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Pancreatic ductal cell antigens are important in the development of invasive insulitis in Non-Obese Diabetic mice

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ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune disease in which insulin producing beta cells of the pancreas are selectively destroyed. Glial Fibrillary Acidic Protein (GFAP) expressed in peri-islet Schwann cells (pSCs) and in the ductal cells of the pancreas is one of the candidate autoantigens for T1D. Immune responses to GFAP expressing cell types precede the islet autoimmunity in Non-Obese Diabetic (NOD) mice. By removing MHC class I from GFAP expressing cell types, we tested the role of autoantigens presented by these cell types in the development of invasive insulitis. Our findings indicate that antigens expressed by pancreatic ductal cells are important in the development of invasive insulitis in NOD mice.

1. Introduction

The immune mediated destruction of beta cells in T1D results in the progressive loss of beta cells, causing insulin deficiency and consequent hyperglycemia (Jayasimhan et al., 2014; van Belle et al., 2011). The presence of multiple auto antibodies and evidence of T cell reactivity to autoantigens indicates multiple targets are involved in disease pathogenesis in both humans and in NOD mice (Han et al., 2013; Roep and Peakman, 2012; Pearson et al., 2016).

Investigations in NOD mice have identified numerous autoantigens and many of these autoantigens including Glutamic Acid Decarboxylase 65 (GAD65), Insulinoma Associated antigen 2 (IA2) and Islet Cell Autoantigen 69 (ICA69) are found not only in beta cells, but also in the cells of the neuroendocrine system (Mallone et al., 2011; Mauvais et al., 2016; Lieberman and DiLorenzo, 2003). It is important to identify the initiating autoantigen to design immunoregulatory strategies to treat the disease in genetically susceptible individuals. To date Proinsulin is the earliest autoantigen that has been identified to drive islet infiltration (Nakayama et al., 2005; Wong, 2005; Wegmann and Eisenbarth, 2000). Mice lacking native proinsulin, and carrying a mutated non-antigenic proinsulin, were protected from developing infiltrates into the islets. However, these transgenic mice had infiltrates into the islet associated pancreatic ductal tissue. This suggests that there are antigenic targets of an autoimmune attack on the islet associated ductal tissue of the pancreas, which precedes islet autoimmunity (Nakayama, 2011).

The possibility that an initiating antigen is expressed in *peri* islet tissues is further supported by the finding that, immune responses to autoantigens expressed in pSCs precede the immune response to beta cells (Winer et al., 2003; Tsui et al., 2008). The early infiltrates seen around the pancreatic ducts and the attack on GFAP expressing pSCs implicates these cells in the autoimmune process. Studies conducted by Razavi et.al also support the involvement of pancreatic sensory neurons in controlling islet inflammation (Razavi et al., 2006). This raises the question of whether an autoantigen present in GFAP positive cells may not trigger an autoimmune response that precedes the immune responses to beta cell antigens (Pang et al., 2017; Kaufman, 2003).

GFAP expressing ductal cells and pSCs of the neuroendocrine pancreas share with islet beta cells a number of autoantigens implicated in T1D (Bouwens and Pipeleers, 1998; Mally et al., 1996a; Gurr et al., 2002; Lieberman and DiLorenzo, 2003). An early immune response against autoantigens expressed in GFAP positive cell types may result in the subsequent cross reactive attack of beta cells (Kaufman, 2003). To understand the role of early upstream antigens expressed in pSCs and in

Abbreviations: DBA, *Dolichos biflorus* Agglutinin; EYFP, Enhanced Yellow Fluorescent Protein; GFAP, Glial Fibrillary Acidic Protein; GFP, Green Fluorescent Protein; H&E, Haematoxylin and Eosin; HBSS, Hanks Balanced Salt Solution; IA2, Insulinoma Associated Antigen 2; ICA-69, 69 kDa Islet cell Autoantigen; MHC, Major Histocompatibility Complex; NOD, Non-Obese Diabetic; OCT, Optimum Cutting Temperature; PBS, Phosphate Buffered Saline; PFA, Paraformaldehyde; pSCs, Peri-Islet Schwann Cells; RBC, Red blood cell; T1D, Type 1 Diabetes; β2M, Beta 2 Microglobulin

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ductal cell types of the pancreas, our lab generated NOD mice lacking MHC class I expression on GFAP expressing cells and assessed the development of insulitis.

2. Materials and methods

2.1. Animals

All animals were maintained under specific pathogen free (SPF) conditions at the Precinct, Animal Centre at the Alfred Hospital (PAC, Melbourne, Australia). All procedures involving mice were performed in compliance with the Animal Ethics guidelines of Monash University and were approved by the Animal Ethics Committee of the Alfred Medical Research and Education Precinct (AMREP).

2.1.1. Generation of transgenic NOD GFAPcre mice

FVB-Tg(GFAP-cre)25Mes/J (Stock number 004600) were purchased from the Jackson Laboratory (Bar Harbor, ME, US) and backcrossed onto NOD/Lt for at least 10 generations. The generated NOD GFAPcre mice express cre recombinase under the control of human GFAP promoter. Hemizygous transgenic mice were identified by PCR based strategy using primers ACT CCT TCA TAA AGC CCT and ATC ACT CGT TGC ATC GAC CG specific for the GFAP and cre gene respectively (Zhuo et al., 2001). Even after 10 generations of backcrossing to NOD, there is a possibility of having a sizeable FVB derived genomic interval surrounding the GFAPcre transgene in NOD GFAPcre mice.

To rule out the possibility of any alterations in disease incidence by this hitchhiking FVB genomic interval, we compared the diabetic incidence in transgene positive NOD mice to conventional NOD mice. Since the pattern of incidence in transgene positive NOD mice and in conventional NOD mice was similar, we did not attempt to sequence the surrounding genomic intervals around the GFAPcre transgene. Endogenous β2M expression in GFAPcre transgene positive mice was removed by crossing to NOD $\beta2M^{-/-}$ mice (Serreze et al., 1994). Homozygous β2M knockout and GFAPcre transgene positive mice were known as NOD GFAPcre mice.

