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Low numbers of CD8+ T lymphocytes in hereditary haemochromatosis are explained by a decrease of the most mature CD8+ effector memory T cells

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Summary

Low CD8+ T lymphocyte numbers have long been described in hereditary haemochromatosis (HH). Recently, two conserved haplotypes localized near the microsatellite D6S105 at the major histocompatibility complex (MHC) class I region were described predicting the clinical expression of HH and the CD8+ T lymphocyte numbers. The A-A-T haplotype was associated with a severe clinical expression of HH and low CD8+ T lymphocyte numbers, while the G-G-G haplotype was associated with a milder clinical expression of HH and high CD8+ T lymphocyte numbers. As CD8+ T lymphocytes are a very heterogeneous population, in this study we analysed the CD8+ subpopulations of naive, central memory (T_{CM}) and effector memory (T_{EM}), and further subsets of CD8+ T_{EM} cells in 47 HH patients and 68 controls. In addition, association studies were conducted between the conserved haplotypes and the CD8+ T cell subpopulations in HH. Variations of the numbers of naive and central memory cells with age were similar between HH patients and controls. For T_{EM} cells and the T_{EM} $CD27^-CD28^-$ subset no effect of age was observed in HH [$R^2 = 0{\cdot}001$, not significant (n.s.) and $R^2 = 0{\cdot}01$, n.s., respectively] contrasting with the increasing of these subpopulations with age in controls ($R^2 = 0{\cdot}09$, $P = 0{\cdot}017$ and $R^2 = 0{\cdot}22$, $P = 0{\cdot}0005$, respectively). Interestingly, patients homozygous for the A-A-T haplotype have lower numbers of CD8+ T_{EM} cells due especially to lower numbers of T_{EM} $CD27^-CD28^-$ ($0{\cdot}206 \pm 0{\cdot}119$ and $0{\cdot}066 \pm 0{\cdot}067 \times 10^6$ cells/ml, respectively) than patients carrying the G-G-G haplotype ($0{\cdot}358 \pm 0{\cdot}195$ and $0{\cdot}246 \pm 0{\cdot}202 \times 10^6$ cells/ml, respectively). This may suggest an inability of HH patients to differentiate the CD8+ T cells into the most mature phenotype.

Keywords: CD8+ T effector memory, CD8+ T lymphocytes, hereditary haemochromatosis, iron overload, MHC class I

Introduction

Abnormalities in the total number of CD8+ T lymphocytes have long been described in hereditary haemochromatosis (HH) [1], a genetic disorder of iron overload where, in spite of the fact that more than 80% of patients are homozygous for the C282Y mutation in the *HFE* gene [2], the clinical expression is highly variable. A large proportion of HH patients have low CD8+ T lymphocyte numbers that are correlated with a more severe expression of the disease, as assessed by high levels of total body iron stores (TBIS)[3,4] and the presence of more HH-related symptoms [5].

The numbers of CD8+ T lymphocytes were shown to be transmitted genetically in association with the major histocompatibility complex (MHC) class I region in humans [6–8]. This was described first in family members of HH patients where CD8+ T cell numbers were correlated significantly in siblings sharing identical *HLA–HFE* haplotypes [6]. More recently, two conserved haplotypes, defined by three single nucleotide polymorphisms (SNPs) and localized at the 6p21.3 region near the microsatellite D6S105, were described in HH patients homozygous for the C282Y mutation [8]. These conserved haplotypes were shown to be predictors of both CD8+ T lymphocyte numbers and the clinical expression of HH [8]. To our knowledge, the possibility that the reported lymphocyte abnormalities could be explained by a particular subpopulation of cells within the CD8+ T lymphocyte pool has never been addressed previously.

The $CD8^+$ T lymphocyte population is highly heterogeneous regarding phenotypic and functional properties. $CD8^+$ T lymphocytes can be grouped into three major subpopulations identified by two cell surface markers, the high molecular weight isoform of the common lymphocyte antigen CD45RA and the lymph node homing receptor CCR7: naive ($CCR7^+$ $CD45RA^+$), central memory (T_{CM}) ($CCR7^+$ $CD45RA^-$) and effector memory (T_{EM}) ($CCR7^-CD45RA^-$ and $CCR7^-CD45RA^+$) [9]. $CCR7^+$ T cells (naive and T_{CM} cells) are characterized by the ability to circulate repeatedly into lymph nodes. The T_{EM} retains the capacity for immediate effector function and lacks CCR7. Further analyses revealed the functional heterogeneity of the $CD8^+$ T_{EM} subpopulation. Different studies used distinct sets of cell surface markers and functional parameters to identify $CD8^+$ T_{EM} subsets. CD27, CD28, CD127 and KLRG1 (killer cell lectin-like receptor G1) are some of the markers that have been used for that purpose. KLRG1 identifies antigen-experienced $CD8^+$ T cells that are impaired in proliferative capacity but are capable of immediate effector functions [10]. CD127 is the interleukin (IL)-7 receptor alpha, the expression of which is down-regulated during $CD8^+$ T_{EM} differentiation [11]. Recently, two independent reports demonstrated clearly the utility of the CD27 and CD28 markers to discriminate four $CD8^+$ T_{EM} subsets with different expression levels of molecules important for $CD8^+$ T cell function (cytokines, chemokines, cytotoxic molecules and several receptors). They allow the division of $CD8^+$ T_{EM} in $CD27^+CD28^+$ [double positive (DP)], $CD27^-CD28^+$ [CD28 single positive (SP)], $CD27^+CD28^-$ (CD27SP) and $CD27^-CD28^-$ [double negative (DN)] [11,12]. Of note, this later subdivision allowed the identification of distinct T_{EM} subtypes with constant characteristics in different individuals. Indeed, although the prevalence of cells with each of these phenotypes varies in different donors, the properties of each of these populations showed no variation between donors even when multiple functional parameters were evaluated at a single-cell level [12]. The progressive increase in the expression frequencies of perforin, granzymes A and B and Fas-L and the decreased expression of CD127, together with the characterization of *ex vivo* killing activity and replicative history analysed by telomere length and level of T cell receptor rearrangement excision circles (T_{RECs}), indicate a progressive degree of differentiation from DP to CD28SP, to CD27SP, and finally to the most differentiated, DN [11,12].

