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Differential Pathways Govern $CD4^+CD28^-$ T Cell Proinflammatory and Effector Responses in Patients with Coronary Artery Disease

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Patients with acute coronary syndromes experience circulatory and intraplaque expansion of an aggressive and unusual $CD4^+$ lymphocyte subpopulation lacking the CD28 receptor. These $CD4^+CD28^-$ cells produce IFN-γ and perforin, and are thought to play an important role in coronary atheromatous plaque destabilization. Aberrant expression of killer Ig-like receptors (KIRs) in $CD4^+CD28^-$ cells is broadly thought to be responsible for their cytotoxicity, but the mechanisms involved remain poorly defined. We therefore sought to investigate the mechanism and regulation of $CD4^+CD28^-$ cell functionality using T cell clones ($n = 536$) established from patients with coronary artery disease ($n = 12$) and healthy volunteers ($n = 3$). Our functional studies demonstrated that KIR2DS2 specifically interacted with MHC class I-presenting human heat shock protein 60 (hHSP60) inducing cytotoxicity. Further investigations revealed the novel finding that hHSP60 stimulation of TCR alone could not induce a cytotoxic response, and that this response was specific and KIR dependent. Analysis of $CD4^+CD28^-2DS2^+$ clones ($n = 162$) showed that not all were hHSP60 cytotoxic; albeit, their prevalence correlated with coronary disease status ($p = 0.017$). A higher proportion of clones responded to hHSP60 by IFN-γ compared with perforin ($p = 0.008$). In this study, for the first time, we define the differential regulatory pathways involved in $CD4^+CD28^-$ cell proinflammatory and effector responses. We describe in this study that, contrary to previous reports, $CD4^+CD28^-$ cell recognition and killing can be specific and discriminate. These results, in addition to contributing to the understanding of $CD4^+CD28^-$ cell functionality, may have implications for the monitoring and management of coronary artery disease progression. *The Journal of Immunology,* **2008, 181: 5233–5241.**

It is established that $CD4^+CD28^-$ cells preferentially expand in patients with acute coronary syndromes (ACS)[2] and subjects with rheumatoid vasculitis (RV) (1–6). These T cells differ from classic $CD4^+CD28^+$ T cells regarding gene expression and function, including their ability to produce high levels of IFN-γ and perforin (5–7). $CD4^+CD28^-$ cells, alongside other proinflammatory mechanisms, are therefore considered to play an important role in the events leading to coronary artery plaque destabilization (8). The cytolytic function of $CD4^+CD28^-$ cells has been attributed to their aberrant killer Ig-like receptor (KIR) expression, usually found on NK cells and infrequently on $CD8^+$ T cells (9, 10).

KIRs belong to a multigenic family, which are encoded by a minimum of 12 independent loci within the leukocyte receptor complex on chromosome 19q13.4 (11, 12). The regulation of KIR expression on $CD4^+CD28^-$ cells is poorly understood, although in NK cells KIR expression is dependent on IL-15 and begins after lineage commitment (13). The surveillance function of NK cells is tightly regulated by two classes of inhibitory and activating KIRs that recognize MHC class I molecules and that can transmit relevant inhibitory or activating signals (14). Engagement of the inhibitory receptors with MHC class I molecules presenting self peptides initiates the inhibitory cascade leading to the activation of protein tyrosine phosphatases (15, 16). These enzymes antagonize activated kinases, which are recruited by the activating KIRs to generate effector responses (17, 18). Thus, the absence of MHC class I complexes from autologous cells or their class I presentation of nonself peptides results in withdrawal of the self-tolerance by the inhibitory receptors leading to lysis of such cells by the activating KIRs (19). The main ligands for the inhibitory and activating KIRs are class I alleles encoded by the HLA-B or HLA-C locus (20), and various allotypes of the encoded molecules are shown to interact with a structurally different set of KIRs. For instance, members of the KIR2D family interact with HLA-C-encoded molecules (20). It is therefore the balance between the prevalence and function of the activating receptors in relation to their inhibitory homologues that determines the effector NK cell activity.

