Research Article

Murine Aortic Smooth Muscle Cells Acquire, Though Fail to Present Exogenous Protein Antigens on Major Histocompatibility Complex Class II Molecules

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In the present study aortic murine smooth muscle cell (SMC) antigen presentation capacity was evaluated using the Eγ-GFP/Y-Ae system to visualize antigen uptake through a GFP tag and tracking of Eγ peptide/MHCII presentation using the Y-Ae Ab. Stimulation with IFN-γ (100 ng/mL) for 72 h caused a significant ($P < 0.01$) increase in the percentage of MHC class II positive SMCs, compared with unstimulated cells. Treatment with Eγ-GFP (100 μg/mL) for 48 h induced a significant ($P < 0.05$) increase in the percentage of GFP positive SMCs while it did not affect the percentage of Y-Ae positive cells, being indicative of antigen uptake without its presentation in the context of MHC class II. After IFN-γ-stimulation, ovalbumin-(OVA, 1 mg/mL) or OVA323–339 peptide-(0.5 μg/mL) treated SMCs failed to induce OT-II CD4+ T cell activation/proliferation; this was also accompanied by a lack of expression of key costimulatory molecules (OX40L, CD40, CD70, and CD86) on SMCs. Finally, OVA-treated SMCs failed to induce DO11.10-GFP hybridoma activation, a process independent of costimulation. Our results demonstrate that while murine primary aortic SMCs express MHC class II and can acquire exogenous antigens, they fail to activate T cells through a failure in antigen presentation and a lack of costimulatory molecule expression.

1. Introduction

Atherosclerosis is an immunoinflammatory process [1, 2] in which smooth muscle cells (SMCs) play a critical role [3–5]. SMCs produce a broad range of immunoinflammatory mediators contributing to vascular inflammation [6] and participate in the formation of arterial tertiary lymphoid tissue in experimental atherosclerosis [7]. Human SMCs express class II major histocompatibility complex molecules (MHC class II) in atherosclerotic plaques [8] and following IFN-γ stimulation [9–11]. In addition, SMC MHC class II expression increases following vascular injury in rodent models [12]. However, the possibility that SMCs can act as antigen presenting cells (APCs) and consequently activate vascular T cell response remains, to date, controversial. In mice it has been demonstrated that brain microvessel SMCs/pericytes can induce a proliferation of syngenic CD4+ T cells in vitro in a MHC class II dependent manner [13]. SMCs/pericytes were able to process and present exogenous antigens to T cell hybridoma [14] and preferentially activated Th1 T cell clones as compared with Th2 T cells of the same antigen specificity [15]. In contrast to syngeneic cocultures using wild type CD4+ T cells, microvascular SMCs did not support proliferation of antigen specific T cell receptor (TCR) transgenic CD4+ T cells [16]. Others demonstrated that murine SMCs pulsed with antigen increased the expression of the IL-2 receptor on T cells but were not able to induce T cell proliferation [17].
Human saphenous vein SMCs expressing MHC class II molecules were unable to activate allogeneic memory T cells [18] and failed to effectively support T cell proliferation to the polyclonal activator, phytohemagglutinin [19]. This inability resulted from a defect in costimulatory function, particularly the lack of OX40 ligand (OX40L) [19]. SMCs from different tissues may behave differently; for example, cultured human airway smooth muscle cells were capable of presenting the superantigen, staphylococcal enterotoxin A, via MHC class II molecules to CD4+ T cells [20]. More selective approaches are required to investigate SMC antigen presentation capacity.

Here we utilized the Exα-GFP/Y-Ae model that allows visualization of antigen uptake through a GFP tagged Ex peptide and tracking of antigen presentation using the Y-Ae Ab. The Ex-GFP protein is internalized and processed by APCs to generate Ex peptide for presentation on MHC class II. The monoclonal Ab Y-Ae detects Ex only when bound to MHC class II molecules (I-Aα) [21–24]. We demonstrate that while murine primary aortic SMCs express MHC class II and can acquire exogenous antigens, they fail to activate T cells through a failure in antigen presentation and a lack of costimulatory molecule expression.