In the present study we describe the $CD8^+$ T lymphocyte subpopulations of naive, T_{CM} and T_{EM} cells and further subsets of T_{EM} cells (the DP, CD28SP, CD27SP and DN cells) in a group of 47 HH C282Y homozygous patients in comparison to 68 controls. Additionally, we evaluate the possible association between the recently described conserved haplotypes (the A-A-T and G-G-G) and the $CD8^+$ T cell subpopulations in HH patients.

Methods

Population studied

HH patients. Forty-seven HH patients, all homozygous for the C282Y mutation of the *HFE* gene, were included in this study. Patients were identified between 1985 and 2008 and were followed-up regularly at the Hemochromatosis Outpatient Clinic of Santo António Hospital, Porto and Predictive and Preventive Genetic Centre, Porto. They were all Caucasians from the north of Portugal. Twenty-six were males with mean age 51 $\pm$ 13 years (range 16–69) and 21 were females with mean age 53 $\pm$ 14 years (range 19–71). Thirty-seven patients were ascertained as probands (22 males and 15 females) detected in the context of a suggestive clinical picture of haemochromatosis or detected accidentally after a routine test. Ten (four males and six females) were family members detected in the context of systematic family screening programmes.

Clinical characterization of the subjects included in this study has been described in detail previously [4,7,13,14]. The clinical parameter used in this study as a measure of the severity of the disease was the level of total body iron stores (TBIS) determined by quantitative phlebotomies [15]. This parameter was available in 36 HH patients (the remaining patients were treated in other centres, and it was not possible to estimate TBIS accurately).

Control population. Sixty-eight apparently healthy subjects were studied as controls. These included 56 blood donors recruited from the Blood Bank of Santo António Hospital, Porto and 12 unrelated subjects selected for an age older than 60 years, in order to have a control population age-matched with patients. The healthy population included 39 males with mean age 49 $\pm$ 13 years (range 22–72) and 29 females with mean age 51 $\pm$ 13 years (range 26–74).

Informed consent and approval of the study

The study was approved by the ethical committee of Santo António Hospital, including a written informed consent obtained from patients and controls according to the Helsinki declaration.

Genetic characterization of HH patients

HH patients were all homozygous for the C282Y mutation of the *HFE* gene. HH patients were genotyped previously, or genotyped in the course of the present study, for three SNPs localized in the following genes: piggyBac transposable element derived 1 (PGBD1), zinc finger protein (ZNF) 193 and ZNF165 [8]. These SNPs are localized in the 6p21.3 region along 500 kb and are approximately 400 kb centromeric to the microsatellite D6S105 at the MHC class I region. SNP genotyping has been described in detail elsewhere [8].

