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Smoking activates cytotoxic CD8$^+$ T cells and causes survivin release in rheumatoid arthritis

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ABSTRACT

CD8$^+$ T cells have an emerging role in RA. Resent research indicates a causal relationship between the non-exhausted state of CD8$^+$ T cells, defined by lost function of PD-1, and development of arthritis. We investigated how smoking contributes to the non-exhausted phenotype of CD8$^+$ T cells and cause survivin release to serum.

We compared serum survivin levels between smokers and non-smokers in 252 RA and 168 healthy subjects. Nicotine effects on CD8$^+$ T cells were studied in peripheral blood of smoking women, bone marrow of nicotine treated mice and in sorted CD8 spleen cells *in vitro* using flow cytometry and quantitative PCR.

Smoking increased the frequency of survivin release in serum of healthy women (OR 3.64, $p = 0.025$) and in RA patients (OR 1.98, $p = 0.039$). CD8$^+$ T cells of smokers gained a non-exhausted PD-1 deficient phenotype. Expression of the cytotoxic marker CD107 correlated to survivin levels in serum. In the experimental setting, nicotine exposure led to an accumulation of non-exhausted PD-1$^-$IL-7R$^+$ CD8$^+$ T cells in the bone marrow that is abundant with survivin producing cells. The production of the cytolytic protein perforin in bone marrow correlated to serum survivin levels. *In vitro* stimulation of nicotinic receptors on murine CD8$^+$ T cells induced repressive transcription factors T-bet and Blimp-1 in support of the non-exhausted phenotype.

We conclude that nicotine contributes to autoimmunity by supporting the non-exhausted state of CD8$^+$ T cells resulting in the release of survivin. This presents a new mechanism by which smoking may contribute to the pathogenesis of RA.

1. Introduction

The CD8$^+$ T cells are a major lymphocyte subgroup recognized by their cytotoxic effector functions. Activated CD8$^+$ T cells are known to identify and kill cells with MHC class I molecules presenting pathogenic antigens, thereby protecting against infection and cancer. Identification triggers the release of perforin and granzymes that will disrupt the cell membrane and induce apoptosis of the target cell [1]. The cytotoxic activity of CD8$^+$ T cells is controlled by receptors expressed on the cell surface, where the inhibitory receptor Programmed Cell Death 1 (PD-1) plays a leading role [2]. PD-1 activation leads to dephosphorylation of mediators downstream the T cell receptor, thereby inhibiting stimulatory signals following target cell recognition

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[3]. Chronic infection with persistent antigen exposure induces abnormally high expression of PD-1. This impairs the immune response and is referred to as the state of exhaustion [4]. The transcriptional and phenotypic profile of exhausted $CD8^+$ T cells reveal that exhausted cells, in addition to upregulating PD-1, lose the potential to form memory cells, distinguished by expression of the inteleukin-7 receptor (IL-7R) [5]. Exhausted $CD8^+$ T cells loose the capacity to eliminate target cells and inhibition of PD-1 has been approved as a therapeutic strategy to reverse the exhausted state in conditions where a robust immune response is desirable [6].

The $CD8^+$ T cells have been given relatively little attention in autoimmunity, but this seem to be a matter of change [7]. Rheumatoid arthritis (RA) is a canonical autoimmune disease with inflammatory attacks to the joints, often resulting in their permanent damage [8]. In early RA, the number of peripheral blood $CD8^+$ T cell is increased [9] and the $CD8^+$ T cytokine production relate to disease activity [10]. In addition, there is a strong association between seropositive RA and polymorphism in the MHC class I locus [11], supporting a role for $CD8^+$ T cells in autoimmunity. However, reports on $CD8^+$ T cells in RA are inconsistent, and a lower frequency of $CD8^+$ T cells has been described in circulation of arthralgia patients who develop RA and in patients with early RA [12]. Other reports imply no difference in frequency of $CD8^+$ T cells in patients with established RA [10,13]. Exhaustion of $CD8^+$ T cells is reasoned to be a mechanism that sustains peripheral tolerance by controlling the activity of autoreactive cells [14], indicating that induced exhaustion could have a therapeutic potential in autoimmune disease [15]. A consequence of this idea is that the opposite phenomenon, non-exhausted memory-like T cells defined by PD-1 deficiency and high expression of IL-7R, may promote autoimmunity. Indeed, there is a growing amount of evidence supporting a role for PD-1 deficiency in RA. Decreased PD-1 expression has been reported in $CD4^+$ and $CD8^+$ T cells from peripheral blood of RA patients. Low PD-1 expression was associated with higher C-reactive protein levels and disease activity score, indicating a protective role of PD-1 in RA [16]. In fact, it seems like lost PD-1 function contribute to the development of RA. Firstly, polymorphism of the PD-1 gene that affects binding to its enhancer region has been shown in association with RA in Swedish [17] and in Chinese [18] cohorts. Secondly, the use of anti-PD-1 antibodies for treatment of melanoma has resulted in several reported cases of inflammatory arthritis [19–21]. In the experimental setting it was shown that PD-1 deficient mice develop arthritis at a higher incidence and severity [22].

Survivin is an inhibitor of apoptosis protein that can be measured in serum of RA patients to predict aggressive autoimmune disease. Survivin was initially attracting attention due to its overexpression in tumour cells, but several recent studies supports a role for survivin in the immune system [23–25]. Its role in RA was recently discovered when high levels of survivin were found in plasma of both RA patients and pre-symptomatic patients who had not yet developed the disease [26]. In early RA the presence of high serum survivin levels was associated with a poor prognosis and poor response to anti-rheumatic treatment [27,28]. Hence, it is known that the release of survivin is associated with destructive autoimmune processes, but the understanding of what events cause the release is very limited. Survivin can be localised in both nucleus and cytoplasm [29] but is rarely found extracellularly in healthy individuals. The only mechanism for survivin secretion described in the literature is exosomal transport from tumour cells [30]. In this study we hypothesised that extracellular survivin in RA is released when $CD8^+$ cytotoxic T cells target cells with high expression of survivin. This proposal is based on two main arguments: firstly, $CD8^+$ T cells targeting survivin expressing tumour cells is a well known phenomenon in the field of cancer research. The activation of cytotoxic $CD8^+$ T cells by survivin derived peptides aids destruction of survivin presenting tumour cells [31] and has reached clinical trial as a therapeutic strategy for treatment of malignant glioma and metastatic melanoma [32,33]. Secondly, previous results from our lab show that activating survivin specific lymphocytes through vaccination of arthritic mice results in higher serum survivin levels [34], demonstrating the relevance for this mechanism in arthritis.

