressive response. Prior to the ovalbumin (OVA) challenge, OVA-sensitised mice were treated (*i.v.*) with saline (sham), immunostimulatory sequence oligodeoxynucleotide (ISS-ODN)-stimulated OVA-Mϕ (ISS-ODN/OVA-Mϕ), or ISS-ODN-stimulated keyhole limpet haemocyanin (KLH)-Mϕ (ISS-ODN/KLH-Mϕ). a) The airway hyperresponsiveness to aerosolised methacholine was measured before (□) and after (■) OVA inhalation challenge (expressed in enhanced pause: Penh). As representative for the methacholine dose response curve of all groups, the response to 50 mg·mL^{-1} methacholine is depicted. b) Serum OVA-specific immunoglobulin (Ig)E levels prior to (□) and after (■) challenge. The number of eosinophils (□), neutrophils (■) and mononuclear cells (▩) in the c) bronchoalveolar lavage fluid (BALF), and d) interleukin (IL)-5 levels in the BALF after OVA challenge. Values are expressed as the mean±SEM (n=6–8 per group). EU: experimental unit. #: $p<0.05$ compared with the same mice before OVA challenge. *: $p<0.05$ and **: $p<0.01$ compared with sham-treated and OVA-challenged mice. ¶: $p<0.05$ and +: $p<0.01$ compared with mice treated with ISS-ODN/KLH-Mϕ and OVA-challenged.

OVA-Mϕ. After 4 weeks the mice were challenged by OVA inhalation (8 days) and, subsequently, the increase in airway responsiveness to methacholine was measured. In sham-treated mice the airway responsiveness to increased concentrations of methacholine (1.6–25 mg·mL^{-1}) was significantly ($p<0.01$) increased upon OVA inhalation challenge. As an example the airway hyperresponsiveness to 25 mg·mL^{-1} methacholine is depicted in figure 3a. After a 4-week interval between treatment and challenge, *e.g.* after a short-term interval, ISS-ODN-stimulated OVA-Mϕ significantly ($p<0.05$) suppressed the AHR for up to 48%, as compared with sham-treated mice (fig. 3a).

After a long-term interval between treatment and challenge, OVA-Mϕ strongly suppressed (75%, $p<0.05$) the upregulation of serum OVA-specific IgE levels (fig. 3b). *Ex vivo* stimulation of OVA-Mϕ with LPS or ISS-ODN did not further suppress these OVA-specific IgE levels (fig. 3b).

Moreover, after a long-term interval between treatment and challenge ISS-ODN-stimulated OVA-Mϕ suppressed signifi-