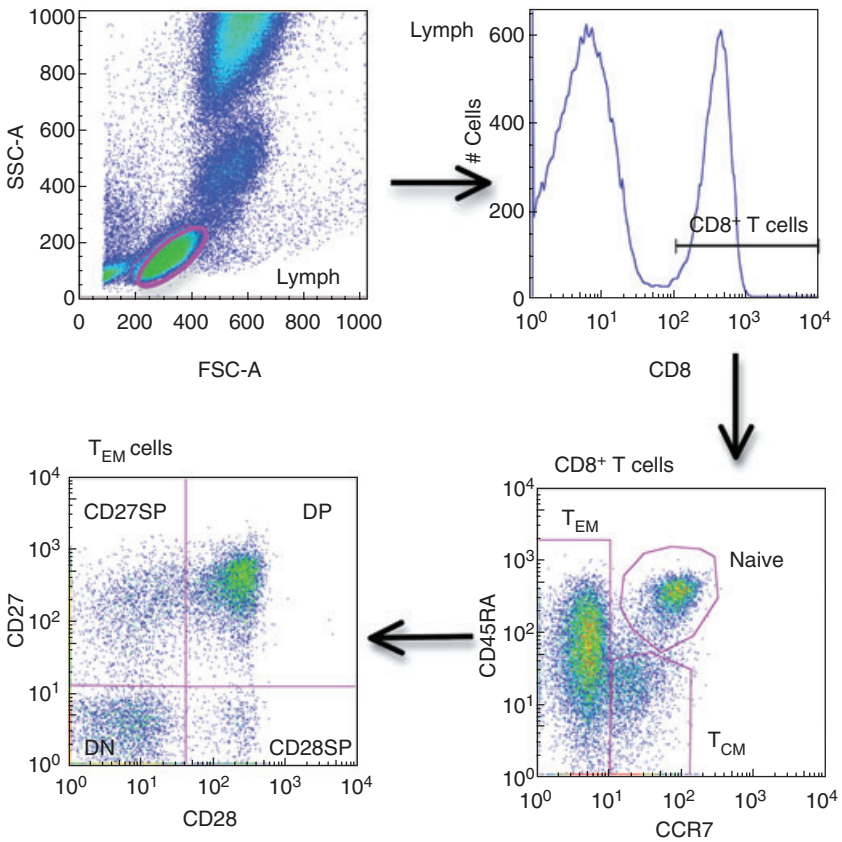

Fig. 1. Gating strategy for $CD8^+$ T subpopulations. Lymphocytes (Lymph) were gated and then selected for $CD8^+$ T cells. The $CD8^+$ T cells were subdivided into three main T cell subpopulations using CD45RA and CCR7: naive, central memory (T_{CM}) and effector memory (T_{EM}). The T_{EM} $CD8^+$ T cells were plotted against CD27 and CD28 to analyse the T_{EM} subsets: $CD27^+CD28^+$ [double positive (DP)], $CD27^-CD28^+$ [CD28 single positive (SP)], $CD27^+CD28^-$ (CD27SP) and $CD27^-CD28^-$ [double negative (DN)].

Haplotypes defined by the three SNPs genotyped were inferred using the program PHASE (http://www.stat.washington.edu/stephens/software.html), as described previously [8,16]. Two conserved 500 kb haplotypes were identified: the ancestral haplotype defined by PGBD1-A, ZNF193-A, ZNF165-T, designated the A-A-T haplotype, found in homozygoty in 39 HH patients, and the conserved haplotype defined by PGBD1-G, ZNF193-G, ZNF165-G, designated the G-G-G haplotype, found in heterozygoty in seven HH patients. A haplotype different from these two was found in one patient only and was defined by PGBD1-A, ZNF193-G and ZNF165-G. The combination of the two inherited conserved haplotypes (A-A-T × A-A-T or A-A-T × G-G-G) defined his genotype in each patient.

$CD8^+$ T cell subpopulations in HH and controls

Total lymphocyte counts were determined in an automatic blood cell counter (Advia 120; Hematology Systems, Bayer®, Tarrytown, NY, USA). The $CD8^+$ T lymphocyte naive ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$), T_{EM} ($CCR7^-$), T_{EM} $CD27^+CD28^+$ (DP), T_{EM} $CD27^-CD28^+$ (CD28SP), T_{EM} $CD27^+CD28^-$ (CD27SP) and T_{EM} $CD27^-CD28^-$ (DN) subpopulations were determined by flow cytometry. The gating strategy is shown in Fig. 1: lymphocytes were gated and then selected for $CD8^{high}$; the $CD8^+$ T cells were subdivided into the main T cell subpopulations using CD45RA and CCR7 markers and the T_{EM} $CD8^+$ T cells were plotted against CD27 and CD28 to analyse the T_{EM} subsets (Fig. 1). The following fluorochrome-labelled monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-labelled anti-CCR7 (R&D Systems, Minneapolis, MN, USA), allophycocyanin (APC)-Alexa Fluor 750-labelled anti-CD8a, APC-labelled anti-CD45RA, phycoerythrin (PE)-labelled anti-CD27 and PECy7-labelled anti-CD28 (eBiosciences, San Diego, CA, USA).

For each experiment 100 µl of unprocessed fresh peripheral blood was stained in 3 ml conic tubes with the corresponding fluorochrome-labelled antibodies and incubated for 10 min on ice in the dark. The red blood cells were then lysed with 1 ml lysis solution (Becton Dickinson, San Jose, CA, USA) and washed three times in phosphate-buffered saline (PBS). After the final wash, cells were fixed in a final volume of 400 µl with PBS containing 0·1% paraformaldehyde. At least 300 000 cells were acquired in a FACSAria (Becton Dickinson) and analysed using FlowJo software (TreeStar, Ashland, OR, USA).

Total numbers of each $CD8^+$ T cell subpopulation were calculated based on the total numbers of lymphocytes. Analyses of $CD8^+$ T cell subpopulations were performed in both percentages and total numbers with concordant results. Therefore, results are described focusing upon total number of cells.

Statistical analysis

Group means were compared by a parametric [Student's *t*-test, analysis of variance (ANOVA)] or non-parametric (Mann–Whitney *U*- and Kruskal–Wallis) tests as appropriate according to each variable distribution. The χ^2 test was used to test the fitness of data to the normal distribution. Because no statistically significant differences were found according to gender, all analyses were performed in males and females together in both HH patients and controls.

Simple linear regressions were run first in order to determine the relative contribution of the different $CD8^+$ subpopulations (naive, T_{CM}, T_{EM} and T_{EM} subsets) to the total number of peripheral blood $CD8^+$ T lymphocytes in both HH patients and controls. Simple linear regressions were also used to study the impact of age on the different $CD8^+$ T cell subpopulations both in controls and HH patients. The R^2 and *P*-values were used as statistical measures.