2. Materials and Methods

2.1. Animals. C57BL/6 mice (Harlan, Shardlow, UK) were used to prepare SMCs and dendritic cells (DCs). OT-II (CD45.1) mice bred in house were used as donors of Tg αβ-actin (+) mice for transgenic mice expressing the mouse alpha-(CD45.1) mice bred in house were used as donors of Tg αβ-actin (+) mice for transgenic mice expressing the mouse alpha-chain and beta-chain T cell receptors that pair with the CD4 coreceptor and is specific for chicken ovalbumin 323–339 in the context of I-A b. Animals were maintained on a 12/12-hour light/dark cycle with free access to food and water and all the procedures were performed in accordance with local ethical and UK Home Office regulations.

2.2. Cell Cultures and Cocultures. Murine primary SMCs were derived from the thoracic aorta of C57BL/6 mice as previously described [25, 26] and grown in DMEM supplemented with L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco, Paisley, UK). Before initiation of the assays, the SMCs were starved into DMEM supplemented with 0.1% fetal bovine serum for 48 hours [25, 27]. Cells were characterized by immunofluorescence microscopy using FITC labeled anti-smooth muscle α-actin (α-SMA) monoclonal antibody (Ab) (clone 1A4; Sigma-Aldrich, Dorset, UK). Studies were performed with cells at passages 3–6. OVA specific TCR transgenic OT-II CD4+ T cells were isolated from OT-II/CD45.1 mice using the MicroBead-based CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bisley, UK) according to manufacturer’s instructions and grown in complete RPMI (containing L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin). The DO11.10-GFP hybridoma cells [28] were grown in complete RPMI containing geneticin (0.5 mg/mL, Sigma-Aldrich) as previously described [29]. DCs were obtained by flushing the bone marrow of C57BL/6 mice and grown in complete RPMI containing 10% granulocyte-macrophage colony stimulating factor (GM-CSF) for 7 days [30]. All cells were kept in a humidified incubator at 37° C in 5% CO2.

Murine SMCs were cultured in 48 multiwell plates until 80% confluence. Subsequently cells were stimulated with IFN-γ (100 ng/mL; R&D Systems, Abingdon, UK) for 72 h to enhance their MHC class II expression and then treated with OVA (1 ng/mL; InvivoGen, Toulouse, France) or OVA323–339 peptide (0.5 μg/mL; InvivoGen) overnight. Isolated OT-II CD4+ T cell or DO11.10-GFP hybridoma cell preparations were then introduced into the murine SMC cultures at a 1:5 ratio, for 24, 48, and 72 h or 24 h, respectively. OVA-treated DCs, cocultured with both OT-II CD4+ T cells and DO11.10-GFP hybridoma cells at the same ratio of SMCs, were used as positive control. Subsequently, OT-II CD4+ T cells or DO11.10-GFP hybridoma cells were collected by rinsing the cocultures three times followed by staining and preparation for flow cytometric analysis. For the analysis of costimulatory molecule expression murine SMCs were cultured in 6 multiwell plates and stimulated with IFN-γ (100 ng/mL) for 72 h before flow cytometry. In a separate set of experiments, SMCs were stimulated with IFN-γ (100 ng/mL) for 72 h and then treated with fluorescein labeled-chicken OVA (FITC-OVA, 1 mg/mL, Molecular Probes) overnight. Subsequently, the supernatant was removed and the cells washed with PBS. The FITC-OVA uptake was visualized using the EVOS FL Cell Imaging System (Life Technologies Ltd., Paisley, UK).

2.3. AlphaGFP Preparation and Treatment. To assess the ability of murine SMCs to act as APCs, we employed the Eα-GFP/Y-Ae system as previously described [22–24]. A recombinant Escherichia coli strain expressing the Eα-GFP fusion protein was grown to midlog phase before induction of protein expression. Protein expression was induced by addition of isoprpyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich) to a final concentration of 1 mM and cultures were incubated overnight at 30° C with agitation (200 rpm). The Eα-GFP fusion protein was purified from the bacterial lysates using HisPur Cobalt Spin Columns (Thermo Scientific, Loughborough, UK) and endotoxin was removed using Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific). Murine SMCs were cultured in 6 multiwell plates, as described above, stimulated with IFN-γ (100 ng/mL) for 72 h, and then treated with Eα-GFP (100 μg/mL). After 1, 24, and 48 h of treatment, cells were collected for flow cytometric analysis. DCs cultured under the same conditions and treated with Eα-GFP (100 μg/mL) for 24 h were used as a positive control.