In the present study we investigate if smoking, and nicotine in particular, shift the phenotype of $CD8^+$ T cells toward a non-exhausted state and promotes the release of survivin.

2. Materials and methods

2.1. Patients

In total, 184 female and 68 male patients of working age with established RA [35] were included in the study between November 2011 and September 2013. Patients were recruited at the Rheumatology units of the Sahlgrenska University Hospital in Gothenburg and the Nothern Älvsborg County Hospital in Uddevalla, Sweden. All but 17 patients were treated with methotrexate (MTX). 81 patients combined MTX with biologics, 18 with other disease modifying drugs. 8 patients used biologics as monotherapy. Oral corticosteroids were used by 23 patients (2.5–15 mg/day). All patients completed a structured questionnaire regarding their smoking habits, medication, and concomitant diseases. At inclusion, all patients underwent clinical examination performed by experienced rheumatologists. Healthy controls consisted of 95 females and 73 males and were recruited from a population study on asthma, the West Sweden Asthma Study (WSAS) [36]. They were examined between 2009 and 2012 using extensive questionnaires regarding their life style, smoking habits, physical examination including lung function tests. Healthy subjects did not report respiratory or autoimmune diseases, or other diseases with systemic inflammation.

Phenotype and transcriptional profile of the peripheral blood $CD8^+$ T cells was analysed in 17 female RA patients (mean age 58.8 (45–76) years, disease duration 14.4 (2–44) years, and in 10 healthy females (mean age 57.9 (49–80) years). At the time of blood sampling, all but 3 patients were treated with MTX (mean 18 (10–25) mg/week) combined with sulfasalazine and hydroxychloroquine in 2 patients; with anti-TNF in 10 patients; with rituximab in 1 patient; and with abatacept in 2 patients.

The study was approved by the Ethical Committee of the Sahlgrenska University Hospital (WSAS, diary no. 593-08; RA, diary no. 659-11). The study was carried out in accordance with the Declaration of Helsinki and patients gave informed written consent prior to participation.

2.2. Mouse models

Two independent experiments were performed. In the first experiment, 20 male, 10 weeks old, DBA1 mice purchased from Taconic Europe A/S (Ry, Denmark) were used. In the second experiment, 38 female, 12 weeks-old Balb/c mice purchased from Charles River (Scanbur, Karlslunde, Denmark) were ovariectomised. Mice were immunised with 100 μg chicken collagen II (Sigma-Aldrich) in complete Freud's adjuvant (Sigma-Aldrich) injected in the tail root. Mice received a booster with incomplete Freud's adjuvant (Sigma-Aldrich) at day 21 in the first experiment. Nicotine (Sigma-Aldrich, St. Louis, MO, USA) was administered in drinking water (0.03%) continuously and control mice drank regular tap

water. The experiments were terminated on days 43 and 28, respectively and samples of serum, BM, spleen and lymph nodes (LN) were collected.

All mice were kept under standard condition with regard to light and temperature and were fed laboratory food and water *ad libitum* at the animal facility at the department of Rheumatology and Inflammation research, University of Gothenburg. The ethical committee of the University of Gothenburg approved the animal studies (diary no. 272–2010 and 50–2014).

2.3. Cell preparation and culture

Cells were cultured at 37 °C and 5% CO_2 in medium supplemented with 10% foetal bovine serum (Sigma-Aldrich), 4 mM Glutamax (Gibco), 50 μM β-mercaptoethanol (Gibco) and 50 μg/mL gentamycin (Sanofi-Aventis, Paris, France).

Human mononuclear cells of the peripheral blood (PBMC) were prepared by gradient centrifugation on Lymphoprep (Fresenius Kabi, Oslo, Norway) as described [37]. Cells were re-suspended in RPMI medium and cultured in 12-well plates pre-coated with anti-CD3 antibodies (1 μg/ml, R&D Systems, McKinley Place, MN, USA) at a concentration of 10^6 cells per well. After 3 days, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 30 nM, Sigma-Aldrich) and ionomycin (500 nM, Sigma-Aldrich) for 1 h in the presence of a protein transport inhibitor (GolgiPlug, Becton Dickinson (BD), Franklin Lakes, NJ, USA).

Mouse femoral bones were flushed with phosphate buffered saline (PBS) to collect BM cells. Spleens and LNs were mashed through a 70 mm cell strainer to obtain a single cell suspension as described [38]. Cells were re-suspended in Iscove's modified Dulbecco's medium. After 44 h and 72 h of incubation, supernatants were collected. For quantitative polymerase chain reaction (qPCR) analysis, cells were lysed in Buffer RLT (Qiagen, Valencia, CA, USA) supplemented with β_2-mercaptoethanol (Sigma-Aldrich) (RLT/β_2-ME).

2.4. Cell sorting

Human $CD8^+$ PBMC were isolated using the Human $CD8^+$ T cell enrichment kit (Stemcell Technologies, Vancouver, BC, USA) according to the manufacturer's instructions, obtaining a purity of 81–92%. Sorted cells were stimulated for 1 h in PMA/ionomycin, and lysed in 350 μl RLT/β_2-ME for qPCR analysis.

Mouse spleen $CD8^+$ T cells were isolated using the Easy-step isolation kit (Stemcell Technologies) according to the manufacturer's instructions. $CD8^+$ cells were activated with anti-CD3 antibodies in presence of nicotine (10 nM, Sigma-Aldrich), interleukin-7 (IL-7 10 nM, Peprotech, Rocky hill, NJ, USA) or both for 3 days before harvested for qPCR and flow cytometry analysis.

2.5. Flow cytometry

Cells were blocked with human normal γ-globulin (Beriglobin, CSL Bhering, King of Prussia, Pennsylvania, USA), or Fc-block (BD Biosciences, San Jose, CA, USA) if murine cells. Cells were incubated with antibodies targeting extracellular proteins (all antibodies summarised in Tables S1 and S2). Cells were fixated with Cytofix/cytoperm (BD Biosciences) and left over night in the blocking/permeabilisation solution. Next day cells were incubated with antibodies for intracellular proteins. For biotinylated antibodies fluorochrome-conjugated streptavidin was used (PE streptavidin, BD). Acquisition was performed with FACSCanto II using the FACSDiva software (BD Biosciences) and data was analysed with FlowJo software (Tree Star, Ashland, OR, USA). Gating was performed using isotype controls and fluorescence minus one (FMO).