Differences in $CD8^+$ T cell subpopulations between controls and HH patients were analysed by comparing average values according to defined age groups (subjects < 40 years, 40–59 years and > 60 years). To analyse further the differences in $CD8^+$ T cell subpopulations between controls and HH patients, stepwise multiple regressions were performed. For this, each $CD8^+$ T cell subpopulation was set as dependent variable and the variables 'subject status' (patients *versus* controls as dummy variable), age and the interaction of age with 'subject status' were set as independent variables. The overall fit of multiple regression models was indicated by the full regression R^2 and the *F*- and *P*-values for each variable in the model.

Differences found between patients and controls were analysed according to the patient's genotype (A-A-T × A-A-T or A-A-T × G-G-G). Patients were grouped according to the combination of the two inherited conserved haplotypes: (i) patients homozygous for the A-A-T haplotype ($n = 39$); and (ii) patients heterozygous for the G-G-G haplotype ($n = 7$). Statistical analyses consisted of comparisons of each subpopulation of $CD8^+$ T lymphocytes between patients homozygous for the A-A-T haplotype and patients carrying the G-G-G haplotype.

All statistical tests were performed at the 0·05 level of significance and all *P*-values are two-sided. Data were analysed by Statgraphics (Statgraphics Graphics System, version 7·0, Warrenton, VA, USA) or SPSS (Statistical Package for Social Sciences, version 16·0, Chicago, IL, USA) software.

Results

Impact of the different $CD8^+$ T cell subpopulations in the total number of peripheral $CD8^+$ T lymphocytes

$CD8^+$ T cell subpopulations of naive, T_{CM} and T_{EM} and further subsets of T_{EM} cells (DP, CD28SP, CD27SP and DN cells) were determined in HH patients and controls (Fig. 1, Material and methods). In order to determine the relative contribution of each $CD8^+$ T cell subpopulation to the variation of the total pool of $CD8^+$ T cells, we performed partial correlations between the total number of naive, T_{CM} and T_{EM} cells and the size of the $CD8^+$ T cell pool in both HH patients and controls. As shown in Fig. 2, the $CD8^+$ T cell subpopulation that contributes most to the variation of the total number of peripheral $CD8^+$ T cell pools was the T_{EM} subpopulations, both in HH patients ($R^2 = 0{\cdot}82$, $P < 0{\cdot}000001$) and controls ($R^2 = 0{\cdot}72$, $P < 0{\cdot}000001$) (Fig. 2c). The $CD8^+$ T subpopulation of naive and T_{CM} contribute only 38% each to the variation of the total $CD8^+$ T cell pool in HH patients and 12% and 23%, respectively, in controls (Fig. 2a and b). Additional partial correlations were performed between the total number of T_{EM} subsets and the size of the T_{EM} pool. Within the T_{EM} subpopulation, the major contribution to its variation was the T_{EM} DN in both HH patients ($R^2 = 0{\cdot}73$, $P < 0{\cdot}000001$, Fig. 2d) and controls ($R^2 = 0{\cdot}78$, $P < 0{\cdot}000001$, Fig. 2d). The other T_{EM} subsets contributed less than 40% to the variation of the T_{EM} pool, in both patients (for DP $R^2 = 0{\cdot}32$, $P = 0{\cdot}0001$; for CD28SP $R^2 = 0{\cdot}32$, $P = 0{\cdot}0001$; for CD27SP $R^2 = 0{\cdot}33$, $P = 0{\cdot}00008$) and controls (for DP $R^2 = 0{\cdot}17$, $P = 0{\cdot}0003$; for CD28SP $R^2 = 0{\cdot}38$, $P < 0{\cdot}000001$; for CD27SP $R^2 = 0{\cdot}40$, $P < 0{\cdot}000001$). In summary, these results show that the relative size of the $CD8^+$ T cell pool in the peripheral blood is determined mainly by the prevalence of $CD8^+$ T_{EM} cells, in particular by the DN subset.

$CD8^+$ T lymphocyte subpopulations in HH patients and controls

Variation of the $CD8^+$ T lymphocyte subpopulations with age in controls. A detailed description of the total numbers of $CD8^+$ T lymphocytes and its subpopulations in subjects according to defined age groups is given in Table 1. Correlations of the total numbers of $CD8^+$ T lymphocyte subpopulations and T_{EM} subsets with age are illustrated in Figs 3 and 4. There was no statistically significant variation in the absolute numbers of $CD8^+$ T lymphocytes with age in controls (Table 1). In terms of the subpopulations of $CD8^+$ T lymphocytes, however, there was a statistically significant decrease with age in the absolute numbers of naive cells ($R^2 = 0{\cdot}42$; $P < 0{\cdot}000001$, Fig. 3a) and an increase in absolute numbers of T_{EM} cells ($R^2 = 0{\cdot}09$, $P = 0{\cdot}017$, Fig. 3c) (see also Table 1). No effect of age was observed in the T_{CM} pool (Fig. 3b and Table 1).

Further analyses of the $CD8^+$ T_{EM} subsets indicate that the T_{EM} DP cells were the predominant $CD8^+$ T_{EM} subset among adults younger than 60 years (Fig. 4a, Table 1). In contrast, the absolute numbers of the T_{EM} DN were the predominant subpopulation in subjects older than 60 years and the size of this subpopulation increased statistically with age ($R^2 = 0{\cdot}22$; $P = 0{\cdot}0005$, Fig. 4b and Table 1).

Given the low representation of T_{EM} CD28SP and CD27SP subsets these were not considered for further analyses.