The activation status of $CD4^+CD28^-$ cells is thought to be governed by both the TCR and KIR components of the cells (7–9, 21, 22). $CD4^+CD28^-$ cells from patients with ACS and RV are reported to exhibit a predominant expression of the activating KIR2DS2 (9, 23). Analysis of the KIR2D repertoire has revealed that a predominant population of $CD4^+CD28^-$ cells in these patients expresses the activating KIR2SD2 in the absence of its opposing inhibitory KIR2DL2 and KIR2DL3 homologues (9, 23). This has been taken to suggest that $CD4^+CD28^-$ cells may be

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[2] Abbreviations used in this paper: ACS, acute coronary syndrome; CAD, coronary artery disease; CSA, chronic stable angina; HCMV, human CMV; hHSP, human heat shock protein; HSP, heat shock protein; KIR, killer Ig-like receptor; LDH, lactate dehydrogenase; mpc, mean percentage cytotoxicity; NSTE, non-ST elevation; RV, rheumatoid vasculitis; UA, unstable angina.

endowed with nondiscriminate cytolytic function that can break self-tolerance and lead to vascular injury (23). In NK cells, KIR2DS2 is thought to bind HLA-C polymorphism with low affinity, although several reports have shown differences in expression and function of KIR2DS2 expressed on $CD4^+CD28^-$ cells and NK cells (19, 21–23). To date, mechanisms governing $CD4^+CD28^-$ cell functionality remain unclear.

We previously reported that in 54% of ACS patients, total peripheral blood $CD4^+CD28^-$ cells were reactive to autologous human heat shock protein (hHSP)60, prompting IFN-γ and perforin responses on exposure to target cells pulsed with this Ag (7). In this study, we present a comprehensive investigation into the pathways instigating these responses using $CD4^+CD28^-$ clones and analyzing the role of KIR2DS2 and other activating KIRs in killing hHSP60-presenting autologous cells.

Our primary aim was to investigate whether $CD4^+CD28^-$ cells could be activated by hHSP60 via MHC class I pathway and respond as a result of KIR-MHC/hHSP60 peptide engagement. We further sought to find whether this response could be generated by interaction of hHSP60 with any other activating KIRs or was selective and skewed toward a particular KIR phenotype. We assessed whether acquisition of autoreactive KIRs by $CD4^+CD28^-$ cells correlated with disease presentation. We finally studied the pathways of KIR- and TCR- mediated effector and cytokine responses in $CD4^+CD28^-$ cells following hHSP60 stimulation.

Materials and Methods

Patients

We studied 12 gender- and age-matched (mean age ± SD; 64 ± 2.3) coronary artery disease (CAD) patients; 10 had non-ST elevation (NSTE) ACS (unstable angina (UA) or NSTE myocardial infarction) and 2 had chronic stable angina (CSA). UA patients were in Braunwald class IIIB, with diagnostic ST segment or T wave changes. NSTE myocardial infarction was defined according to established criteria (24). CSA was defined as typical exertional chest pain relieved by rest and/or sublingual glyceryl trinitrate with symptoms unchanged for at least 3 mo and angiographically documented coronary artery stenosis >50% diameter reduction in at least one major coronary artery. We also studied three healthy asymptomatic volunteers in whom we ruled out the presence of carotid atherosclerosis, as assessed by carotid artery ultrasound, and endothelial dysfunction, as assessed by brachial artery flow-mediated dilatation ultrasound techniques. Standardized clinical data, including risk factors for CAD, such as smoking history, family history, hypertension, diabetes mellitus, hypercholesterolemia and previous coronary events, body mass index, and cardiovascular treatment, were assessed in each patient. Patients with evidence of systemic or cardiac inflammatory diseases (with the exception of CAD) were not included. Approval from the Local Research Ethics Committee was granted for this study, and all individuals gave written informed consent form.