2.6. Measurements of survivin levels

Survivin levels in serum samples and cell culture supernatants were measured using a matched-antibody pair (rabbit anti-human survivin) and recombinant standard by a sandwich enzyme-linked immunosorbent assay (ELISA, DYC647, R&D Systems, Minneapolis, MN). The detection limit of the assay was 0.1 ng/mL. The cut-off level of 0.45 ng/mL was set as previously reported [39], and was used to distinguish between survivin positive and negative patients.

2.7. Preparation of ribonucleic acid (RNA) and gene expression analysis

RNA was prepared from cell lysates with the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. The concentration and quality of the RNA were evaluated with a NanoDrop spectrophotometer (Thermo Scientific, USA) and Experion™ RNA StdSens Analysis chip (Bio-Rad Laboratories, Hercules, CA, USA). Complementary deoxyribonucleic acid (cDNA) synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA). Gene transcription was assessed using SYBR Green qPCR Mastermix (SABiosciences, Qiagen) and ViiA™ 7 Real-Time PCR (Applied Biosystems). Primers are listed in Tables S3 and S4. Melting curves for each reaction were performed between 60 °C and 95 °C. The results were calculated as a fold change compared to controls with the ddCq-method in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and presented in heat maps using Gene-e software (www.broadinstitute.org).

2.8. Statistical analysis

Descriptive data are presented as the median, the interquartile range, the number and the percentage. The patient material was stratified on smoking history, comparing current and former smokers to never smokers. Differences between groups were assessed by the Mann-Whitney *U* test or paired *t*-test. The Pearson's test was used to analyse correlations. All tests were two-tailed and conducted with 95% confidence. Statistical analyses were performed using GraphPad Prism v.6, SPSS v.22 and OpenEpi.org [40].

3. Results

3.1. Smoking contributes to levels of survivin in serum

To study if smoking contributes to enrichment of survivin, serum levels of survivin were measured in 252 RA patients and 168 healthy controls (Table 1). Smoking was associated with a significantly increased risk of being survivin-positive both for RA patients and for healthy subjects (Fig. 1A). The estimated risk for a smoking woman to be survivin-positive was 3.64 times higher among the healthy controls (Fig. 1A). The risk for a smoking healthy man to be survivin-positive was comparable to that of a non-smoking. As expected, the risk of being survivin-positive was higher for RA patients (47.6% survivin positive) compared to the age matched healthy controls (14.9% survivin positive), OR 5.2[3.18–8.50], $p < 10^{-7}$ (Table 1). In RA patients, this smoking-associated risk for being survivin-positive was similar for women and men. Importantly, the absolute levels of survivin were higher in RA patients only if they had smoked (Fig. 1B).

3.2. Smoking is associated with PD-1 deficiency on $CD107^+CD8^+$ T cells in peripheral blood

Since $CD8^+$ T cells are likely mediators of extracellular release of

Table 1
Demographic characteristics of rheumatoid arthritis (RA) patients and healthy controls.

Patients	Women		Men	
	RA (n = 184)	Control (n = 95)	RA (n = 68)	Control (n = 73)
Non-smokers, n	68	52	16	44
Age, y	46.9 [21–66]	57.4 [41–74]	48.8 [26–66]	58.0 [41–77]
Survivin positives, n	25 (37.8%)	5 (9.6%)	6 (37.5%)	5 (11.4%)
Smokers and ex-smokers, n	116	43	52	29
Age, y	54.4 [22–71]	53.2 [42–71]	55.8 [27–69]	54.3 [40–72]
Survivin positives, n	61 (52.6%)	12 (27.9%)	28 (53.8%)	3 (10.3%)

survivin, we ask if smoking contributes to an activated phenotype of $CD8^+$ T cells. Flow cytometry analysis of the peripheral blood revealed that the percentage of $CD8^+$ cells positive for CD107, defining active cytotoxic T cells, correlated to the serum levels of survivin (Fig. 2A). Additionally, a smaller proportion of the $CD107^+CD8^+$ cells of smokers expressed of PD-1 (Fig. 2B). This smaller PD-1^+ population was consistent for the smoking healthy subjects and for RA patients. The transcriptional analysis of isolated $CD8^+$ cells showed that healthy smokers tended to have higher transcription of the interleukin-7 receptor (IL-7R) compared to non-smokers (Fig. 2C). Among the non-smokers, RA-patients had higher expression of IL-7R than healthy controls. The levels of IL-10 mRNA was similar in all groups (Fig. 2D).

3.3. Nicotine increases survivin release from the BM of arthritic mice

The effect of continuous oral administration of nicotine on serum survivin levels was investigated in collagen-immunised mice. We found that mice treated with nicotine had higher levels of survivin in serum compared to control mice (Fig. 3A). To study possible sources of the extracellular survivin in serum, we measured spontaneous release of survivin from cultured unstimulated cells of the BM, LN and spleen. Supernatants of the