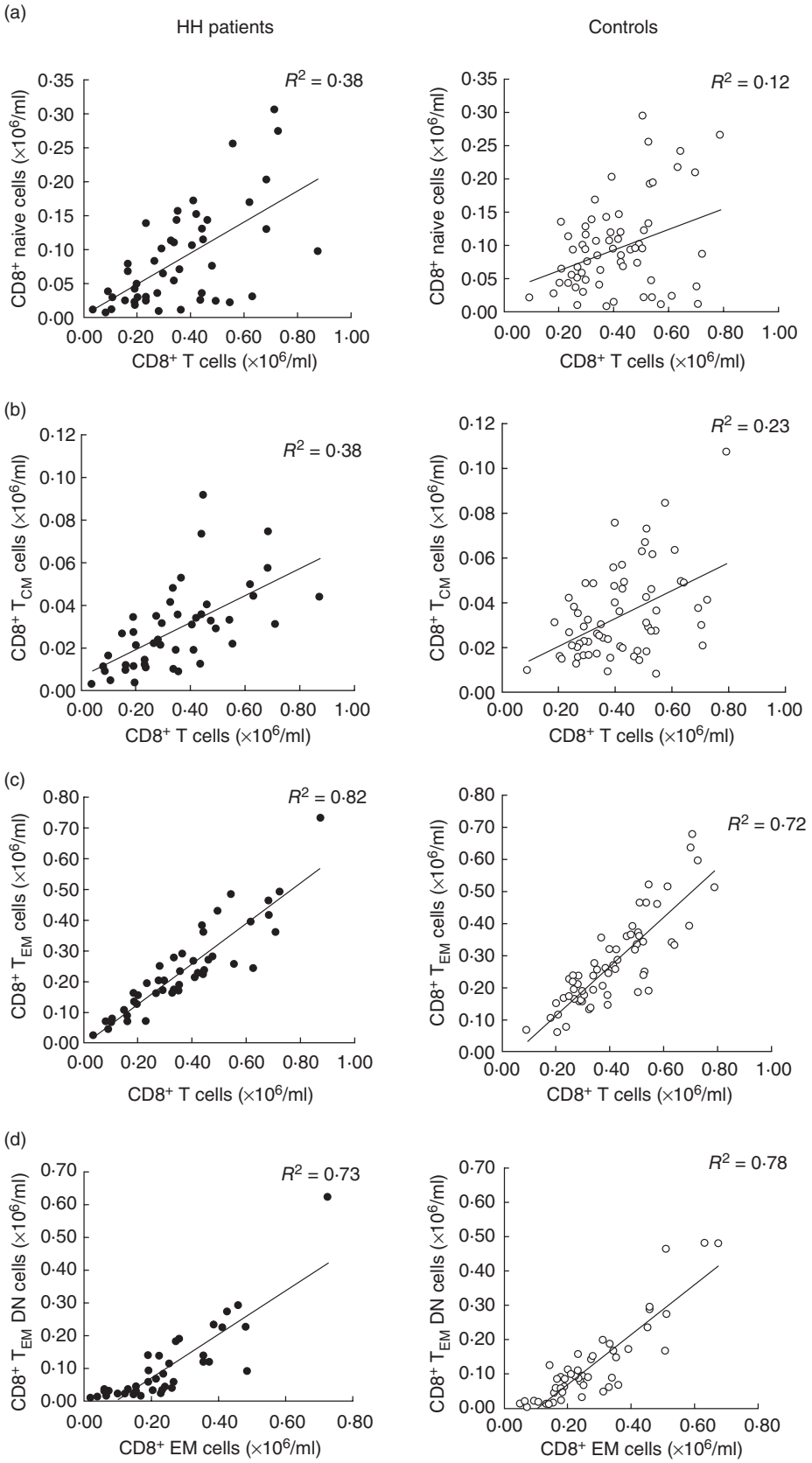

Fig. 2. Contribution of the different $CD8^+$ T cell subpopulations to the total number of peripheral blood $CD8^+$ T lymphocytes both in hereditary haemochromatosis (HH) patients and controls. Correlations of $CD8^+$ T lymphocytes with (a) naive T cells ($CCR7^+CD45RA^+$), (b) central memory cells (T_{CM}) ($CCR7^+CD45RA^-$) and (c) effector memory cells (T_{EM}) ($CCR7^-$). Correlations of $CD8^+$ T_{EM} cells with (d) T_{EM} double negative cells ($CD28^-CD27^-$) (T_{EM} DN).

Table 1. Total numbers of $CD8^+$ T lymphocyte major subpopulations of naive, central memory and effector memory (T_{EM}), and the four $CD8^+$ T_{EM} subsets according to age groups in hereditary haemochromatosis (HH) and control subjects.

