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A 'High-Mobility Low-Cost' Phenotype Defines Effector-Memory CD8$^+$ T-cells

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Abstract

T-cells move / crawl randomly (‘random-walk’), a characteristic thought to be integral to their function. Using migration-assays and time-lapse microscopy we found that, directly *ex vivo*, the lymphnode homing chemokine receptor CCR7 separates $CD8^+$T-cells with high ($CCR7^-$) versus low ($CCR7^+$) frequency of cells exhibiting random crawling-activity. Surprisingly, quantified in micro-calorimetry assays, $CCR7^-CD8^+$T-cells –despite higher ‘crawling-frequency’– produced less heat than their $CCR7^+$ counterparts and also contained less ATP. Identifying non-lymphoid homing $CD8^+$T-cells as ‘high-mobility low-cost-cells’ suggests that our immune system has evolved an energy-efficient means to screen non-lymphoid compartments for antigen.

Introduction

Phenotypic and immune-functional analyses have defined differences in the nature of $CD8^+$T-cell populations, differing migrational behavior being one of the key distinctions among subsets. Naïve and central-memory $CD8^+$T-cells express the lymphnode homing chemokine receptor CCR7 and screen lymphoid tissue for cognate antigen. Effector-memory $CD8^+$T-cells, by contrast, lack expression of CCR7 and primarily home into non-lymphoid tissues.[1-5] Screening for cognate antigen via random lymphocyte motility (‘random-walk’ activity) –an energy consuming biological feature– has been shown to be characteristic of both, lymphoid as well as non-lymphoid homing $CD8^+$T-cells.[6-11] Here we sought to determine how random lymphocyte motility (‘random-walk’) and 'energy consumption' is distributed among human $CD8^+$T-cells.

Materials and Methods

Isolation of peripheral blood mononuclear cells and CD8$^+$T-cells, FACScan analysis and FACS sorting

Detailed procedures are outlined in Supplemental Data.

Migration assay

Bulk lymphocytes or sorted CD8$^+$T-cell subsets were resuspended at 6x10^5 cells/mL in RPMI1640 supplemented with 5% bovine serum albumin and loaded in duplicates into 5μm pore size polycarbonate transwell inserts (Costar®). 1mL R5 (GIBCO™, LuBioScience GmbH, Luzern, Switzerland) was added to the lower well. After 4h or 16h of incubation at 37°C and 5% CO_2, cells from both compartments were collected and stained with the appropriate antibodies.

Calorimetry

Bulk lymphocytes and FACS sorted CCR7$^-$ and CCR7$^+$CD8$^+$T-cells were resuspended at 1.2x10^6 or 2.4x10^6 cells/mL in R10. After a calibration period of 24 hours, heat production of bulk and sorted lymphocytes was recorded every minute during 12 hours using an isothermal calorimeter (Thermal Activity Monitor 3102 TAM III, TA Instruments, New Castle, DE; USA).

ATP quantification

FACS sorted CD8$^+$T-cell subsets were resuspended at 1x10^6 cells/mL in purified water, transferred to cryotubes (Nunc A/S, Roskilde, Denmark) and snap frozen in liquid nitrogen. After thawing, cells were incubated for 10min. in boiling water and centrifuged for 5min. at 20,000xg. The supernatant was kept on ice until ATP measurement was performed according to the manufacturer's protocol (FL-AA, Sigma-Aldrich).

Statistical analysis

Results were tested for normality using D'Agostino&Pearson omnibus normality test. Student's t-tests, Mann-Whitney U-tests and Wilcoxon's signed rank-tests were performed using Prism4 software (GraphPad Software, Inc. San Diego, CA, USA). P-values<0.05 were considered statistically significant. Results are given as mean±standard deviation (SD), or median and range, as appropriate.