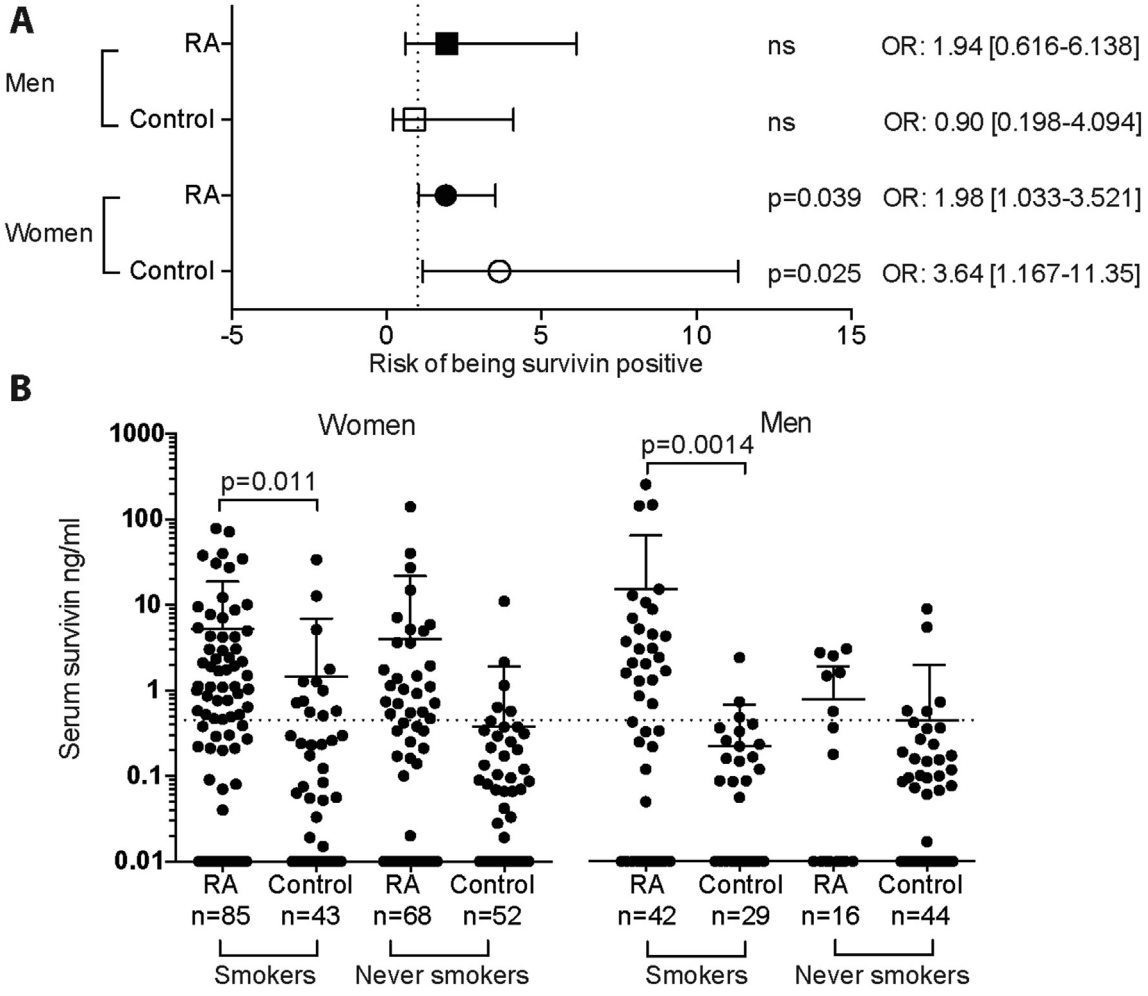

Fig. 1. Smoking contributes to high levels of survivin in serum. Serum survivin levels were analysed between the smokers and non-smokers of healthy controls (women, n = 95 and men, n = 73) and rheumatoid arthritis (RA) patients (women, n = 184 and men, n = 68). **A**: The increased risk for serum survivin positivity in smokers is presented as the odds ratio (OR) with 95% confidence interval. **B**: The dot plot presents the absolute levels of serum survivin within the studied groups. The dotted line represents the cut-off level for serum positivity at 0.45 ng/mL. Comparison between the groups was calculated by the Mann-Whitney *U* test.

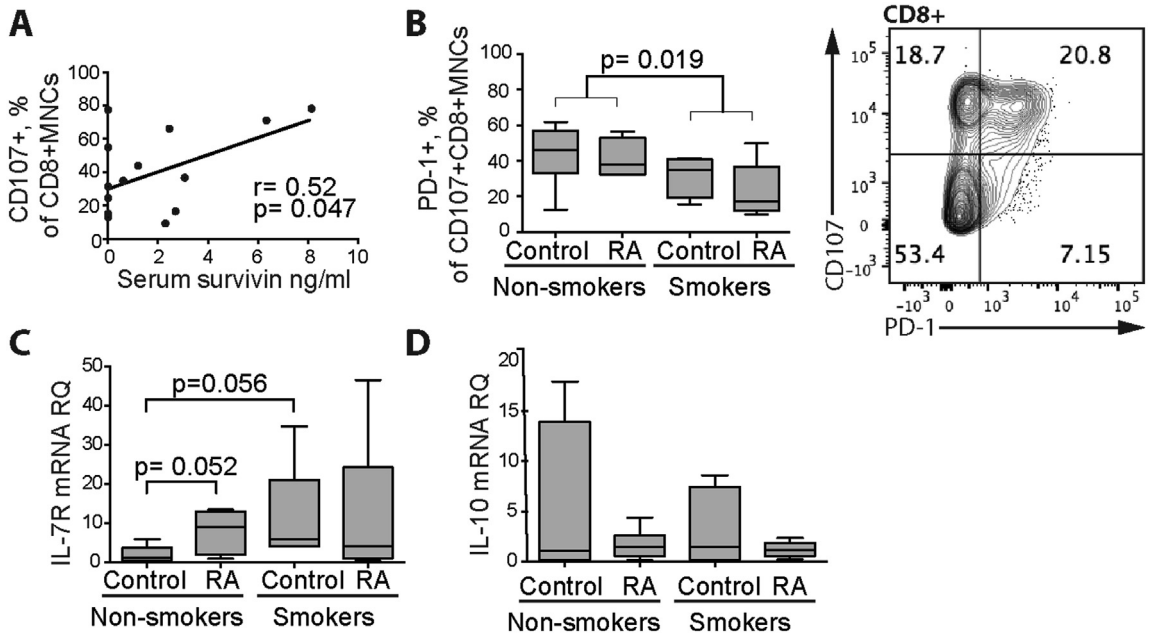

Fig. 2. Smoking associates with PD-1 deficiency on CD8$^+$ T cells in the peripheral blood. A + B. Mononuclear leukocytes were isolated from the peripheral blood of female RA patients (6 smokers and 4 non-smokers) and healthy controls (4 smokers, 6 non-smokers). Cells were cultured for 3 days in presence of anti-CD3 antibody and stimulated for 1 h in PMA/ionomycin. **A**: Correlation between the number of CD107$^+$ CD8$^+$ cells and serum survivin levels is shown. **B**: Phenotype of CD8$^+$ T cells was analysed by flow cytometry using the presented gating strategy. Representative flow cytometry plot of CD8$^+$ cells separated by their expression of CD107 and PD-1, and frequency of PD-1$^+$ cells in the CD107$^+$ population is shown. **C**, **D**: CD8$^+$ cells were isolated from the patients and controls described above and activated with PMA/ionomycin for 1 h. Gene transcription, IL-7R mRNA (C) and IL-10 mRNA (D), was measured by qPCR and presented as relative quantity (RQ) of the control non-smokers. Boxes indicate interquartile range, horizontal lines indicate median, and whiskers indicate min to max range. Comparison between the groups was performed using the Mann-Whitney *U* test and Pearson's test to analyse correlations.