2.1.2. Generation of NOD GFAPcre.flβ2Ma mice (class I GFAP bald mice)

NOD.flβ2Ma mice carrying a floxed β2Ma transgene (Hamilton-Williams et al., 2003; Hamilton-Williams et al., 2001) on an endogenous β2M knockout background were crossed with NOD GFAPcre mice to generate NOD GFAPcre.flβ2Ma mice. The NOD $GFAPcre^{+}$ $\beta2M^{-/-}$ $fl\beta2Ma^{+}$ (class I GFAP bald mice) exhibit ductal cell and neuronal cell specific deletion of MHC class I whereas NOD $GFAPcre^{-}\beta2M^{-/-}fl\beta2Ma^{+}$ (Control mice) are class I GFAP normal mice respectively. 100–160 day old female NOD class I GFAP bald and NOD class I GFAP normal mice were used for insulitis studies.

2.1.3. Generation of Reporter mice (NOD $GFAPcre^{+}EYFP^{+}$)

C57BL/6.Gt(ROSA)26Sor.EYFP mice (gift by Dr. David Izon at St Vincent's Institute for Medical Research, Melbourne, Australia) carries a loxP-flanked STOP sequence followed the Enhanced Yellow Fluorescent Protein (EYFP) gene (Srinivas et al., 2001). C57BL/6.Gt(ROSA)26Sor.-EYFP mice were backcrossed to NOD/Lt for at least 10 generations. Crossing the mutant NOD EYFP mice to NOD GFAPcre mice generated the reporter mice (NOD $GFAPcre^{+}EYFP^{+}$), exhibiting EYFP expression in GFAP driven cre expressing tissues. To analyze the pattern of cre expression, tissues extracted from reporter and control (NOD $GFAP-cre^{-}EYFP^{+}$) mice were analyzed via immunohistochemistry and flow cytometry.

2.2. Flow cytometry

2.2.1. Pancreatic ductal cell isolation and staining

The pancreas from the reporter and control mice was dissected and single cell suspensions were prepared as described by Maximilian Reichert et al. (Reichert et al., 2013). The pancreatic single cell suspensions were incubated with biotinylated *Dolichos biflorus* Agglutinin (DBA) (Vector Labs, Burlingame, CA, US) (Xiao et al., 2013), washed and labelled with streptavidin conjugated to PerCP (BD Biosciences, USA). Following a second wash, cells were fixed in 4% paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS), permeabilized using 0.1% saponin and stained with polyclonal guinea pig anti-insulin antibodies (Dako, Carpinteria, CA) and secondary goat anti guinea pig Alexa Fluor 647 antibody (Thermo Fisher scientific, USA). The stained cells were acquired on FACS Calibur (BD Biosciences) and the data were analyzed using FlowJo software (Version 7.6.5). Establishment of the gate was based on the staining profiles of the negative control. We also attempted to stain for MHC class I molecules in pancreatic ductal cells. Despite multiple attempts, we were unable to view the MHC class I expression on ductal cells of the pancreas. We believe that the prolonged enzymatic digestion method used in isolating ductal cells, may have damaged the surface proteins which annihilated the detection of MHC class I molecules using antibody dependent flow cytometric methods.

2.2.2. Splenocytes isolation and staining

Red Blood Cell (RBC) lysed spleen single cell suspensions from the reporter and control mice were stained for CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70) and CD11c (HL3) with anti mouse monoclonal antibodies from BD Biosciences. The stained cells were acquired on a FACS Calibur and data was analyzed using FlowJo software. Establishment of the gate was based on the staining profiles of the negative control.

2.2.3. Brain cell isolation

The brain was dissected from reporter and control mice and collected in Hanks Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+}). The brain hemispheres were mechanically dissociated and passed through a 70 μm cell strainer. The suspension was centrifuged at 250*g* for one minute and the collected pellet resuspended in HBSS containing 0.5% trypsin EDTA (Gibco, USA). Following 15 min incubation at 37 °C with intermittent shaking, the cell suspension was centrifuged, washed in HBSS and passed through a 70 μm strainer for analysis via flow cytometry.

2.3. Histology

2.3.1. Immunofluorescence

Pancreatic tissue from the reporter and control mice was fixed overnight in 4% PFA, immersed in 20% sucrose solution for 8 h and then snap frozen in Optimum Cutting Temperature (OCT) compound (Tissue-Tek). 5 μm tissue sections were cut and stained using Alexa Fluor 555 conjugated rabbit antibody against Green Fluorescent Protein (GFP) (Invitrogen, Carlsbad, CA, USA). Stained sections were visualized using a fluorescent microscope (Olympus BX61) at 20× magnification. Images were captured in AnalySIS 5.0 software and analyzed using Image J.

2.3.2. Histochemistry

Paraffin embedded pancreatic tissue from 100 to 160 day old class I GFAP Bald and GFAP normal mice was cut at three levels (100 μm apart) and each 5 μm section stained with Haematoxylin and Eosin (H&E). The degree of mononuclear cell infiltration was determined in 10–60 islets from each pancreas, in a blinded fashion. Insulitis severity was scored on a scale of 0–4 as previously described (Hamilton-Williams et al., 2003). The proportion of islets exhibiting invasive insulitis or disease free was determined and expressed as a percentage of total islets scored. H&E stained sections were visualized using the Olympus BX51 microscope at 20× magnification.

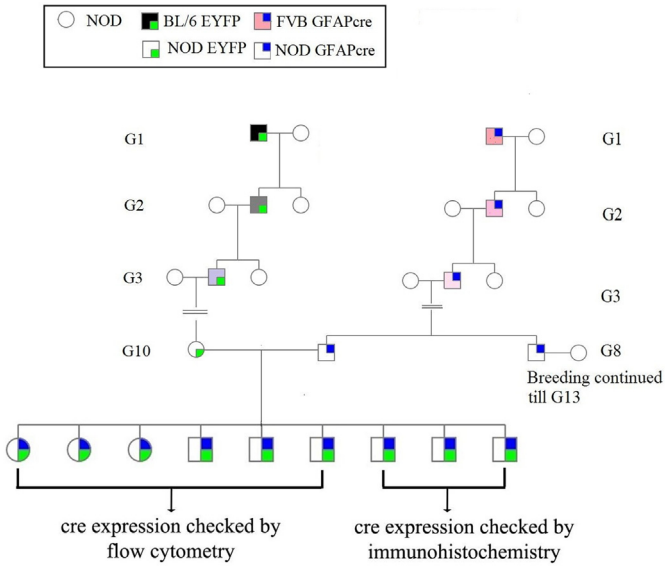


Fig. 1. Genogram shows the breeding strategy for NOD GFAPcre and reporter mice. FVB.GFAPcre mice were crossed to NOD mice for at least 10 generations to obtain NOD GFAPcre mice. At generation eight the GFAPcre mice were crossed to NOD EYFP mice to generate reporter mice. The pattern of cre expression in reporter mice was determined by flow cytometry and immunohistochemistry.