		Age groups (years)			
		< 40	40–59	≥ 60	
		n = 17 *n* = 7	*n* = 34 *n* = 25	*n* = 17 *n* = 15	*P*
$CD8^+$ T cells	Controls	0·42 ± 0·15	0·42 ± 0·14	0·39 ± 0·18	n.s.
	HH patients	0·39 ± 0·17	0·41 ± 0·20	0·26 ± 0·16*	0·04
Naive	Controls	0·145 ± 0·066	0·097 ± 0·062	0·038 ± 0·027	< 0·001
	HH patients	0·132 ± 0·048	0·106 ± 0·080	0·029 ± 0·020	< 0·001
Central memory	Controls	0·030 ± 0·013	0·039 ± 0·021	0·029 ± 0·021	n.s.
	HH patients	0·035 ± 0·023	0·030 ± 0·021	0·023 ± 0·014	n.s.
Effector memory	Controls	0·235 ± 0·133	0·268 ± 0·103	0·315 ± 0·180	n.s.
	HH patients	0·208 ± 0·119	0·271 ± 0·160	0·173 ± 0·092*	n.s.
	Controls	*n* = 8	*n* = 28	*n* = 16	
	HH patients	*n* = 7	*n* = 21	*n* = 13	
T_{EM} DP	Controls	0·0790 ± 0·0353	0·1177 ± 0·0559	0·0776 ± 0·0362	0·02
	HH patients	0·0928 ± 0·0443	0·1187 ± 0·0577	0·0723 ± 0·0405	0·04
T_{EM} CD28SP	Controls	0·0077 ± 0·0046	0·0166 ± 0·0101	0·0186 ± 0·0203	n.s.
	HH patients	0·0193 ± 0·0131	0·0127 ± 0·0127	0·0092 ± 0·0060	n.s.
T_{EM} CD27SP	Controls	0·0329 ± 0·0139	0·0331 ± 0·0160	0·0302 ± 0·0174	n.s.
	HH patients	0·0239 ± 0·0174	0·0376 ± 0·0189	0·0263 ± 0·0242	n.s.
T_{EM} DN	Controls	0·0594 ± 0·0624	0·0999 ± 0·0741	0·1867 ± 0·1581	0·02
	HH patients	0·0714 ± 0·0711	0·1177 ± 0·1469	0·0708 ± 0·0620*	n.s.

$CD8^+$ T lymphocyte subpopulations are expressed as 10^6 cells/ml and were defined as shown in Fig. 1. CD27SP, $CD27^+$ single positive; CD28SP, $CD28^+$ single positive; DN, double negative; DP, double positive; n.s., not significant; T_{EM}, effector memory.
P: statistical significance of the differences between age groups in controls or in HH patients, determined by Kruskal–Wallis or analysis of variance test as appropriate. Shadowed areas and * represent statistical significant differences ($P < 0·05$) between controls and HH patients within each age group, determined by the Mann–Whitney *U*- or Student's *t*-test, as appropriate.

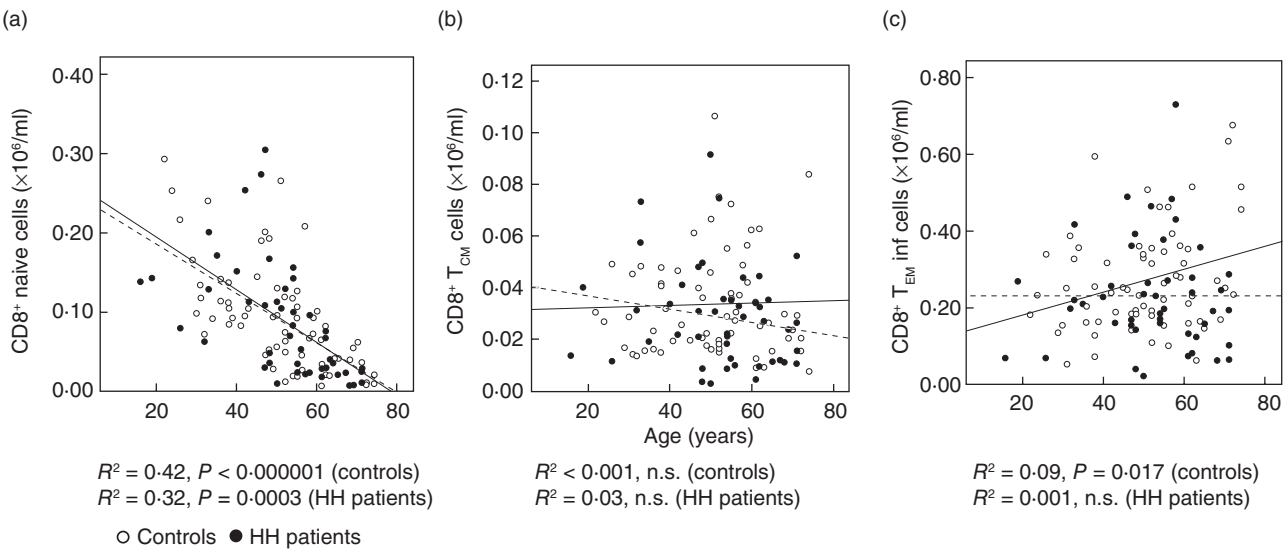

Fig. 3. Correlations of the three major $CD8^+$ T cell subpopulations with age in healthy donors (open circles, *n* = 68) and hereditary haemochromatosis (HH) patients (full circles, *n* = 47). (a) Naive T cells ($CCR7^+CD45RA^+$), (b) central memory cells (T_{CM}) ($CCR7^+CD45RA^-$), (c) effector memory cells (T_{EM}) ($CCR7^-$). The R^2 and significance of the correlation (*P*) is shown for each correlation. Solid lines represent controls and dashed lines represent HH patients.