nicotine treated mice contained higher levels of survivin in the cultures of BM and spleen cells (Fig. 3B). Survivin levels were also increased in the LN cultures (2.60 vs. 19.4 pg/mL, p = 0.17). Comparing survivin levels in the supernatants we found a ten-fold excess of survivin in cultures of BM cells compared to LN cells. Importantly, from the *in vivo* experiment we could correlate the intensity of survivin expression (MFI) in BM directly to serum survivin levels (r = 0.46, p = 0.0040).

Furthermore, BM cells of nicotine treated mice had higher survivin expression level and survivin mRNA production compared to controls (Fig. 3C). Flow cytometry analysis of the BM reveals that the majority of the survivin$^+$ cells express IgM, CD93 or both (Fig. 3D). The CD93$^+$IgM$^+$ population was increased by nicotine treatment (Fig. 3D).

3.4. Nicotine induces enrichment of PD-1 deficient CD8$^+$ T cells in BM

Since our results suggest the BM to be a plausible source of survivin, we focused our analysis of CD8$^+$ T cells in the BM. The

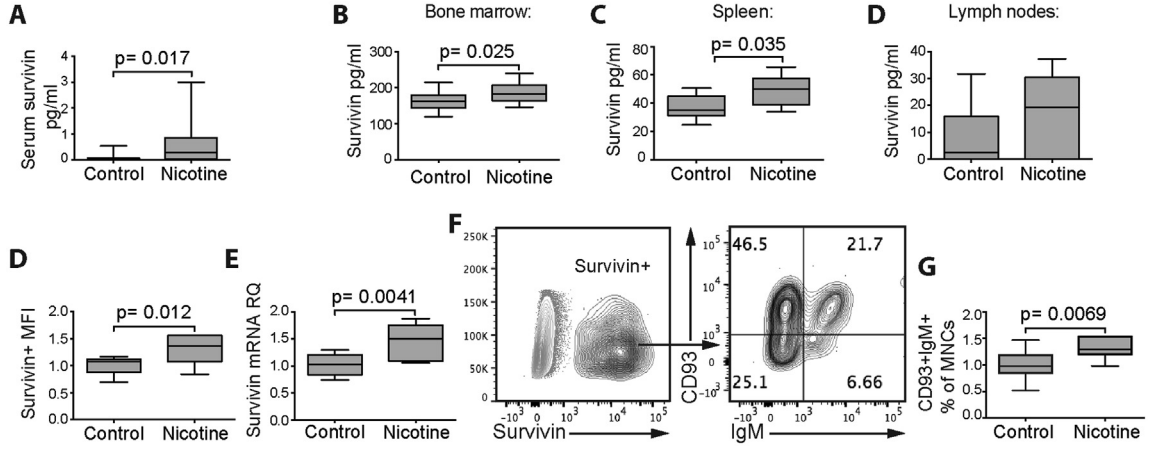

Fig. 3. Nicotine increases survivin release from the bone marrow (BM) of collagen-immunised mice. Female ovariectomised Balb/c mice (n = 28) were immunised with collagen and continuously treated with 0.03% nicotine in their drinking water for 28 days. Survivin levels were measured by ELISA. **A**: Survivin levels were higher in serum of nicotine treated mice. **B-D**: Single cell cultures were prepared from the BM (B), spleens (C) and lymph nodes (D) and cultured (1×10^6/ml) under unstimulated conditions for 72 h. Survivin levels were measured in supernatants. **D**, **E**: Survivin amount in freshly isolated mononuclear leukocytes from the BM was analysed by flow cytometry and presented as median fluorescent intensity (MFI, D) and by mRNA levels measured with qPCR (E). **F**: Phenotype of the survivin-positive cells in the BM with respect to the expression of IgM and CD93 is shown in the contour plot. Gating strategy of survivin positive cells show its separation from the fluorescence minus one (FMO) staining control. **G**: Percentage of survivin$^+$ cells expressing CD93 and IgM. Boxes represent interquartile range, horizontal lines indicate median, and whiskers indicate min to max range. Comparison between groups was performed using the Mann-Whitney *U* test.

$CD8^+$ population in the nicotine treated mice was 1.48 times larger compared to controls (Fig. 4A) and correlated both to the intensity of survivin expression in BM ($r = 0.62$, $n = 38$, $p < 0.0001$) and to the serum survivin levels ($r = 0.50$, $p = 0.028$). In $CD8^+$ cells, nicotine treatment induced a significant reduction in PD-1 and induction of IL-7R (Fig. 4A), inversely correlating to each other ($r = -0.705$, $p < 0.0001$). The size of the $PD\text{-}1^-CD8^+$ population correlated to the survivin mRNA levels and the survivin expression levels in BM (Fig. 4B), and to the serum levels of survivin ($r = 0.49$, $p = 0.032$). The acquisition of an activated phenotype was further confirmed by the use of cytotoxic marker CXCR3. We observed that nicotine enlarged $CXCR3^+$ population in reverse proportion to PD-1 expression (Fig. 3C). Gating on $CXCR3^+$ cells specifically, we saw that the reduction in PD-1 expressing cells was larger in the $CXCR3^+$ cells compared to $CXCR3^-$. Transcriptional analysis of the BM cells showed higher mRNA levels of T-bet and perforin (Fig. 4D) that correlated with serum survivin levels (T-bet, $r = 0.45$, $p = 0.054$, perforin, $r = 0.50$, $p = 0.029$) and the size of $PD\text{-}1^-CD8^+$ population (T-bet, $r = 0.56$, $p = 0.012$; perforin, $r = 0.58$, $p = 0.0079$), linking serum survivin and loss of PD-1 to cytotoxic effector T cell markers.