2.4. Statistical analysis

All data were represented as means ± SEM. Statistical difference was determined by using the unpaired *t*-test for column comparisons. Significance was accepted at two tailed *p* values ≤ .05 and analyzed with Graphpad prism software (version 7.02).

3. Results

3.1. Characterization of EYFP expression

3.1.1. Detection of EYFP expression in pSCs and ductal cells of reporter mice

NOD GFAPcre mice were generated by backcrossing the FVB GFAPcre mice to NOD mice for 10 generations (Fig. 1). The FVB X NOD GFAPcre mice at generation eight were crossed to NOD EYFP mice that carried a stop codon flanked by lox sites. The resultant NOD GFAP-cre$^+$EYFP$^+$ reporter mice lost the stop codon and thus expressed EYFP. The expression of EYFP indicating cre expression was determined by flow cytometry (Fig. 2a) and immunohistochemistry (Fig. 2b). Flow cytometric analyses of brain cells isolated from reporter mice showed EYFP expression in 28 ± 1.6% of the cells. EYFP was also detected in fixed pancreatic cryosections using fluorescence microscopy. The pattern of fluorescence was consistent with the morphology of pancreatic pSCs (Fig. 2b (VIII) white arrows) and ductal cells (Fig. 2b (VIII) red arrows) of the pancreas.

3.1.2. EYFP expression was not detected in immune cells of reporter mice

Ectopic expression of cre in off-target tissue types has variably been

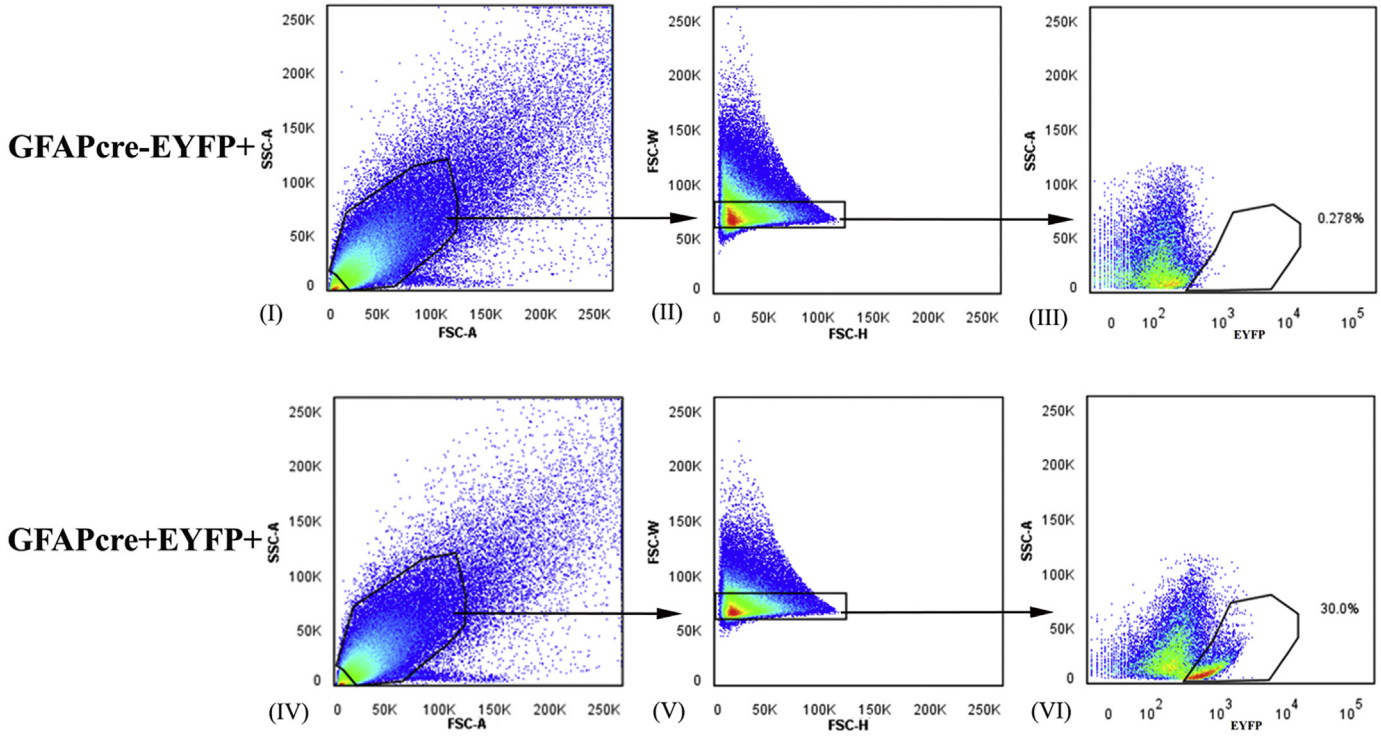


Fig. 2. a. Detection of EYFP in brain cells indicates cre expression. Flow cytometric analysis of brain cells isolated from control (top panel) and reporter mice (n = 3) showing gating strategy used and EYFP expression. Dot plot shows brain single cell suspension gated in forward and side scatter. First doublets were excluded from live gate (I, IV) based on FSC-H and FSC-W (II, V) then EYFP expressing cells were gated (x axis) vs side scatter (y axis) (III, VI). Brain cells in reporter mice express EYFP (VI) and the absence of expression in control mice (III). b Detection of EYFP in pancreatic neuroendocrine and ductal cells indicates cre expression. Fluorescent microscopic analysis showing cre expression in morphologically identified pancreatic neuroendocrine pSC and ductal cells of the reporter mice (n = 3). Pancreatic tissue sections from reporter (VII, VIII, IX) and control mice (I, II, III) were stained using a rabbit antibody against GFP conjugated to Alexa Fluor 555 and nuclear stained with DAPI. White arrows (VIII and IX) point to EYFP expressing pSC and red arrows point to EYFP expressing ductal cells. Stained brain sections from reporter mice (IV, V, and VI) were used as a positive control for EYFP signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