Results and Discussion

'Random-walk' activity of CD8$^+$T-cells

Distinct anatomical compartments are screened for cognate antigen by specialized subsets of T-cells.[1-5] 'Antigen screening efficiency' of T-cells –both in lymphoid and non-lymphoid organs– is thought to be increased by random cellular movement ('random-walk').[6,7,10,12-15] Aiming at assigning random crawling or 'random-walk' activity to distinct-cell-populations we compared the phenotype of CD8$^+$T-cells *ex vivo* with that of CD8$^+$T-cells that –in absence of a chemotactic gradient– migrated across a 5μM pore. Intriguingly, in a time-dependent manner the phenotype of migrating CD8$^+$T-cells became skewed towards cells lacking expression of the lymphnode homing chemokine receptor CCR7 (*ex vivo* CD8$^+$T-cells: median CCR7$^-$ 36.9 % [range 28.8-52.9%], median CCR7$^+$ 55.7% [range 36.6-72.7%], n=9; 4h migrated CD8$^+$T-cells: median CCR7$^-$ 70.9% [range 23.3-96.5%], median CCR7$^+$ 29.2% [range 3.6-76.8%], n=6; 16h migrated CD8$^+$T-cells: median CCR7$^-$ 91.5 % [range 82.3-98.3 %], median CCR7$^+$ 5.6% [range 1.7-17.6%], n=9 (Fig.1A). The increase in the median percentage of CCR7$^-$ cells among migrating cells, compared to cells *ex vivo*, was statistically significant (after 16h migration; $p<0.001$). CD8$^+$T-cells were also labeled for expression of the maturation-marker CD45RA.[5,16] No significant difference was observed in the accumulation of CD45RA$^-$ vs. CD45RA$^+$CCR7$^-$ CD8$^+$T-cells (Fig.1A).

The observed difference in the phenotype of migrating cells may be explained by internalization of CCR7, rather than differences in 'random-walk' activity of CCR7$^-$ vs. CCR7$^+$CD8$^+$T-cells. To control for this possibility we sorted CCR7$^-$ and CCR7$^+$CD8$^+$T-cells and (a) quantified migration by relating the starting number of cells to the number of cells that had migrated after 16h of incubation, and (b) visually compared movement activity of CCR7$^-$ and CCR7$^+$CD8$^+$T-cells in time-lapse microscopy assays. Supporting their higher intrinsic crawling or random movement activity, the percentage of migrating cells, as compared to CCR7$^+$CD8$^+$T-cells, was almost 3-fold higher among sorted CCR7$^-$ CD8$^+$T-cells (median % of cells migrating after 16h: CCR7$^-$ 27.7% [range 23.7-49.0%], n=5, CCR7$^+$ 10.1% [range 1.3-14.4%], n=6, $p=0.004$). In line with the migration pattern of CCR7$^-$ and CCR7$^+$CD8$^+$T-cells in migration chambers, time-lapse microscopy of sorted CCR7$^-$ and CCR7$^+$CD8$^+$T-cells also revealed –at each recorded time point– a higher number of *crawling* cells per high power field among CCR7$^-$CD8$^+$T-cells (crawling defined as flattening of the cell-stoma with extension of pseudopodia) (mean % of crawling cells/high-power field among CCR7$^-$ vs. CCR7$^+$CD8$^+$T-cells; 11.5±2.04% vs. 1.1±0.47% [$p<0.001$]) (Suppl. Figure and Movie).

We also used CD27 and CD28, two additional phenotypic markers of CD8$^+$T-cell-differentiation, to characterize cells accumulating in migration assays.[3] As for the selective accumulation of CCR7$^-$ CD8$^+$T-cells, selective accumulation of cells with a so called 'late differentiated' phenotype was observed (*ex vivo* CD8$^+$T-cells: median CD27$^-$CD28$^-$ 20.5% [range 6.9-25.9%], n=6; 16h migrated CD8$^+$T-cells: median CD27$^-$CD28$^-$ 52.8% [range 35.0-73.8%], n=6). This increase in median percentage of CD27$^-$CD28$^-$CD8$^+$T-cells among migrating cells compared to cells *ex vivo* was statistically significant ($p=0.03$) (Fig.1B).

By comparing the size (forward scatter histogram) of migrating vs. non-migrating cells we also tested –and dismissed– the hypothesis that accumulation of $CD8^+$T-cells with an effector-memory or 'late differentiated' phenotype merely reflects selection for smaller cells (Fig.1C). Together these experiments identified robust differences between phenotypically distinct subsets of $CD8^+$T-cells with regards to their crawling/'random-walk' activity. The observed difference may imply that stochastic detection of cognate antigen *at a given rate* requires higher 'random-walk' activity in structurally more variable non-lymphoid tissues than within anatomically highly defined secondary lymphnodes.