KIR genotyping

The presence of KIR2DS2, 2DL2, and 2DL3 genes in all selected individuals was established before inclusion. DNA extraction was conducted on 100 μg of each blood sample using Flexi Gen DNA extraction kit (Qiagen) and used in PCRs using a previously published protocol and PCR primers for 2DS2, 2DL2, and 2DL3 genes (25).

Assessing total peripheral blood $CD4^+CD28^-$ cell reactivity to hHSP60

PBMCs were separated by Lymphoprep (Nycomed), and hHSP60 stimulation was conducted, as we previously described (7). $CD4^+CD28^-$ and $CD4^+CD28^+$ fractions were then separated either magnetically using MACS $CD4^+$ isolation kit on LS and MS columns, according to the manufacturer's instructions (Miltenyi Biotec), or by FACS sorting, as we previously described (7). Cellular RNA was then extracted from each fraction using the TRIzol reagent, followed by cDNA synthesis with Superscript II reverse transcriptase and random hexamers (Life Technologies) (7). The activation status of separated fractions was analyzed by assessing the upregulation of IFN-γ and perforin transcriptions using RT-PCR, as we described previously (7). Ag reactivity of $CD4^+CD28^+$ fraction in each volunteer indicated previous exposure.

Patients with ACS (i.e., NSTE myocardial infarction and UA) were divided into two groups. Six patients had total peripheral blood $CD4^+CD28^-$ cells giving detectable levels of IFN-γ and perforin mRNA upregulation upon hHSP60 stimulation (7) (group 1), and four whose total $CD4^+CD28^-$ fractions gave no detectable levels of reactivity to Ag exposure by RT-PCR analysis (7) (group 2).

$CD4^+CD28^-$ T cell cloning

Cloning was performed at a dilution of 0.5 cell/well of 96-well plates, according to a published protocol (26). Each well contained 1×10^5 irradiated allogeneic feeder cells, 2.5 μg/ml PHA, and 40 U/ml rIL-2 (Sigma-Aldrich) with IMDM (Life Technologies) supplemented with 5% human AB serum, 2 mM L-glutamine, and 5000 U/ml penicillin/streptomycin (Sigma-Aldrich). Cultures were incubated at 37°C for 4 days before fresh medium was added. Wells containing single clones were identified on days 12–14 and used for expansion.

Expansion of $CD4^+CD28^-$ clones

For functional assays, each clone was subcultured under the conditions described above, but in the absence of PHA. The medium was replaced on day 3, and each clone was further subcultured on day 6. The expansion was continued until sufficient replicas of each clone were available for experiments.

KIR phenotyping of $CD4^+CD28^-$ cell clones

The KIR repertoire was analyzed by RT-PCR following RNA extraction from each clone and cDNA synthesis (7) and using a previously described amplification protocol (25).

Analysis of KIR-mediated cytotoxicity of $CD4^+CD28^-$ clones

Target cell preparation and Ag pulsing. Autologous monocyte-derived target cells were prepared from PBMCs using a previously described methodology (27). Target cells were then pulsed with 5 μg/ml hHSP60 in the presence or absence of MHC class I Ab blocking (10 μg/ml) at 37°C overnight (7). Negative controls included unpulsed target cells and cells treated with isotype control Abs (7). Target cells pulsed with 5 μg/ml human CMV (HCMV) proteins (Abingdon Oxon), 5 μg/ml hHSP70 (Bioquote), or 10 μg/ml PHA (Sigma-Aldrich) were used as controls (7).

Reactivity analyzed by perforin analysis. HHSP60-pulsed and control target cells were reseeded into 96-well plates at a density of 3×10^4 cells/well. $CD4^+CD28^-$ cell clones expressing different KIRs were added to the target cells at an E:T ratio of 30:1 in 100 μl final volumes. After 5 h of incubation at 37°C, perforin mRNA transcription was assessed for each well by RT-PCR (7). Each well containing the same number of target and effector cells was used for RNA extraction, and then 1 μg of cDNA was used for each RT-PCR.