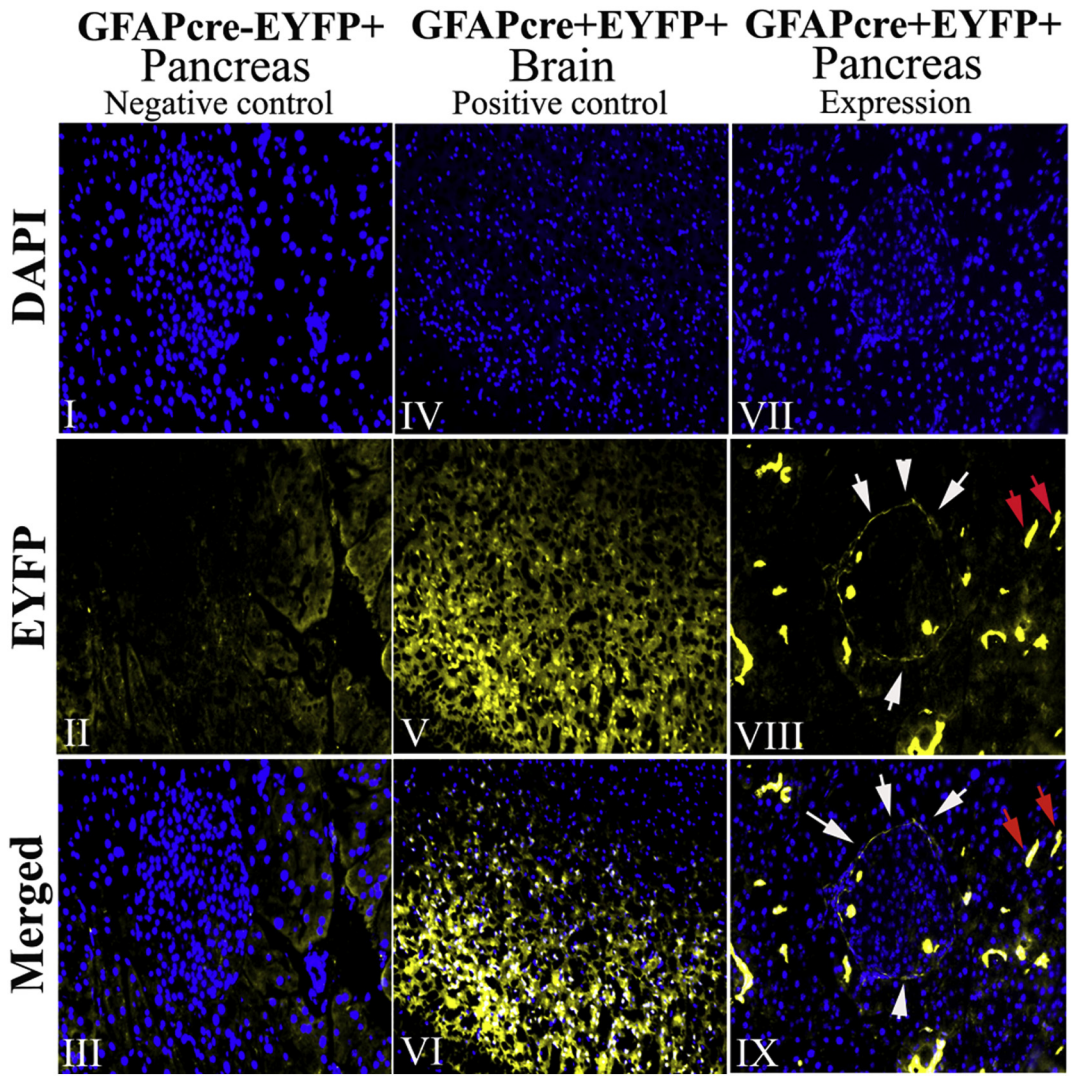


Fig. 2. (*continued*)

reported with the use of cre-lox systems (Smith, 2011; Harno et al., 2013). The specificity of the promoter dictates the fidelity of its expression and therefore the pattern of cre mediated recombination and EYFP expression. It was important to assess the pattern of cre expression in GFAPcre mice. This is because, in class I GFAP bald mice any non-specific expression of cre in immune cell types, would have resulted in deletion of β2Ma and loss of MHC class I molecules from these cell types. This would have confounded the interpretation of results.

EYFP expression was not detected in CD4 T cells, CD8 T cells (Fig. 3a) dendritic cells or macrophages (Fig. 3b) isolated from reporter mice ($n = 5$). The absence of EYFP expression in the entire splenocyte population (Supplementary Fig. 1A) indicates that GFAPcre mice are an appropriate model in which to study the role of MHC class I expression on pSCs and ductal cells in the initiation of insulitis.

3.2. Severity of insulitis is significantly reduced in the absence of MHC class I on GFAP expressing cell types

Class I GFAP bald mice were generated by crossing the NOD GFAPcre mice to NOD.flβ2ma mice (Fig. 4). These mice were used to assess whether MHC class I presented self-antigens, expressed by GFAP positive cells, were necessary for the initiation and progression of insulitis. Histological analyses of pancreatic sections showed insulitis was significantly reduced in mice lacking MHC class I molecules on GFAP expressing cells compared with class I sufficient age and sex matched control mice (Fig. 5a). Class I GFAP bald mice had more disease free islets (70 ± 5%) compared with class I sufficient controls (41 ± 8%) (Fig. 5b). Conversely the percentage of islets with invasive insulitis was also significantly reduced in class I GFAP bald mice. Class I GFAP bald mice had 9.5 ± 2% compared with MHC class I sufficient controls (32 ± 9%) (Fig. 5c). These data suggest that the loss of MHC class I from GFAP expressing cells somehow protected the mice from insulitis. Since a subset of GFAP positive cells expresses insulin, we therefore wanted to test whether the significant reduction of invasive insulitis seen in these mice is due to the absence of MHC class I mediated presentation of the autoantigen insulin.