Heat production and ATP content of $CD8^+$T-cell subsets

Microcalorimetry has previously been used to measure the heat-flow of bulk human lymphocytes.[17] We adapted this methodology to assess heat production of unstimulated $CCR7^-$ and $CCR7^+CD8^+$T-cell subsets. Heat-flow signals were cell-concentration dependent (data not shown). Intriguingly, $CCR7^-CD8^+$T-cells produced a median of only 68nW/10^6 cells (range 0-530nW) (n=8), whereas $CCR7^+CD8^+$T-cells produced a median of 390nW/10^6 cells (range 2-1050nW) (n=8), corresponding to a 3.2 to 22.9-fold higher energy consumption by the latter subset (p=0.039) (Fig.2A).

Next we measured ATP contents of $CD8^+$T-cell subsets using a robust luciferin-based assay. The following $CD8^+$T-cell-populations were sorted and their ATP content quantified: $CCR7^+CD45RA^+$, $CCR7^+CD45RA^-$, $CCR7^-CD45RA^-$, and $CCR7^-CD45RA^+$. No differences in ATP contents were detected between $CD45RA^+$ and $CD45RA^-$ subsets, both within the $CCR7^-$ and $CCR7^+$ populations (data not shown). However, $CCR7^-CD8^+$ T-cells consistently contained less ATP than their $CCR7^+$ counterparts ($CCR7^-CD8^+$T-cells: median ATP content 299±62nmol/10^6 cells, $CCR7^+CD8^+$T-cells: median ATP content 378±62nmol/10^6 cells, n=10, p=0.025) (Fig.2B). These data seem plausible in that higher ATP contents can support the increased energy requirements/heat production –as documented by calorimetry– of $CCR7^+CD8^+$T-cells.

Cellular locomotion relies on transient interactions of motor proteins with microtubules or actin filaments, which then activates the binding and hydrolysis of ATP.[18] Higher crawling/'random-walk' activity thus requires more energy. Somewhat to our surprise, therefore, $CD8^+$T-cells with a high frequency of crawling cells appear to use *less energy* than those with a low frequency of crawling cells. As any biologically costly system, immunity has evolved to minimize energy expenditure in performing its task,[19-21] and screening for cognate antigen within lymphoid and non-lymphoid/peripheral tissues is bound to comply with this principle. Evolution of compartmentalized immunity thus seems to have resulted in a *energy-efficient means* to screen peripheral anatomical sites for reappearance of cognate antigen.

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Authorship

Contribution: G.Z. designed and performed experiments, analyzed data and helped write the report; P.G. designed and performed experiments and analyzed data; O.G. and A.T. designed and supervised experiments; A.S. performed and analyzed experiments; A.D.L. designed and supervised migration experiments and helped write the report, C.H. initiated the study, designed and supervised the research, analyzed data and wrote the report.