Nicotine influences the expression of transcription factors involved in PD-1 regulation by $CD8^+$ T cells. To further investigate the mechanism by which $CD8^+$ T cells acquired the $PD\text{-}1^-IL\text{-}7R^+$ phenotype, $CD8^+$ cells were isolated from the spleens of collagen-immunised mice. $CD8^+$ cells were activated with anti-CD3 and cultured in presence of nicotine (10 nM), IL-7 (10 nM) or both.

Transcriptional analysis of nicotine treated $CD8^+$ cells showed an inhibition of PD-1 in parallel with a significant up-regulation of nicotinic acetylcholine receptors alpha 4 and alpha 7 (nAChRα4/α7) and IL-7R. Nicotine up-regulated the positive regulators of PD-1 transcription IRF9, FoxO1, NFAT2, and Bcl-6 (Fig. 5A, upper panels). mRNA levels of negative regulators of PD-1, T-bet and Blimp-1, were not affected by nicotine. These effects of nicotine could be tracked in the presence of IL-7. The effects of IL-7 opposed those of nicotine and were recognized by down-regulation of IL-7R and nAChRα4/α7 combined with inhibition of the transcription factors IRF9, FoxO1, NFAT2, and Bcl-6 and also Blimp-1, STAT3 and STAT5b (Fig. 5A, lower panels). In difference to nicotine, IL-7 induced no significant change in transcription of PD-1. The transcriptional profiles were independent of competing stimulation with either nicotine or IL-7 and suggest response of separate cell

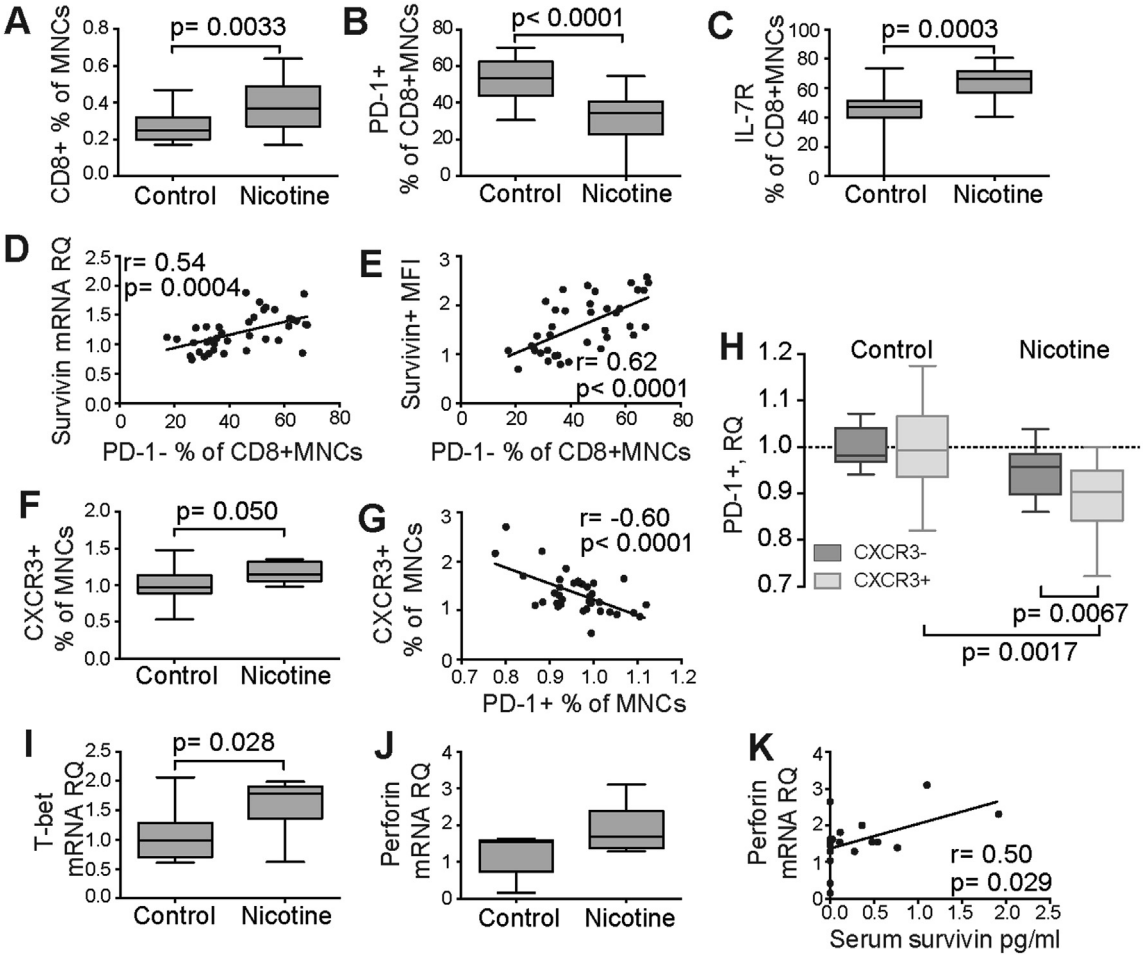

Fig. 4. Nicotine induces enrichment of PD-1 deficient $CD8^+$ T cells in the bone marrow (BM). Female ovariectomised Balb/c mice ($n = 38$) were immunised with the type II collagen and continuously treated with 0.03% nicotine in their drinking water for 28 days. Phenotype of freshly isolated mononuclear cells (MNC) of the BM was analysed by flow cytometry. Transcriptional analysis was performed by qPCR analysis and presented in relative quantity (RQ) to the control group. **A**: Proportion of $CD8^+$ cells. **B**, **C**: $PD\text{-}1^+$ (B) and $IL\text{-}7R^+$ (C) cells in the $CD8^+$ population. **D**, **E**: Correlation between the number of $PD\text{-}1^-CD8^+$ cells and survivin levels expression levels presented by mRNA levels (D) and by median fluorescent intensity (MFI) (E). **F**: CXCR3+ population of the total MNC. **G**: Negative correlation between the expression of CXCR3 and PD-1 in MNC. **H**: A change of PD-1 expression in the $CXCR3^+$ and $CXCR3^-$ cells is shown as relative quantity to control. **I**, **J**: levels of T-bet (I) and perforin (J) mRNA ($n = 19$). **K**: Correlation between perforin mRNA levels in BM and serum survivin levels ($n = 19$). Boxes represent interquartile range, horizontal lines indicate median, and whiskers indicate min to max range. Comparison between groups is performed using the Mann-Whitney *U* test. Correlations were analysed by the Pearson's test.