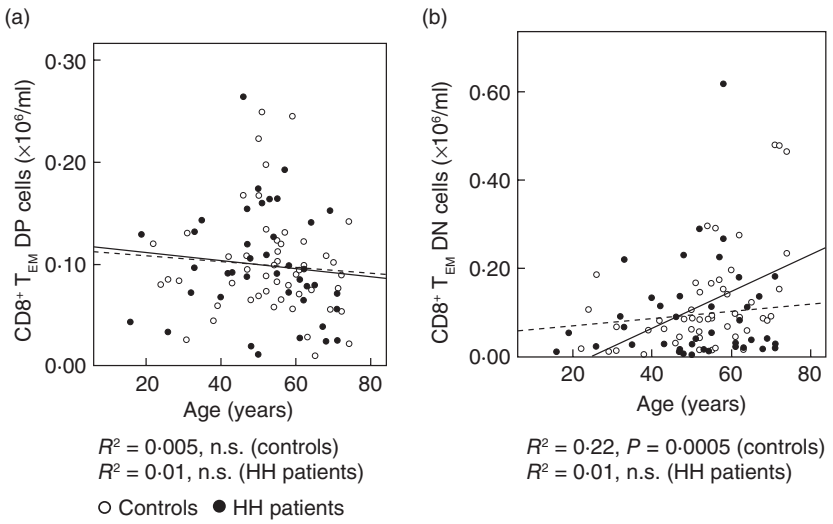

Fig. 4. Correlations of the CD8+ effector memory T cell subsets (T_{EM}) with age in healthy donors (open circles, $n = 52$) and hereditary haemochromatosis (HH) patients (full circles, $n = 41$). (a) T_{EM} double positive cells ($CD28^+CD27^+$) (T_{EM} DP) and (b) T_{EM} double negative cells ($CD28^-CD27^-$) (T_{EM} DN). The R^2 and significance of the correlation (P) is shown for each correlation. Solid lines represent controls and dashed lines represent HH patients.

Variation of the CD8+ T lymphocyte subpopulations with age in HH patients. Similar to what was observed in the control group, the number of CD8+ naive T cells decreased with age in HH patients ($R^2 = 0{\cdot}32$, $P = 0{\cdot}0003$) (Fig. 3a) and there was no effect of age in the number of CD8+ T_{CM} cells (Fig. 3b). In contrast to what was found in controls, no effect of age was observed for the CD8+ T_{EM} cells in HH patients (Fig. 3c). In a similar manner, no effect of age was observed in the T_{EM} DN subpopulation in HH patients contrasting with the accumulation of this subpopulation with age found in the control group (Fig. 4b).

Comparison of the CD8+ T lymphocyte subpopulations between HH patients and controls. A detailed comparison of CD8+ T cell subpopulations between patients and controls according to defined age groups is given in Table 1. In general, HH patients have average numbers of CD8+ T lymphocytes lower than control subjects, this difference being statistically significant for subjects older than 60 years ($0{\cdot}26 \pm 0{\cdot}16$ and $0{\cdot}39 \pm 0{\cdot}18 \times 10^6$/ml, $P = 0{\cdot}02$) (Table 1). The variation in the number of naive and T_{CM} cells with age was similar between HH and control subjects (Table 1 and Fig. 3). However, for T_{EM} cells a difference was found between patients and controls: HH patients failed to increase the number of T_{EM} cells with age (Table 1 and Fig. 3c). Among the T_{EM} subpopulation the difference is also clear between HH patients and control subjects in the most differentiated subtype, the T_{EM} DN: again, HH patients failed to increase the number of T_{EM} cells with age (Table 1 and Fig. 4). The significance of the age-dependent differences in CD8+ T lymphocyte subpopulations between HH patients and controls was analysed further by stepwise multiple regressions, where the number of each of the CD8+ T subpopulations was considered a dependent variable against the independent variables: age, 'subject status' (HH patients *versus* controls as dummy variable) and the interaction of age with 'subject status' (see Statistical analysis). For the CD8+ naive subpopulation, the only variable entering the model was age ($F = 66{\cdot}7$, R^2 for the full regression = 0·37 and $P < 0{\cdot}000001$) and no effect was observed in the variable 'subject status', thus confirming that there are no significant differences between HH patients and controls in the age-dependent total numbers of the CD8+ naive subpopulation (illustrated in Fig. 3a). For CD8+ T_{EM} cells, in contrast, a significant effect was observed in the interaction of age with 'subject status' ($F = 5{\cdot}56$, R^2 for the full regression = 0·04 and $P = 0{\cdot}0202$), confirming that the size of this cell subpopulation increases with age in controls but not in patients (illustrated in Fig. 3c). For the T_{EM} DN subset a significant effect was also observed in the interaction of age with 'subject status' ($F = 4{\cdot}8$, $R^2 = 0{\cdot}04$, $P = 0{\cdot}03$), confirming that the size of this cell subpopulation increases with age in controls but not in patients (illustrated in Fig. 4b).

CD8+ T_{EM} subpopulations in HH patients according to patient genotype

Confirming our previous results [8], patients homozygous for the A-A-T haplotype ($n = 39$) have a more severe expression of haemochromatosis in terms of iron stores, calculated after removal of iron by phlebotomies (TBIS = $7{\cdot}34 \pm 4{\cdot}47$ g, $n = 30$) in comparison with patients carrying the G-G-G haplotype ($n = 7$) with lower clinical expression in terms of iron stores (TBIS = $4{\cdot}03 \pm 2{\cdot}79$ g, $n = 6$).