2.4. Flow Cytometry. Aliquots of cells were washed and resuspended in Fc block (2.4G2 hybridoma supernatant) for 25 mins at 4°C to block Fc receptors. Subsequently, cells were incubated with Abs (in PBS containing 2% FBS) for 30 mins at 4°C, washed twice and then, where necessary, incubated with streptavidin for additional 20 mins at 4°C. Following washing, cells were analyzed on a FACScalibur using CellQuest-Pro (BD Biosciences, Oxford, UK), or on
SMC treatment with E

Using the E

3. Results

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able to present the E

that, although SMCs internalize the antigen, they are not

24h (Figure1(a)). Asshownin Figure1(b),

similarity Abs: Y-Ae-Bio (specific for I-E

followed by streptavidin-PerCP. OT-II CD4

M5/114.15.2), anti-CD11c-APC (clone: N418), anti-CD54-PE

FITC (clone: 16-10A1), anti-CD44-FITC (clone: IM7), anti-OX40L-Bio

that cytomere,

expression. A significant (P < 0.01) increase in CD44

therefore, treated with E

3.2. SMCs Fail to Induce OT-II CD4+ T Cell Activation

and Proliferation. We next assessed the ability of SMCs to

activate OVA-specific transgenic CD4+ T cells. In preliminary

experiments by using FITC-OVA we confirmed the uptake of

the model antigen by SMCs (data not shown). Using CFSE

to track proliferation, we evaluated the number of Tg T
cells undergoing proliferation after 72 h of coculture with SMCs or

bone marrow derived DCs, used as positive control. The propor-
tion of dividing T cells (expressed as percentage of CFSE-

cells) was approximately 0.5–1% in both presence and absence of
cocultured unstimulated SMCs (Figure 2).

Neither stimulation with IFN-γ nor treatment with OVA or

OVA323–339 peptide of SMCs affected the proliferation of OT-

II CD4+ T cells. In contrast, coculture with OVA-treated DCs

dramatically increased the proportion of dividing OT-II CD4+ T
cells by around 20% (Figure 2).

We also examined cell surface expression of activation

markers such as CD25, CD44, and CD69 on OT-II CD4+

cells after coculture with SMCs or bone marrow derived

DCs. CD25 and CD69 were detected in approximately 2% of

OT-II CD4+ T cells, alone or cocultured for 24, 48, and 72 h

with unstimulated SMCs, IFN-γ-stimulated SMCs, or IFN-

γ-stimulated SMCs treated with OVA or OVA323–339 peptide.

Moreover, the percentage of CD25 and CD69 positive T
cells did not change after SMC treatment with OVA or OVA323–339

peptide alone, while a significant (P < 0.001) increase was

observed only after coculture with OVA-treated DCs at all of

the time points considered (Figure 3). The percentage of

CD44 positive OT-II CD4+ T cells was about 7% at all of the
time points considered, in both presence and absence of

unstimulated SMCs. Stimulation with IFN-γ and/or treat-

ment of SMCs with OVA or OVA323–339 did not affect CD44

expression. A significant (P < 0.01) increase in CD44

positive OT-II CD4+ T cells was observed after 48 and 72 h

coculture with OVA-treated DCs (Figure 3). These data

demonstrate that antigen-pulsed aortic murine SMCs are not

able to induce antigen-specific T cell activation/proliferation.

3.3. Effect of IFN-γ Stimulation on Costimulatory/Adhesion

Molecules Expression by Murine SMCs. Previous studies

have corroborated the inability of human SMCs to activate memory

T cells with the lack of costimulation [19]. Thus we exam-
in whether murine SMCs express costimulatory/adhesion

molecules at baseline and after IFN-γ (100 ng/mL) stimu-

lation for 72 h. As shown in Figure 4, unstimulated SMCs

did not affect percentage of ICAM-1 (P < 0.01) and CD80 (P < 0.001) positive cells while it did not

affect the percentage of CD44 positive cells. In contrast, only

low levels of OX40L, CD40, CD70, and CD86 expression

detectable in unstimulated SMCs. IFN-γ stimulation

did not increase the percentage of SMCs positive to these

molecules. The failure of SMCs to respond to IFN-γ, in this

case, was selective for the costimulatory molecules because

2.5. CFSE Staining. OT-II CD4+ T cells were labeled with

the fluorescent dye carboxyl fluorescein succinimidyl ester

(CFSE, Molecular Probes) as previously described [31]. The
cells were washed and then cocultured with SMCs or DCs

(used as a positive control) for 72 h. The level of fluores-
cence intensity from the CFSE labeling was measured by

flow cytometry. Incremental loss of CFSE intensity showed

proliferation.