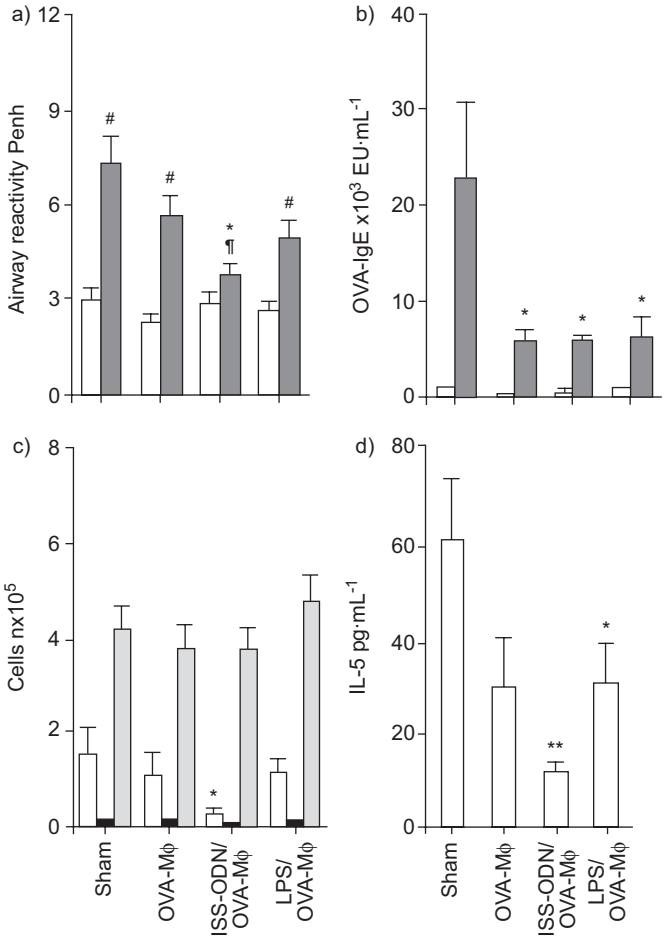

FIGURE 3. Immunostimulatory sequence oligodeoxynucleotide (ISS-ODN)-stimulated ovalbumin macrophage (OVA-Mϕ; ISS-ODN/OVA-Mϕ) induce a long-term immunosuppressive response. OVA-sensitised mice were treated (*i.v.*) with saline (sham), OVA-Mϕ, ISS-ODN/OVA-Mϕ, or lipopolysaccharide (LPS)-stimulated OVA-Mϕ (LPS/OVA-Mϕ). After 4 weeks, these mice were challenged by OVA inhalation. a) The airway hyperresponsiveness to aerosolised methacholine was measured before (□) and after (■) OVA inhalation challenge. As representative for the methacholine dose response curve of all groups, the response to 25 mg·mL^{-1} methacholine is depicted. b) OVA-specific immunoglobulin (Ig)E levels in serum prior to (□) and after (■) challenge. c) Number of eosinophils (□), neutrophils (■) and mononuclear cells (▩) in the bronchoalveolar lavage fluid (BALF) after OVA challenge. d) Interleukin (IL)-5 levels in the BALF after OVA challenge. Values are expressed as the mean±SEM (n=6–8 per group). EU: experimental units. ¶: $p<0.05$ and #: $p<0.01$ compared with the same mice before OVA challenge; *: $p<0.05$ and **: $p<0.01$ compared with sham-treated and OVA-challenged mice.

cantly ($p<0.05$) the influx of eosinophils into the lungs for 82%, as compared with sham-treated mice (fig. 3c). In correlation with the numbers of eosinophils, the IL-5 levels in the BALF were significantly suppressed (81%, $p<0.01$) upon treatment with ISS-ODN-stimulated OVA-Mϕ (fig. 3d). LPS-stimulated OVA-Mϕ failed to suppress airway eosinophilia but suppressed significantly ($p<0.05$) the IL-5 levels in the BALF for 51%, compared with sham-treatment (fig. 3d).

DISCUSSION

Though it is suggested that Mϕ play a pivotal role in the activation, as well as suppression of allergic asthma, the mechanism by which they exert these functions is still under debate [3]. Previously, the current authors demonstrated that allergen-loaded Mϕ suppress anti-inflammatory responses in a mouse model of allergic asthma [20, 21]. This current study shows that allergen-loaded Mϕ migrate to the spleen and induce, locally, an allergen-specific and long-lasting immunosuppressive response, hallmarks of a memory lymphocyte response.

Using animal models of allergic asthma, it has been suggested that Mϕ can exert a direct effect on the immunological homeostasis in the airways. Depletion of alveolar Mϕ increased the IgE responses [18] and adoptive transfer of alveolar Mϕ abrogated AHR [19]. Recently, PYNAERT *et al.* [27] showed that spleen Mϕ, loaded with OVA and intratracheal-administered, suppressed airway eosinophilia, probably, by inducing a Th1-mediated counter regulation in the lung [8, 27]. In contrast, in the present study evidence was obtained for an indirect mechanism by which Mϕ can suppress anti-inflammatory responses in the lung.

Using CMFDA-labelled Mϕ, it was observed that intravenously-administered Mϕ were not trapped in the lungs, but migrated to the spleen. Twenty hours after treatment, CMFDA-labelled Mϕ were only detectable in the spleen, while no CMFDA-labelled Mϕ were found in the lungs or lymph nodes. This is in agreement with the homing of intravenous-administered Mϕ and dendritic cells generated *ex vivo*, which transiently migrate to the lungs (within 2–3 h) followed by accumulation in the spleen and hardly in the lymph nodes [28–31]. In addition, >95% of interferon-γ-stimulated Mϕ reach the spleen within 5 h after *i.v.* injection, while less than 3% are detected in lung tissue (personal communication: G. Pynaert and J.Grooten, Dept Molecular Biomedical Research, Ghent University, Ghent, Belgium). The accumulation of allergen-loaded Mϕ in the spleen suggests that the Mϕ induce, locally, an indirect immunosuppressive effect. The additional findings that allergen-loaded Mϕ induce an allergen-specific and long-lasting suppressive response clearly demonstrated that allergen-specific suppressor lymphocytes have to be induced in the spleen. However, it cannot be fully excluded that the small numbers of Mϕ in the lungs are also responsible for the induction of suppressor T-cells. Mϕ have to be activated to produce high levels of IL-10 that, in turn, is essential to suppress OVA-induced airway inflammation [21]. Since OVA-induced airway inflammation is suppressed even 4 weeks after *i.v.* administration of Mϕ and it is rather hypothetical that they will still produce high levels of IL-10 at this time, the present data supports a role for allergen-specific suppressor lymphocytes induced by allergen-loaded Mϕ in the spleen. Furthermore, the pivotal role of donor-Mϕ-derived IL-10 [21] does not suggest a role for resident Mϕ responding to dead or apoptotic administered allergen-loaded Mϕ.