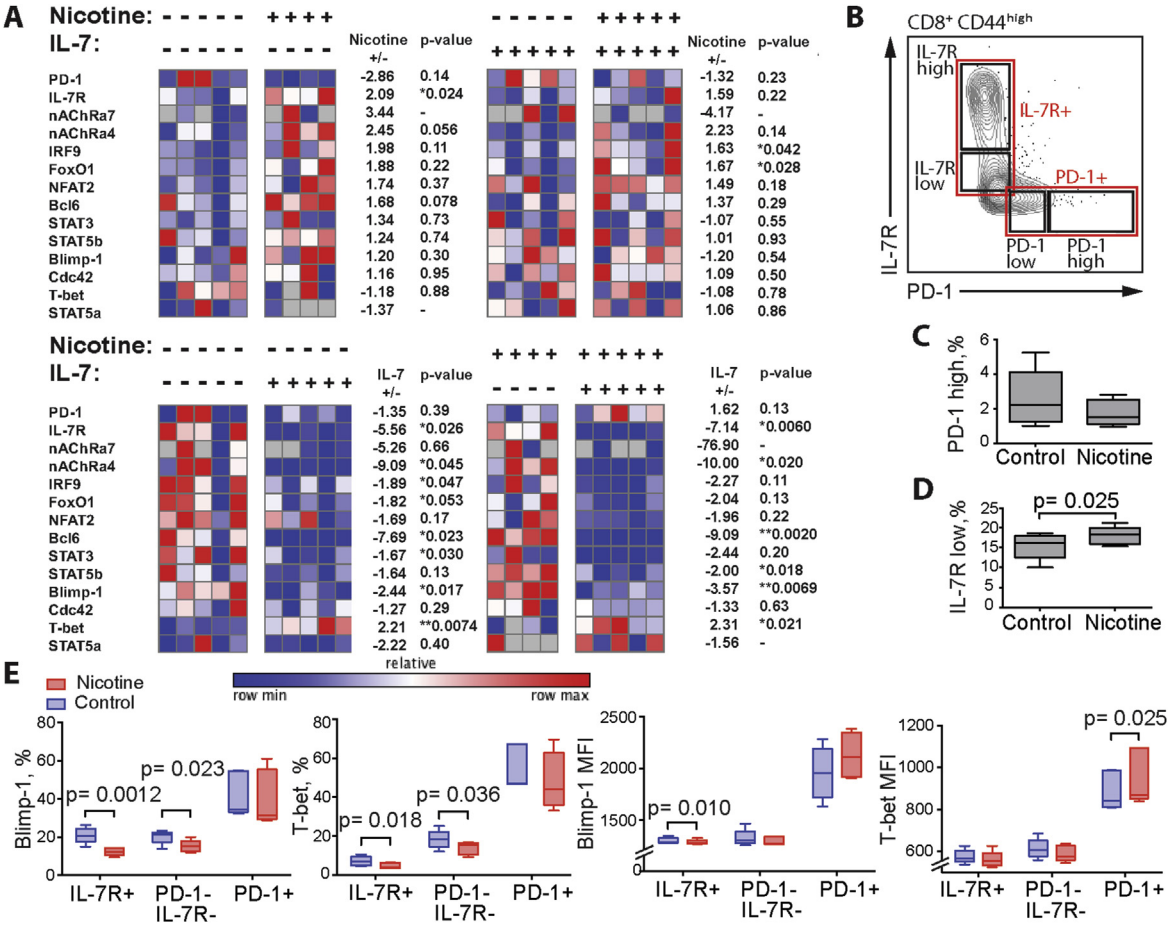

Fig. 5. Nicotine supports the non-exhausted phenotype of CD8$^+$ T cells. CD8$^+$ T cells were isolated by a negative selection from the collagen-immunised mice (n = 5). CD8 cells were activated with anti-CD3 (10 nM) and cultured in presence of nicotine (10 nM), IL-7 (10 nM) or both for 72 h. **A**: Transcriptional analysis of the genes involved in regulation of PD-1 and IL-7R transcription and signalling was done by qPCR. Gene expression heat map presents a change compared to the left-side group. **B**: Gating strategy for FACS analysis. **C**, **D**: Percentage of CD8$^+$ cells with PD-1 high (C) and IL-7R low phenotype. **E**: Intracellular expression level (% and median fluorescent intensity, MFI) of the transcription factors Blimp-1 and T-bet in IL-7$^+$, PD-1$^-$IL-7R$^-$ and PD-1$^+$ populations. Boxes represent interquartile range, horizontal lines indicate median, and whiskers indicate min to max range. Comparison between groups was analysed using the paired *t*-test.

populations.

Flow cytometry analysis of CD44^{hi}CD8$^+$ cells showed that expression of PD-1 and the IL-7R was present in distinctly separate cell populations (Fig. 5B). Flow cytometry analysis also showed a non-significant decrease in the PD-1^{hi} population and an increase in the IL-7R^{lo} population (defined in Fig. 5B). Analysis of the intracellular expression of Blimp-1 and T-bet demonstrated that nicotine reduced the proportion of T-bet and Blimp-1 positive cells in the IL-7R$^+$ and PD-1$^-$IL-7R$^-$ populations, and increased expression level of both transcription factors in the PD-1$^+$ population, significantly in the case of T-bet. These findings confirm that nicotine exposure affect the PD-1 and IL-7R expressing populations differently.