3.3. GFAP positive ductal cells do not express insulin

Our data from reporter mice show that ductal cells are made up of GFAP positive and GFAP negative cell types (Fig. 6(III)). We detected insulin expression in 2.5 ± 0.6% of the ductal cells and identified that 90% of the insulin expressing ductal cells were GFAP negative. Since GFAP positive cells were found not to co-express insulin, the reduction of invasive insulitis seen in class I GFAP bald mice cannot be due to the loss of expression of insulin. It must be due to the loss of the presentation of another autoantigen present in the GFAP expressing ductal cells. There are a number of other autoantigens that ductal cells share with beta cells. It is possible therefore that one of these autoantigens is an early autoantigen that can initiate insulitis.

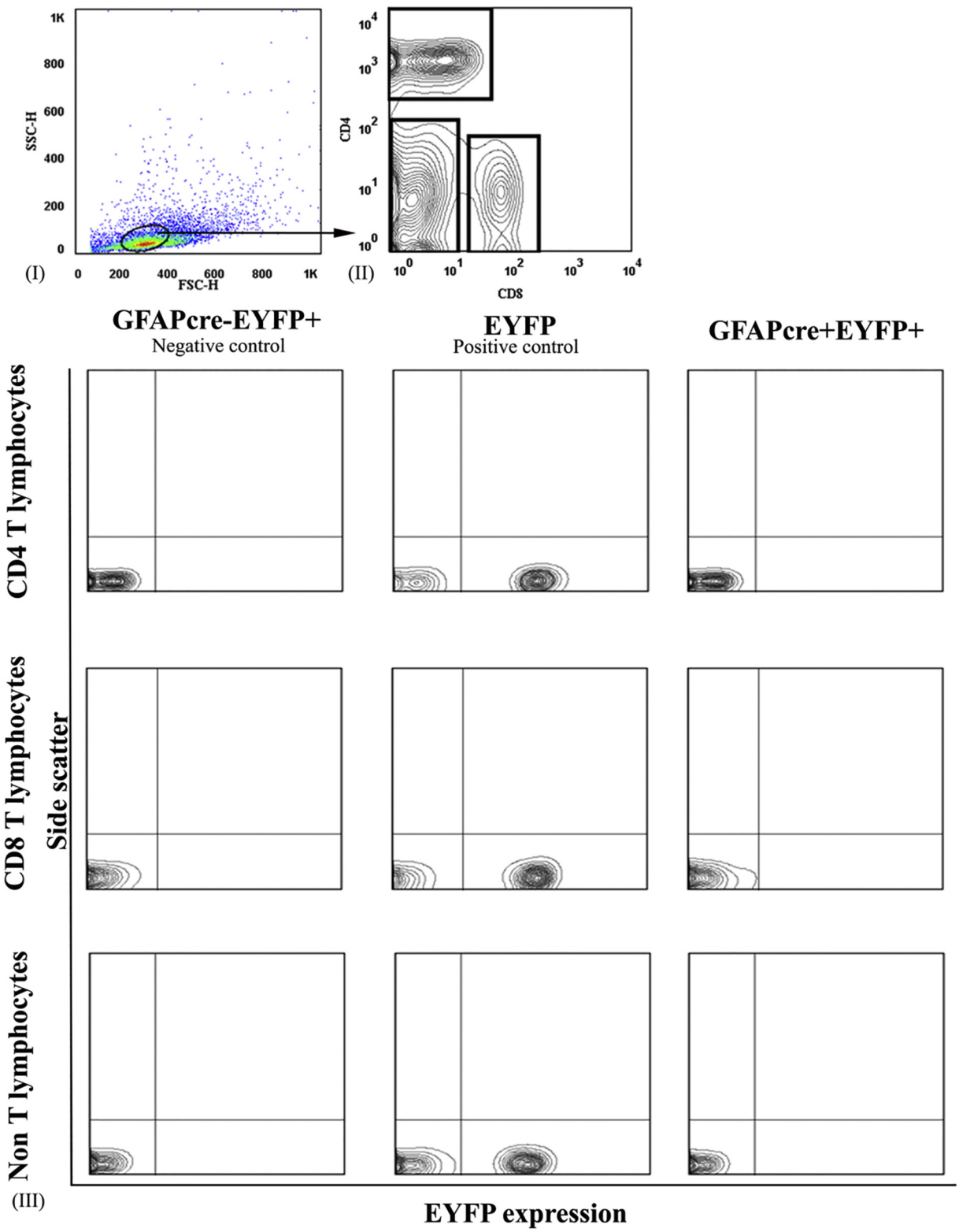
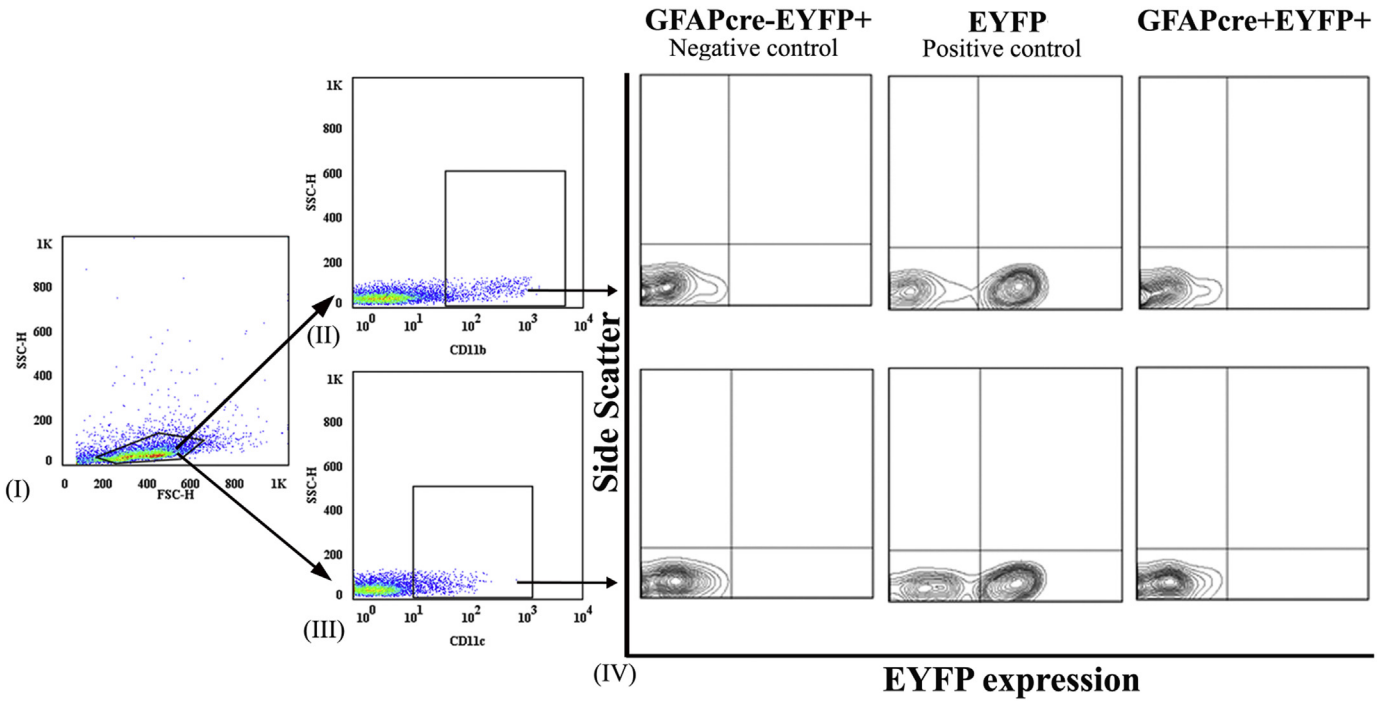