After treatment with allergen-loaded Mϕ, differential effects were observed dependent on the examined parameters (fig. 3). This is in agreement with previous observations [21]. ISS-ODN-stimulated OVA-Mϕ suppress airway inflammation more effectively compared with unstimulated OVA-Mϕ or LPS-stimulated OVA-Mϕ. This suggests that activation with ISS-ODN is needed for an effective induction of allergen-specific suppressor lymphocytes by OVA-Mϕ in the spleen. As activation of Mϕ is not required for suppression of serum OVA-specific IgE levels (fig. 3), it can be speculated that allergen-loaded Mϕ suppress allergen-induced airway manifestations *via*, at least, two different mechanisms of which one is IL-10 dependent [21]. OVA-induced airway hyperresponsiveness was measured using the Penh method, the authors would like to stipulate that Penh values may not correlate with changes in pulmonary resistance [32].

Most presumably a T-cell subset is involved, as the IL-10 production by OVA-Mϕ upon recognition of OVA-specific T-cells *in vitro*, is dependent on major histocompatibility complex class II/T-cell receptor interaction [20]. Upon allergen inhalation, the suppressive T-lymphocyte subset will be allergen-specifically activated (fig. 2) and, thereby, suppresses the Th2-lymphocyte mediated allergic airway inflammation and AHR. These data indicate that therapy with allergen-loaded Mϕ could be used to suppress airway inflammation in an allergen-specific fashion. As a result, the immune system is still functional during therapy and is able to respond to intruders. This suggests that patients that are sensitised to multiple aeroallergens may require Mϕ-based treatment with each allergen, either simultaneously or consecutively. However, it has been shown that multiple vaccinations with immature or mature antigen-presenting cells will not result in toxicity [33].

Although the exact suppressive T-cell subset remains to be elucidated, regulatory T (Treg) cells are likely candidates to be induced by allergen-loaded Mϕ. Treg cells are typically induced in microenvironments with local high levels of IL-10 and antigen presenting cells that present foreign-antigens. Importantly, Treg cells are antigen-specific and have a memory phenotype [34]. Antigen-induced Treg cells play a crucial role in the maintenance of T-cell tolerance against foreign-antigens. This T-cell subset exhibits its suppressive activity by secreting the suppressive cytokine IL-10 (type 1 Treg cells) or TGF-β (Th3 cells) [35]. Studies, using mouse models of allergic asthma, demonstrated that allergen-specific Treg cells can mediate the suppression of allergen-induced airway manifestations [36–38]. In the current study's model no increase of IL-10 levels in the BALF after Mϕ-treatment and subsequent OVA-inhalation challenge was found (data not shown). This suggests that OVA-Mϕ induce a suppressor-lymphocyte subset other than type 1 Treg cells.

The localisation of the allergen-loaded Mϕ in the spleen further supports the concept that Treg cells are induced. Although a few CMFDA-labelled Mϕ were detectable in the T-cell area, most (~90%) CMFDA-labelled Mϕ were localised in the marginal zones of the spleen. Previously, it was demonstrated

that in the marginal zone of the spleen antigen-presenting Mϕ, together with marginal zone B-cells and natural killer T-cells that likely secrete suppressive cytokines such as IL-10 and TGF-β, create a microenvironment that induces antigen-specific Treg cells [39, 40]. In the current study's model, ISS-ODN-stimulated Mϕ migrate to the marginal zones of the spleen and present, there, the allergen to lymphocytes. Since these Mϕ produce high levels of IL-10 [21], it can be hypothesised that a microenvironment is created in which allergen-specific Treg cells could be induced. Experiments in which splenocytes from tolerised mice are transferred to sensitised recipient mice are in progress to establish which suppressor-lymphocyte subset is induced upon interaction with allergen-loaded Mϕ.

Together, it was demonstrated that allergen-loaded macrophages are able to induce, besides direct suppression, an indirect suppression of airway manifestations of asthma in a mouse model. It can be speculated that allergen-loaded macrophages migrate to the spleen and present, locally, the allergen to the immune system while secreting interleukin-10. This causes a microenvironment in which an allergen-specific and long-lasting immunosuppressive response is induced. During the effector-phase, this immunosuppressive response suppresses allergen-induced airway manifestations. The role of macrophages in asthma pathology in general and the specific importance of macrophage-induced immunosuppressive memory responses against asthma manifestations may lead to novel strategies to induce stable suppression of allergen-specific T-helper type 2 responses.

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