Cytotoxicity analyzed by CytoTox96 assay. CytoTox96 Cytotoxicity Assay kit (Promega) was used to assess the killing function of $CD4^+CD28^-$ clones. The kit measures the amount of lactate dehydrogenase (LDH) in the culture supernatant that is released following lysis of the target cells. Thus, assessment of LDH release reflects target cell lysis levels as a measure of T cell cytotoxicity. Clones expressing different KIRs were analyzed for their effector response to different Ags in the presence and absence of MHC class I blocking. The same procedures as described above were used for preparation of target and effector cells as well as for Ag pulsing and class I blocking. The CytoTox96 assay protocol was then followed (Promega). After exposure of target to effector T cells for 5 h, the absorbance values were recorded at 492 nm and cytotoxicity was calculated according to the manufacturer's instruction (Promega).

Perforin response to hHSP60 peptides. The specificity of perforin response to Ag was further investigated using autologous target cells pulsed with hHSP60 peptides. The peptides used (Table I) were from a previously published work (28), and were synthesized by Alta Bioscience (University of Birmingham). Autologous target cells were pulsed with 10 μl/ml of each peptide resuspended in DMSO (28) and used in the assays, followed by perforin transcription measurements, as described earlier.

MHC class I, II, and KIR blocking

The effect of MHC class I and II blocking was analyzed by treating the target cells with 10 μg/ml anti-human MHC class I Abs (7) (HLA-A, B, C, clone W6/32; DakoCytomation) or 10 μg/ml anti-human MHC class II Abs (7) (HLA DR, DP, DQ; DakoCytomation) for 40 min at room temperature before cytotoxicity assay. The effect of KIR2DS2 blocking was investigated by treating the effector cells with different concentrations of the anti-human

Table I. *hHSP60 peptides used for T cell stimulation*[a]

Peptide	Sequence	hHSP60 Position
P1	KFGADARALMLQGVDLLADA	31
P2	NPVEIRRGVMLAVDAVIAEL	136
P3	QDAYVLLSEKKISSIQSIVP	240
P4	QSIVPALEIANAHRKPLVIIA	255

[a] hHSP60 peptides were synthesized by Alta Bioscience and used in functional assays, as described in *Materials and Methods*.

KIR2DS2/L2/L3 Ab (2, 10, and 20 μg/ml, clone DX27; Biolegend) for 1 h at room temperature before target cell exposure. Cells treated with 10 μg/ml isotype control Abs were included in each blocking experiment, as previously described (7).

Specificity of KIR2DS2 with HLA-A, B, C

The specificity of KIR2DS2 interaction with MHC class I molecules was further investigated using 10 μg/ml of each anti-human HLA-A, B (Lab Vision), and HLA-C (Santa Cruz Biotechnology) Ab. Target cells were separately treated with each Ab for 1 h at room temperature before exposure to $CD4^+CD28^-2DS2^+$ clones, as described earlier. Perforin levels were measured to assess the effect of each Ab blocking, and hence, the specificity of class I binding. Isotype control Abs (7) (10 μg/ml) were included in each experiment.

Statistical analysis

Proportions of clones with reactivity were compared between groups using ANOVA, except where there were zero rates when the Kruskal-Wallis test was used. Paired comparison of patient's reactivity to perforin and IFN-γ was done using the Sign test. Two-tailed p values less than 0.05 were considered significant.

Results

KIR genotyping of the patients

Because KIR2DS2-mediated cytotoxicity was the prime focus of this study, patients were genotyped for the presence of 2DS2 and its inhibitory receptor genes. A cohort of patients known from our previous work to express KIR2DS2 gene was genotyped for the presence of 2DL2 and 2DL3 genes, and only those who were positive for both genes were included (Fig. 1).