Fig. 3. a. EYFP expression was not detected in immune cells of reporter mice. Flow cytometric analyses of splenocytes from reporter mice were unable to detect lymphocytes expressing EYFP (n = 5). Dot plots showing live gating of lymphocytes in forward and side scatter (I). Sequential gating on live lymphocytes and contour plots showing CD4 T cells, CD8 T cells and Non T lymphocytes (II). EYFP expression was not detected in CD4 T cell, CD8 T cell and non T lymphocytes in reporter and control mice (III). Spleenocytes from mice known to express EYFP in immune cells were used as a positive control. b. EYFP expression was not detected in macrophages and dendritic cells of reporter mice (n = 5). Dot plots showing live splenocytes gated in forward and side scatter (I). Sequential gating on live splenocytes and dot plots showing macrophages gated using CD11b expression (II) and dendritic cells gated using CD11c expression (III). EYFP expression was not detected in macrophages and dendritic cells in reporter and control mice (IV). Splenocytes from mice known to express EYFP were used as a positive control.



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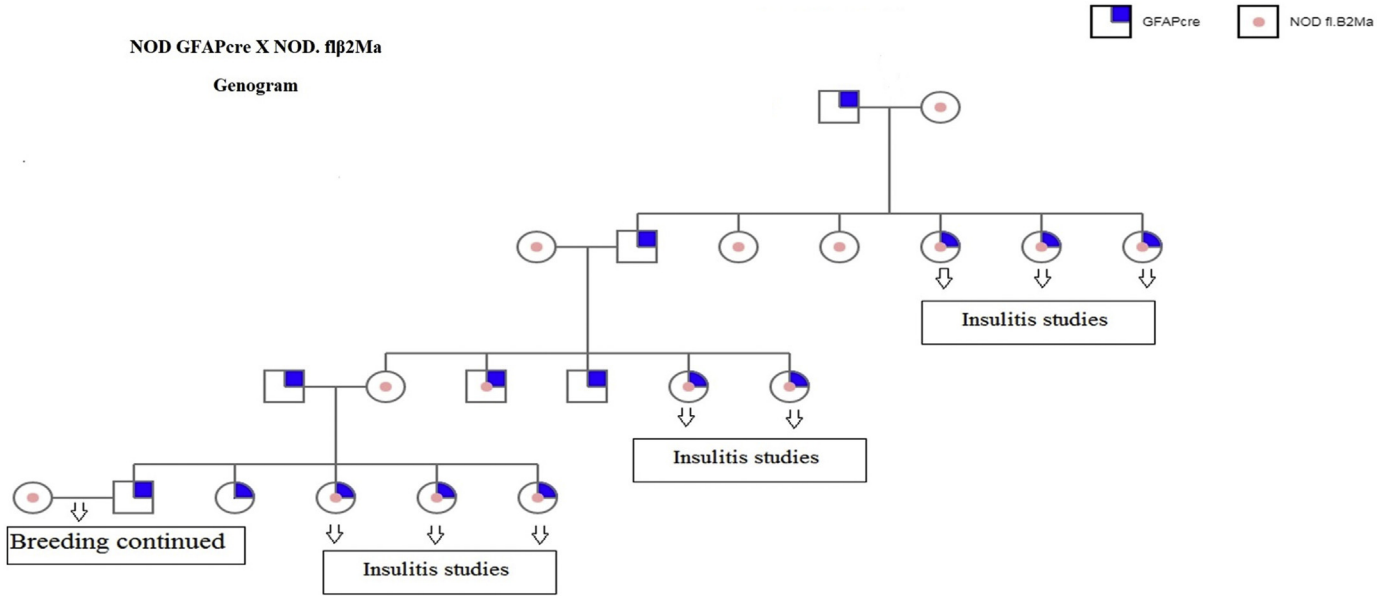

Fig. 4. Genogram shows the breeding strategy in generating female NOD GFAP bald mice. NOD GFAPcre mice on β2M knockout background were crossed with NOD.flβ2Ma mice and the female GFAP bald littermates at 100–160 days of age were chosen for insulitis studies.