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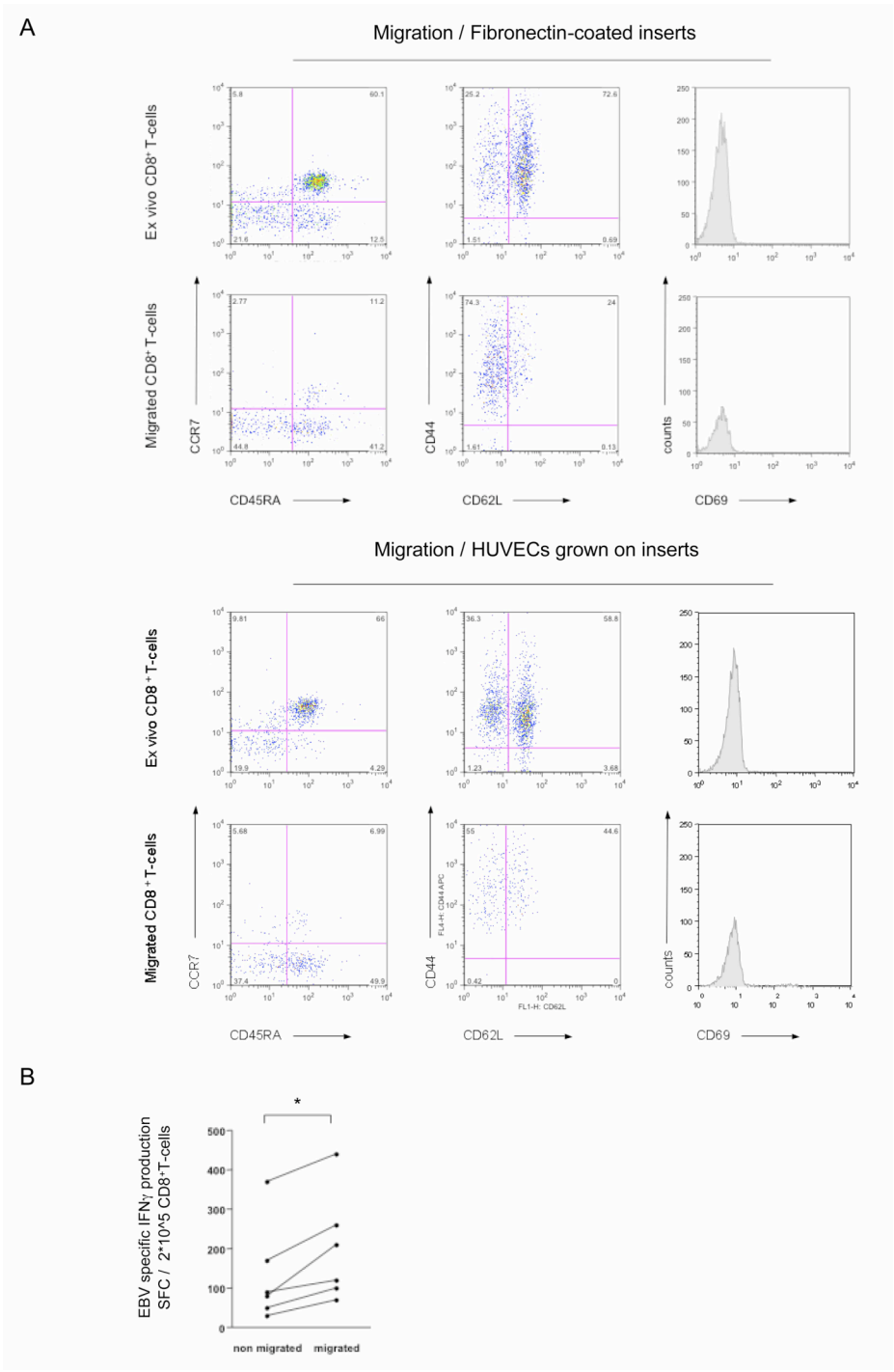

Figure 1. Phenotypic and functional characteristics of randomly moving of CD8$^+$ T cells

(A) In transwell migration experiments, migration of CD8$^+$ T cells through a fibronectin coated 5 μM pore membrane (upper panel) and through HUVECs grown on a transwell membrane (lower panel) was assessed in absence of a chemotactic gradient. *Ex vivo*-expression of CCR7 and CD45RA, CD44 and CD62L, and the activation marker CD69 was compared to expression on migrating cells (16 hours migration). Migrating CD8$^+$ T cells were skewed towards a CCR7$^-$ and CD62L$^-$ phenotype. (B) As assessed in transwell migration experiments, the frequency of EBV-specific CD8$^+$ T cells producing IFNγ was higher amid migrating than non-migrating cells. *P<0.05