As the major differences in the CD8+ T cell subpopulations found between patients and controls were in the subpopulation of CD8+ T_{EM} cells, and among these in the subset of DN cells, we analysed further the impact of the A-A-T and G-G-G haplotypes on the number of these cells. Interestingly, statistically significant differences were found in the

Table 2. Comparisons of the total number of $CD8^+$ T_{EM} lymphocytes and their subsets in hereditary haemochromatosis (HH) patients according to the combination of the two conserved haplotypes, A-A-T and G-G-G.

	HH patients genotype		
	A-A-T × A-A-T ($n = 39$)	G-G-G × A-A-T ($n = 7$)	*P*
Age (years)	52 ± 14	50 ± 10	n.s.
T_{EM}	0·206 ± 0·119	0·358 ± 0·195	0·0075
T_{EM} DP	0·100 ± 0·056	0·104 ± 0·044	n.s.
T_{EM} CD28SP	0·011 ± 0·011	0·019 ± 0·015	n.s.
T_{EM} CD27SP	0·033 ± 0·026	0·023 ± 0·007	n.s.
T_{EM} DN	0·066 ± 0·067	0·246 ± 0·202	0·0002

$CD8^+$ T lymphocyte subpopulations are expressed as 10^6 cells/ml and were defined as shown in Fig. 1. CD27SP, $CD27^+$ single positive; CD28SP, $CD28^+$ single positive; DN, double negative; DP, double positive; n.s., not significant; T_{EM}, effector memory.
P: Statistically significant differences between HH patients homozygous for the A-A-T haplotype and carrying the G-G-G haplotype, determined by the Mann–Whitney *U*- or Student's *t*-test, as appropriate.

average numbers of $CD8^+$ T_{EM} cells and T_{EM} DN cells, according to the genotype (Table 2). These differences were not explained by age differences (Table 2). Patients homozygous for the A-A-T haplotype have lower numbers of the T_{EM} cells (0·206 ± 0·119) and DN cells (0·066 ± 0·067) in comparison to patients carrying the G-G-G haplotype (0·358 ± 0·195 and 0·246 ± 0·202, respectively) (Table 2 and Fig. 5).

Discussion

This study follows previous observations showing that HH patients with a low number of $CD8^+$ T cells present a more severe expression of iron overload [3–5]. As the $CD8^+$ T lymphocyte pool is highly heterogeneous, this study aimed to characterize, for the first time, the $CD8^+$ T cell subpopulations (naive, T_{CM} and T_{EM} and further subsets of T_{EM} cells) in this disease.

The effect of age in the two major $CD8^+$ T cell subpopulations, naive and memory, is well established (reviewed in [17]). Due to thymic evolution and cumulative antigen contact experience there is a decrease in the number of $CD8^+$ naive T cells with a concomitant increase in effector memory pool [18,19]. The effect of age on the different effector memory subsets is shown here for the first time, with evidence of an increase of the most differentiated T_{EM} subset, DN, with age in controls. In HH patients, however, the DN subset did not increase with age, suggesting an inability to differentiate into the most mature phenotype that could justify the overall low number of $CD8^+$ T lymphocytes [4,5]. This is corroborated by previous work showing that the $CD8^+$ T cells of these patients have decreased cytotoxic activity [20] and defective CD8-p56lck activity [21].

Finally, the evidence presented here that defective T_{EM} $CD8^+$ and DN T subpopulations in HH patients are associated with the A-A-T but not the G-G-G haplotypes allows us to speculate that a putative gene in this region could be involved in the regulation of the effector memory pool by impairing directly or indirectly the differentiation and/or survival of the most mature $CD8^+$ T cells.

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Disclosure

The authors declare no conflict of interest.

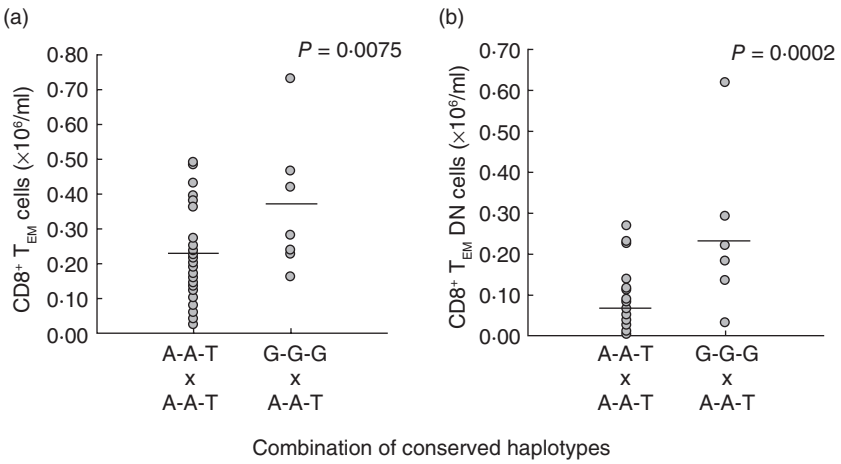

Fig. 5. Distribution of (a) $CD8^+$ effector memory cells (T_{EM}) and (b) T_{EM} double negative cells ($CD28^-CD27^-$) (T_{EM} DN) in hereditary haemochromatosis (HH) patients according to the combination of the conserved haplotypes, which divide patients into two groups: homozygous for the A-A-T haplotype ($n = 39$) and heterozygous for the G-G-G haplotype ($n = 7$).

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