4. Discussion

We aimed to understand the role of MHC class I on GFAP expressing cells in the initiation of disease in NOD mice. Cre-lox technology was used to generate tissue specific knockout mice. Cre was expressed under the control of the GFAP promoter and thus cre mediated loss of MHC class I occurred in GFAP expressing cells including ductal and pSC types. Transgenic expression of cre has occasionally been reported to occur in off-target tissues. We therefore wanted to confirm that cre was not leaking in the immune compartment because any cre mediated loss of MHC class I from immune cells would have confounded the interpretation of results (de Jersey et al., 2007). The absence of any leakiness of cre in the immune compartment confirmed that the model was appropriate to study the role of MHC class I on GFAP expressing cells, in particular the ductal and pSCs of the pancreas.

Utilizing this model we found that the expression of MHC class I on GFAP positive cells was important for the development of insulitis. Not only did the mice lacking MHC class I on GFAP expressing cells have more disease free islets, they were also significantly protected from developing invasive insulitis. This suggests that MHC class I expression on GFAP positive cells is important *early* in the disease process. Because proinsulin is widely held to be the earliest autoantigen, we wondered whether the reduction in insulitis in class I GFAP bald mice may have been due to the loss of MHC class I mediated presentation of insulin peptides expressed by ductal cells of the pancreas.

The decrease in the presence of islets with insulitis, even low level

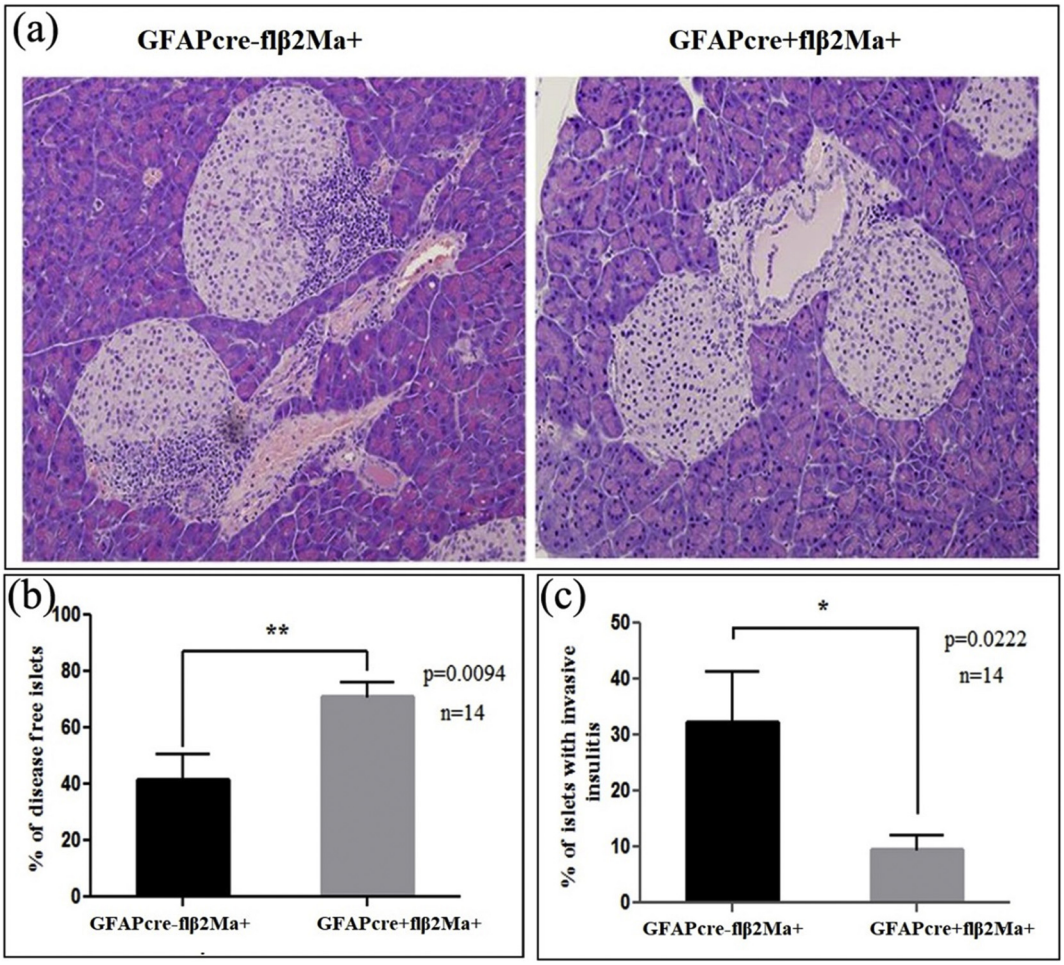

Fig. 5. GFAP bald mice had more disease free islets. The pancreatic sections from NOD GFAP bald mice at 100–160 days of age were analyzed by haematoxylin and eosin staining. Microphotography shows mononuclear infiltrated islets in control and disease free islets in GFAP bald mice (a). Statistical data showing the percentage of disease free islets (b) and islets with invasive insulitis in GFAP bald and control mice (c). 236 islets were scored for GFAPcre-flβ2Ma + group and 265 islets were scored for GFAPcre + flβ2Ma + group respectively.

periductal insulitis in class I GFAP bald mice suggests there has been a loss of immune response to ductal and/or pSC antigens. However, it is unlikely that the protection from insulitis observed in class I GFAP bald mice was due to the inability of ductal cells to present insulin peptides to CD8 T cells. This is because a proportion of ductal cells retained class I expression in GFAP bald mice and also expressed insulin. Thus a subset of ductal cells would have been able to present insulin peptides to CD8 T cells capable of initiating the disease. This begs the question as to which antigen/s GFAP positive ductal and/or pSCs express that may be important for the initiation of insulitis.