2.6. Statistical Analysis. Results are expressed as mean ± SEM

of 3 experiments run in triplicate. The results were statistically

analyzed by the t-test or ANOVA (Two-Tail P value) and the

Bonferroni post hoc test. The level of statistical significance

was P < 0.05 per test.

3. Results

3.1. Assessment of Antigen Uptake/Presentation by SMCs

Using the Ex-GFP/Y-Ae System. Stimulation with IFN-γ

(100 ng/mL) for 72 h resulted in a significant (P < 0.01) 5-
to 6-fold increase in the percentage of MHC class II positive

SMCs compared with unstimulated cells (Figure 1(a)).

Similar results were observed in IFN-γ-stimulated SMCs

subsequently treated with Ex peptide (100 μg/mL) for 1 and

24 h (P < 0.05), while no significant changes were observed

after 48 h of treatment (Figure 1(a)). As shown in Figure 1(b),

SMC treatment with Ex peptide induced an increase in the

percentage of GFP positive cells, both in presence or absence

of IFN-γ-stimulation, being indicative of antigen uptake. The

increase in GFP positive cells observed was significant only

at 48 h (P < 0.05). No significant changes were observed in

the percentage of Y-Ae positive SMCs after IFN-γ-stimulation

and/or treatment with Ex peptide (Figure 1(c)) suggesting

that, although SMCs internalize the antigen, they are not

able to present the Ex peptide in the context of MHC class

II. Treatment of DCs with Ex peptide (100 μg/mL), used as

positive control, caused an increase in the percentage of Y-Ae

positive cells (Figure 1(d)).
Figure 1: SMCs acquire exogenous antigens but fail to present them in the context of MHC class II. Evaluation of antigen uptake/presentation by murine SMCs. SMCs were stimulated with IFN-γ (100 ng/mL) for 72 h and subsequently treated with E-α-GFP peptide (100 μg/mL) for the indicated time points. (a) MHC class II expression. (b) GFP expression. (c) Representative flow cytometry plots showing no positivity of SMCs to the Y-Ae Ab or (d) positivity of DCs, used as a positive control. Results are expressed as mean ± SEM from three separate experiments. *P < 0.05, **P < 0.01, versus unstimulated cells.
Figure 2: SMCs fail to induce OT-II CD4+ T cell proliferation. Representative plots and relative statistical analysis showing the effect of SMCs on OT-II CD4+ T cell proliferation. IFN-γ-stimulated SMCs were treated with OVA (1 mg/mL) or OVA323−339 peptide (0.5 μg/mL) overnight and then cocultured with CFSE-labeled OT-II CD4+ T cells for 72 h. OVA-treated DCs were used as a positive control. Results are expressed as mean ± SEM from three separate experiments run in triplicate. **p < 0.01 versus OT-II CD4+ T cells alone.

the percentage of MHC class II molecules was increased after IFN-γ stimulation under the same conditions (Figure 4).

3.4. SMCs Do Not Activate DO11.10-GFP Hybridoma Cells. The murine DO11.10-GFP hybridoma was originally obtained by stably transfecting a DO11.10 T cell hybridoma with a construct in which GFP expression is under the control of a nuclear factor of activated T cells (NFAT) regulated promoter [28]. Thus, once activated, hybridoma cells, detectable using the KJ1-26 clonotypic antibody, become GFP-positive. DO11.10 hybridoma cells express the TCR recognizing OVA323–339 peptide in the context of either I-A\(^d\) or I-A\(^b\) MHC class II [32] without any requirement for costimulation [29]. Coculture with unstimulated SMCs had no effect on GFP expression by DO11.10-GFP hybridoma cells and similar results were obtained after stimulation with IFN-γ and/or treatment of SMCs with OVA or OVA323−339 peptide. On the contrary, DCs treated with OVA, used as positive control, caused a significant (P < 0.001) increase in GFP expression by hybridoma cells (Figure 5). These data confirm that SMCs are unable to present exogenous protein antigens in the context of MHC class II.
4. Discussion

In the present study, we demonstrated that (1) cultured primary murine SMCs express MHC class II molecules after stimulation with IFN-γ and are able to acquire/uptake antigens; however, they fail to present the peptide antigen in the context of MHC class II, as demonstrated by using the specific Ealpha-(Eγα-)GFP/Y-Ae system; (2) OVA-treated SMCs fail to induce activation/proliferation of OT-II CD4+ T cells, data consistent with a defect in MHC class II-restricted Ag presentation and in the expression of costimulatory molecules, such as OX40L, CD40, CD70, and CD86; (3) SMCs also fail to promote activation of OVA responding DO11.10-GFP hybridoma T cells that do not require any costimulatory signal for activation.