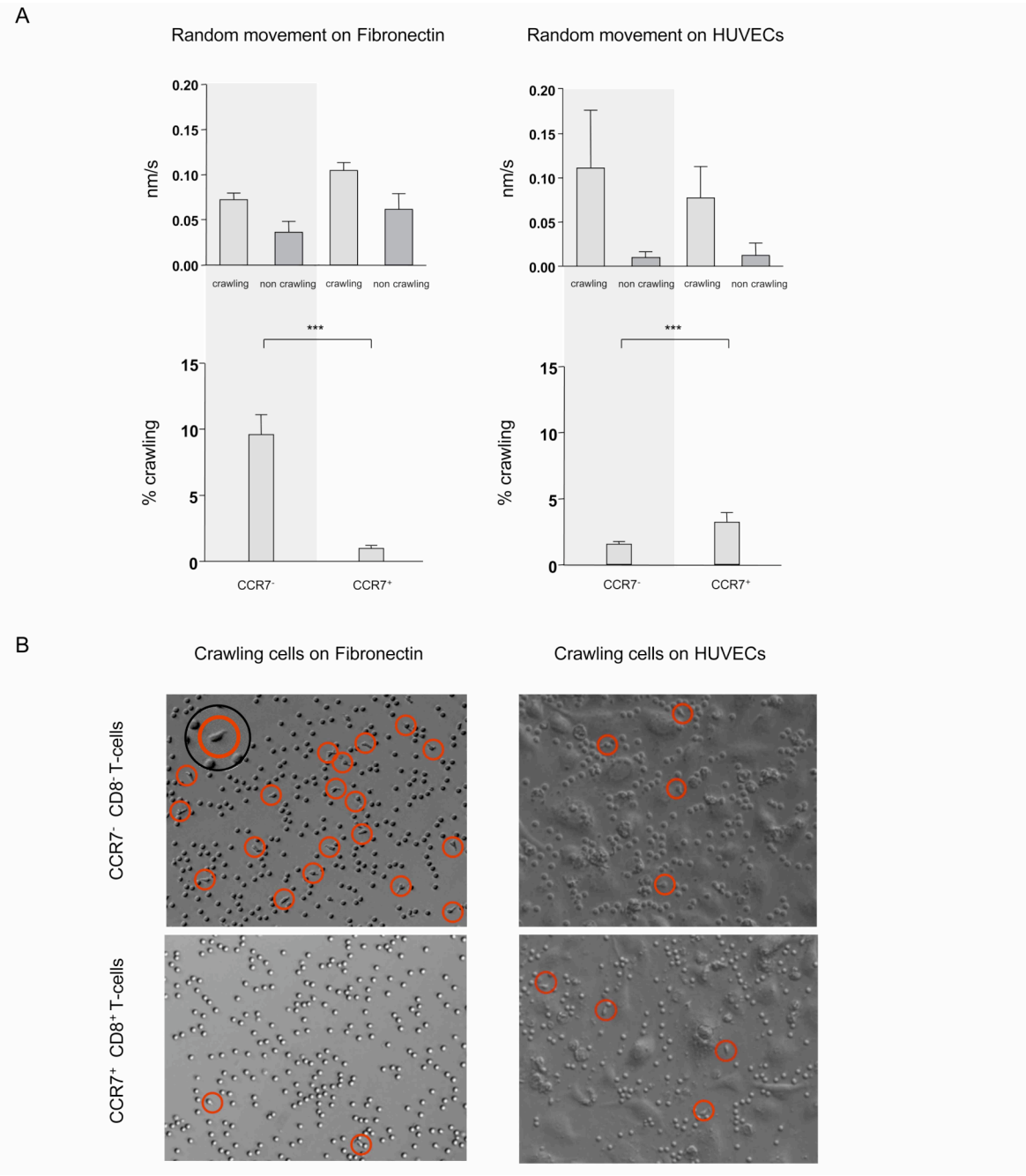

Figure 2. Morphology and motility of CCR7$^-$ versus CCR7$^+$ CD8$^+$ T cells on fibronectin and endothelial cells

Using time-lapse microscopy, the movement-pattern of sorted CCR7$^-$ and CCR7$^+$ CD8$^+$ T cells on fibronectin-coated cell culture plates and on HUVECs was visualized. (A) The velocity of crawling and non-crawling cells was determined analyzing 1 min.-spaced pictures. The average speed of crawling cells was similar among subsets on both fibronectin and HUVECs, whereas non-crawling cells moved slightly more rapid on fibronectin (upper panel). Crawling and non-crawling cells were enumerated on 5 min.-spaced pictures throughout a 1 hour observation period, and the mean percentage of crawling cells compared among CCR7$^-$ and CCR7$^+$ CD8$^+$ T cells. Crawling was a prominent feature of CCR7$^-$ CD8$^+$ T cells on fibronectin (~10% of all cells). On HUVECs the frequency of crawling CD8$^+$ T cells was higher among the CCR7$^+$ subset, but always remained <5% (lower panel). ***P<0.001 (B) On representative time-lapse video-pictures crawling CD8$^+$ T cells on fibronectin and HUVECs are marked with a circle. The insert shows the typical features of a crawling cell at higher magnification.