Ductal cells are beta cell progenitors and as such they share a number of autoantigens with beta cells, including proinsulin, GAD65, ICA69 and HIP/PAP (Inada et al., 2008; Bouwens and Pipeleers, 1998; Gurr et al., 2002; Mally et al., 1996a; Mally et al., 1996b). Thus, it is plausible that the initiation of an immune response to ductal cell antigens may lead to cross reactive attack of beta cells expressing shared antigen/s. Although not widely discussed, the argument that ductal cell antigens may have an important role in the initiation of periductal insulitis has credence. Peri ductal insulitis persisted in mice lacking native proinsulin indicating that the immune response to autoantigens expressed by ductal cells can occur independently of the immune response to proinsulin (Moriyama et al., 2003).

The chronological order of the immune response to autoantigens implicated in T1D is not completely understood. There has long been an assumption that proinsulin is the primary autoantigen, however, in addition to the findings presented here, it has also been shown that the immune response to antigens expressed by pSCs precede the immune response and attack of beta cells (Winer et al., 2003). Our data, and the findings of Michael Dosch et al. (Winer et al., 2003), suggests there may be several alternative autoantigens that could initiate an immune response which culminates in beta cell auto reactivity (Dilorenzo, 2011).

While it is not known what causes the inflammation around the ducts of the pancreas, there are a number of hypotheses which have been proposed. The pro inflammatory milieu of the islets could be driven by chemicals (Bodin et al., 2015), microbial infections (Kondrashova and Hyöty, 2014; de Beeck and Eizirik, 2016), food allergens (Virtanen, 2016; Rewers and Ludvigsson, 2016) or the defective clearance of apoptotic bodies by macrophages (O'Brien et al., 2002). Inflammation is associated with tissue damage that can result in the release of otherwise sequestered self-antigens. Our findings indicate that self-antigens expressed by GFAP positive cells are important in the development of insulitis in NOD mice. Further studies using the Class I GFAP bald NOD mice will help researchers to understand the importance of ductal cell antigens in insulitis progression and diabetes development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.01.001.

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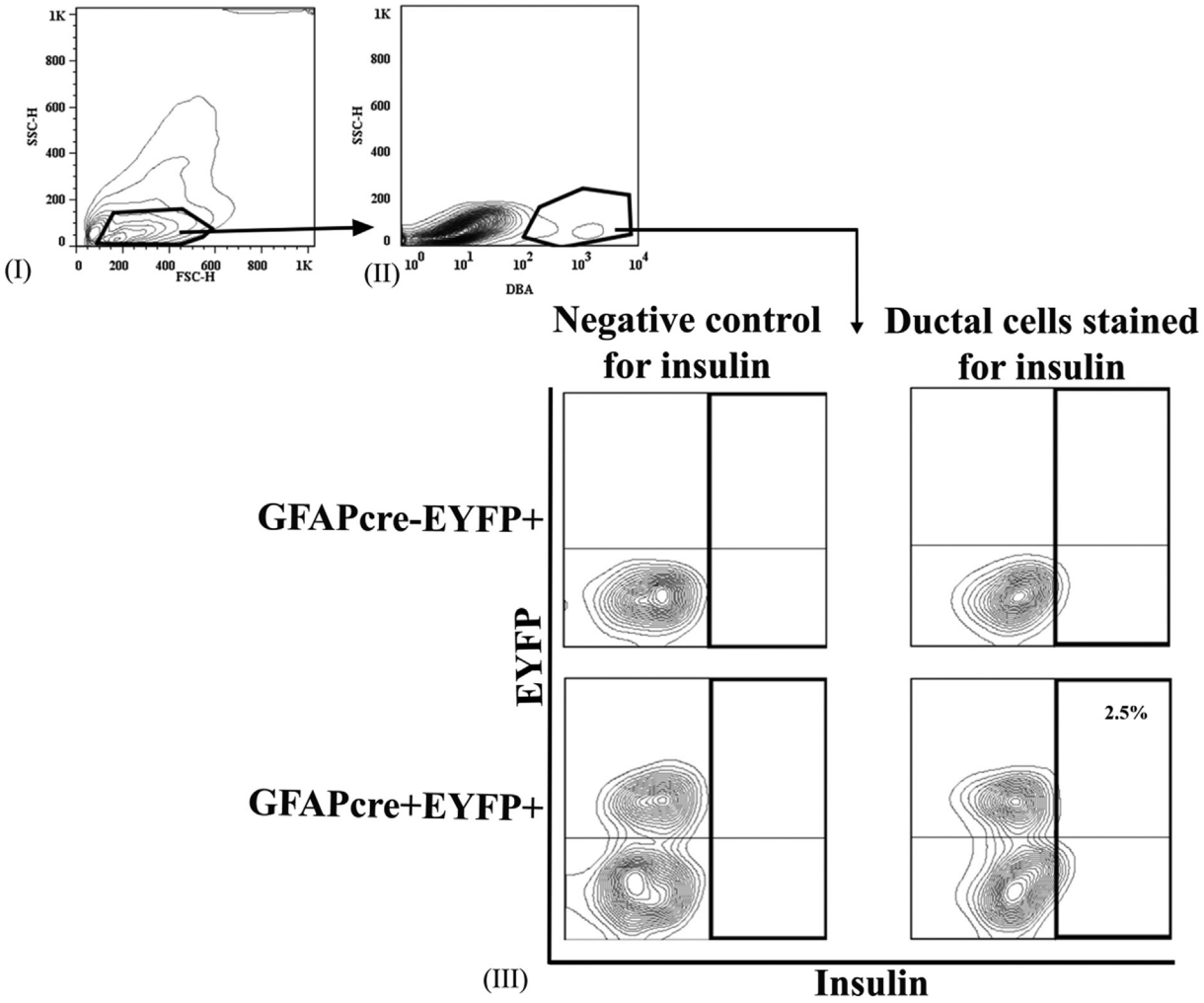

Fig. 6. Insulin positive pancreatic ductal cells do not express EYFP. Contour plots showing pancreatic single cells live gated for low side scatter (I). DBA stained pancreatic ductal cells (II) from reporter and control mice (n = 4). DBA stained ductal cells showing insulin and EYFP expression (III). Out of the 2.5% of insulin expressing ductal cells, > 90% of the cells did not express EYFP.

Competing interest

The authors declare they have no competing interest associated with this manuscript.

Funding

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