The first finding that murine aortic SMCs express MHC class II molecules is in line with previous data showing MHC class II expression in atheroma SMCs [8] and in rodent arteries in response to injury [12], as well as in human SMCs in culture following IFN-γ-stimulation [18]. Murray and colleagues [18] demonstrated that class II molecules on human saphenous vein SMCs were functional, since they induced CD25 expression on resting CD4+ T cells. Additional studies demonstrated that survival and activation of T cells occurred as a result of the specific interaction between TCR on T cells and MHC molecules on SMCs, since treatment with antibodies directed toward MHC class II blocked the proliferation of CD4+ T cells cocultured with syngeneic SMCs [13,16]. On the contrary, in the context of nonspecific generalized T cell stimulation or in the presence of polyclonal activators such as phytohemagglutinin SMCs did not activate CD4+ T cells [18,19].

In order to understand whether an antigen specific stimulation leads to immunological competence of SMCs, engaging MHC molecules, we employed a novel and selective approach such as the Eα-GFP/Y-Ae model that allows visualization of antigen uptake through a GFP tagged Eα peptide and tracking of antigen presentation using the Y-Ae Ab. The Eα-GFP protein is internalized and processed by APCs to generate Eα peptide for presentation on MHC class II. The monoclonal Ab Y-Ae detects Eα only when bound to MHC class II molecules (I-Ab) [21–24]. Eα-GFP treatment of SMCs increased the percentage of GFP positive cells, without affecting the percentage of SMCs positive to
the monoclonal Ab Y-Ae. These results clearly demonstrate that primary aortic murine SMCs fail to present exogenous protein antigens in the context of MHC class II.

Our results also prove the inability of SMCs in inducing OVA specific OT-I CD4+ T cell activation and proliferation. A possible explanation for these observations is that SMCs fail to activate T cells through a failure in antigen presentation and a lack of costimulatory molecule expression. Indeed, although human SMCs express the costimulatory molecules CD44, CD54, CD58, and CD59 [18], they lack OX40L, which is considered essential for T cell activation [19]. We also observed lack of costimulatory molecule expression (OX40L, CD40, CD70, and CD86) on SMC surface following IFN-γ stimulation, supporting the hypothesis that impaired costimulation function contributes to the inability of SMCs to induce T cell activation/proliferation.

In order to analyze this point further, we cocultured SMCs with the DO11.10-GFP hybridoma cells that, in presence of the model Ag OVA, undergo activation without requiring any costimulatory signal [28, 29]. Importantly, SMCs failed to activate DO11.10-GFP hybridoma cells, demonstrating that other mechanisms, apart from a defect in costimulation function, are liable for the limited capacity of SMCs to activate T cells.

One possibility could be that SMCs cannot process protein antigens, rather than not being able to present

**Figure 4:** SMCs lack key costimulatory molecules. Representative flow cytometry histograms and relative graph showing the effect of IFN-γ (100 ng/mL) on costimulatory/adhesion molecules expression in murine SMCs. Red empty histograms: isotype control; gray filled histograms or white columns: unstimulated SMCs; black empty histograms or black columns: IFN-γ-stimulated SMCs. Results are expressed as mean ± SEM from three separate experiments run in triplicate. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus unstimulated cells.
them. Nevertheless, in our experiments, treatment of SMCs with OVA_{323-339} peptide, which does not require any processing to be presented in the context of MHC molecules, did not affect activation/proliferation of neither OT-II CD4^+ T cell nor DO11.10-GFP hybridoma cells. This observation demonstrates that the SMC inability in presentation cannot lie in a defect in the antigen processing; thus further investigations will be necessary to understand the mechanisms underlining this deficiency.

5. Conclusions

In summary, our work demonstrates that while murine primary aortic SMCs express MHC class II and can acquire exogenous antigens, they fail to activate T cells through a failure in antigen presentation and a lack of costimulatory molecule expression. Our results do not preclude the possibility that SMCs could act as APCs, depending on the environment (e.g., in atherosclerotic arteries) and the vascular bed; however, they suggest that antigen presentation...
may not be the key immunological feature of SMCs in the initiation of vascular inflammation.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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