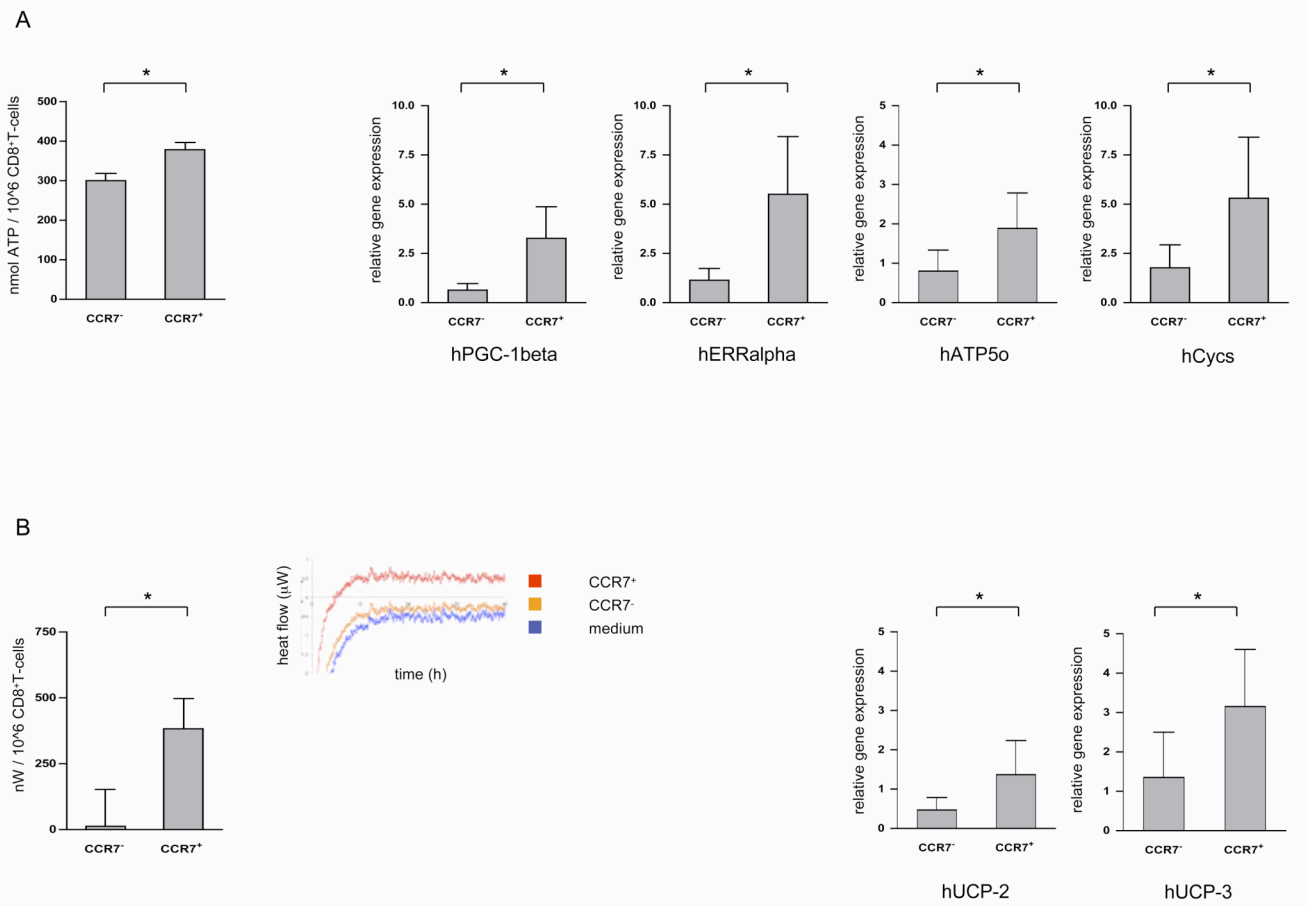

Figure 3. ATP content and energy efficiency of CCR7$^-$ versus CCR7$^+$ CD8$^+$ T cells

(A) ATP contents of sorted CCR7$^-$ and CCR7$^+$ CD8$^+$ T cells were measured in a luminescence based assay (left panel); mRNA levels of PGC-1β, ERRα, Cytochrome C (Cycs) und ATP Synthase (Atp5o) were quantified using RT-PCR technology (right panels). CCR7$^-$ CD8$^+$ T cells contained less ATP and expressed less mRNA for PGC-1β, ERRα, Cycs und Atp5o than their CCR7$^+$ counterparts. *P<0.05 (B) In calorimetric analyses heat production of sorted CCR7$^-$ and CCR7$^+$ CD8$^+$ T cells was quantified. After a calibration period of 24 hours heat production was quantified every minute for 12 hours. The insert shows the heat flow diagram of a representative experiment (left panel). mRNA levels of the uncoupling protein 2 and 3 of the respiratory chain (UCP-2 and UCP-3) were quantified by RT-PCR (right panels). CCR7$^-$ CD8$^+$ T cells contained significantly less mRNA for both UCP-2 and UCP-3 than their CCR7$^+$ counterparts. *P<0.05