4. Discussion

Smoking is an established environmental risk factor for RA. The risk for RA can be doubled in smokers, depending on the exposure over time [41]. Cigarette smoke exhibits variable effects on the immune system that could be associated with the pathogenesis of RA. This includes oxidative stress induced by nicotine and high content of free radicals in cigarette smoke [42,43], local inflammation in bronchial mucosa induced by smoke [44], and increased citrullination of proteins triggering production of arthritis-specific autoantibodies [45]. The risk for serum survivin positivity is also higher in smokers [41,46]. This coincidence makes it highly interesting to study the release of survivin as a result of lost tolerance in smoking individuals. In the present study we show that cigarette smoking increases the risk of being survivin positive, especially among women. The study confirms our earlier report of independent patient and healthy cohorts demonstrating an association between smoking and enhanced serum survivin levels [46]. In an experimental setting we demonstrate that nicotine is a component of cigarette smoke that causes increased serum survivin levels and we correlated serum survivin levels to the expression of survivin in BM cells. Cell cultures from BM released survivin at higher levels compared to cultures from LN. The BM is an organ rich in survivin positive cells [23] and we propose the BM to be a probable source of the extracellular survivin found in serum during RA. In a different study the source of extracellular survivin in the synovial fluid of RA patients was discussed, suggesting fibroblast-like synoviocytes and peripheral blood leukocytes as potential sources [47]. It is likely that CD8$^+$ T cells target survivin expressing cells in various locations, but to achieve measurable and stable levels of survivin in serum of RA patients we speculate that the main source need to be rich in cells with a consistent expression of survivin. In BM, the transcription and expression of survivin was increased in response to nicotine. The ability of nicotine to induce survivin expression has

been previously reported in cultured lymphocytes [48] and in lung and colon cancer cells [49,50]. Consequently, smoking increases the pool of survivin-positive cells that could serve as the source of serum survivin.

Analysis of $CD8^+$ T cell phenotype in peripheral blood of smoking and non-smoking women revealed a correlation between the surface expression of the degranulation marker CD107 by $CD8^+$ T cells and serum survivin levels, thereby linking cytotoxic activity to the extracellular survivin. Further support to increased activity of the $CD107^+CD8^+$ cells in smokers is found in reduced expression of the inhibitory receptor PD-1. This observation was consistent with a distinct decrease in PD-1^+ $CD8^+$ cells in BM of nicotine treated mice. In mice, expression of CXCR3, T-bet and perforin was used to ensure that the increased proportion of PD-1^-CD8^+ cells represent an accumulation of non-exhausted, aggressive cells, rather than an infiltration of naïve cells that would naturally not express PD-1. The size of the PD-1^- population in mice correlated to survivin expression in the BM. An appealing explanation to these correlations is that survivin specific $CD8^+$ T cells lacking inhibitory receptor PD-1 migrate and accumulate in tissues with high expression of survivin. Because limited function of PD-1 relates to development of arthritis; nicotine-induced limitation of PD-1 expression could possibly contribute to the association between smoking and RA.

The down-regulation of PD-1 on $CD8^+$ cells coincided with an increased expression of IL-7R, both in smoking women and the BM of nicotine treated mice. Similar change in the balance between PD-1 and IL-7R was achieved by nicotine in $CD8^+$ spleen cells *in vitro*. This added to the interpretation that $CD8^+$ T cells convert to a non-exhausted phenotype in presence of nicotine. The non-exhausted phenotype has been previously associated with auto-reactivity in T cells. Blockade of IL-7R reverted autoimmune diabetes in two independent studies and the effect was attributed to the accompanying up-regulation of PD-1 that limit T cell auto-reactivity [51,52]. In conclusion, we demonstrate that nicotine promote a non-exhausted phenotype associated with loss of tolerance in T cells.

Flow cytometry analysis revealed that the expression of PD-1 and IL-7R in combination is rare. It has been previously demonstrated that activation of IL-7R in diabetic mice limited the expression of PD-1 in $CD4^+$ T cells [52]. Moreover, stimulation of human $CD8^+$ cells with PD-1 ligand resulted in limited expression of IL-7R [15], suggesting that the mechanism causing PD-1 and IL-7R to stay concordant works both way. In our study, we see no effect of isolated IL-7R activation by IL-7 on PD-1 transcription. In contrast, cells treated with IL-7 in combination with nicotine had a higher PD-1 transcription level in comparison to cells stimulated with nicotine alone. This could be a result of the drastic down regulation of nicotinic receptors caused by the IL-7 stimulation rendering the cells less responsive to nicotine.

Attempting to further elucidate the molecular mechanism linking nicotine stimulation to the PD-1^-IL-$7R^+$ phenotype we performed a transcriptional analysis of transcription factors involved in regulation of PD-1 and IL-7R in $CD8^+$ cells stimulated with nicotine *in vitro*. We found that nicotine increased expression levels of repressive transcription factors T-bet and Blimp-1 within the PD-1^+CD8^+ cells, offering a possible explanation to the decrease in PD-1 mRNA synthesis. This finding was consistent with the increase of T-bet transcription observed in BM cells of nicotine treated mice. In contrast, nicotine limited expression Blimp-1 and T-bet in the IL-$7R^+CD8^+$ cells, which might have facilitated the expression of IL-7R. Previous studies have shown that T-bet binds to the IL-7R locus and repress its transcription on $CD4^+$ T cells [53]. Additionally, both T-bet and Blimp-1 deficient mice have a drastic enlargement of the IL-$7R^{hi}$ population within $CD8^+$ T cells [54], supporting our interpretation.

We found that nicotine induced the mRNA production of FoxO1, IRF9, NFAT2 and Bcl6. This was unexpected since these transcription factors are known to support PD-1 expression. However, FoxO1 has a binding site at the IL-7R promoter and stimulate the transcription of IL-7R in naïve $CD8^+$ T cells [55]. In fact, the transcription factors regulating PD-1 and IL-7R largely overlap [56] and it is possible that we would need to observe them in the PD-1^+ and IL-$7R^+$ populations separately to fully understand their contribution to the change in phenotype observed during nicotine stimulation.

5. Conclusions

We have shown that nicotine stimulates both the production of survivin in BM and its release to the circulation, resulting in higher serum survivin levels in smokers. We have learned that nicotine cause $CD8^+$ T cells to adopt a non-exhausted PD-1^-IL-$7R^+$ phenotype associated with loss of tolerance and development of arthritis. Because $CD8^+$ T cells are likely to contribute to release of survivin into serum, we propose a connection between the phenotype of $CD8^+$ T cells and enrichment of survivin in serum. We suggest that nicotine induce the non-exhausted phenotype of $CD8^+$ T by stimulation of nicotinic receptors, increasing the expression of inhibitory transcription factors in the PD-1^+ population. Non-exhausted $CD8^+$ T cells accumulate in the BM that is rich in survivin expressing cells and targeting of these cells result in enhanced serum survivin levels. The study adds new insight in mechanisms by which smoking contributes to serum survivin and to the pathogenesis of rheumatoid arthritis.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2016.12.009.

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