KIR profiling of $CD4^+CD28^-$ T cell clones

KIR2DS2 is believed to be the prominent receptor involved in $CD4^+CD28^-$ cell cytotoxicity. It was therefore essential to determine the expression pattern of KIR2DS2 and its inhibitory receptors KIR2DL2 and 2DL3 on $CD4^+CD28^-$ T cells from patient and control groups before functional studies. We established $CD4^+CD28^-$ cell clones from our study populations following FACS analysis (7) or magnetic purification: 332 clones from 10 patients

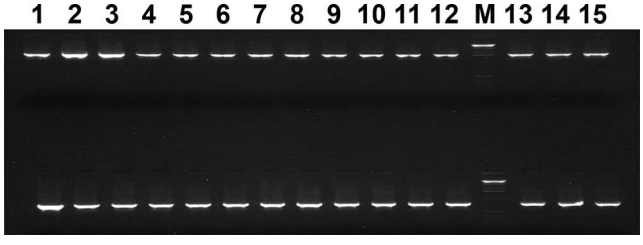

FIGURE 1. KIR2DL2 and KIR2DL3 genotyping. DNA was extracted from each patient's blood sample using FlexiGen kit (Qiagen), and the presence of KIR2DL2 and 2DL3 genes was determined by PCRs. *Top panels 1–12* and *13–15* represent a 1800-bp fragment of KIR2DL2 gene from ACS and CSA patients, respectively. The parallel *bottom panels* represent a 798-bp fragment of KIR2DL3 in the same order as the *top panels*.

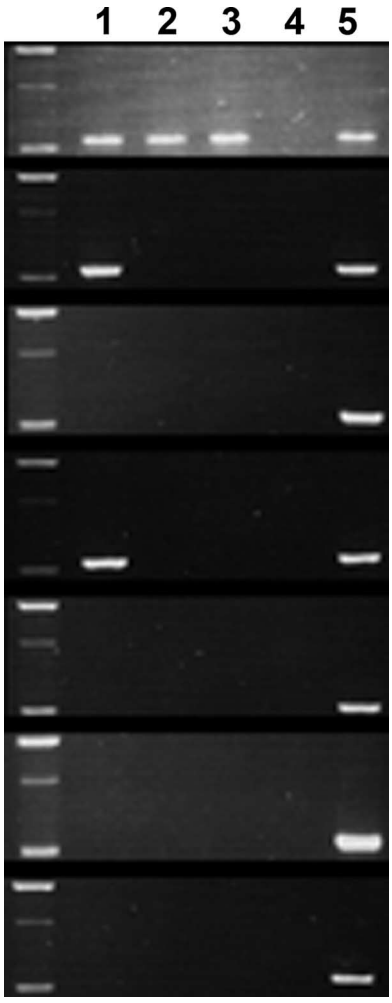

FIGURE 2. Reactivity of KIR2DS2 and other activating KIRs against hHSP60. The specificity of hHSP60-induced KIR2DS2 activation of $CD4^+CD28^-$ cells was demonstrated using clones expressing variant isoforms of the activating KIR against different Ags. Thirty T cell clones expressing KIR2DS1, 2DS2, 2DS3, 2DS4, and 3DS1 (6 of each) were individually tested for reactivity against cells pulsed separately with 5 μg/ml hHSP60, hHSP70, or HCMV at an E:T ratio of 30:1 (gel sections 3–7 from top). Assessment of perforin levels was used as marker of T cell response. Untreated target cells and cells treated with PHA were used as negative and positive controls. *Panels 1–5* represent clone responses to hHSP60, hHSP70, HCMV, untreated cells, and PHA, respectively. Total $CD4^+CD28^-$ fraction and $CD4^+CD28^-$ cell clone expressing KIR2DS2 were reactive to hHSP60-pulsed target cells, but not to other Ags (gel sections 2, 4 from top). None of the clones expressing the other receptors (2DS1, 2DS3, 2DS4, and 3DS1) reacted with any of the Ags. Total $CD4^+CD28^+$ fraction was also analyzed, which confirmed previous exposure and, hence, reactivity of the patients to all three Ags (gel section 1).

with ACS, 91 clones from 2 patients with CSA, and 113 clones from 3 healthy individuals. Patients with ACS were divided into two groups depending on their total peripheral $CD4^+CD28^-$ cell reactivity against hHSP60, as described in *Materials and Methods*.

KIR expression profile on each T cell clone was then determined by assessing the presence of KIR transcripts. As previously reported (9), a predominant presence of KIR2DS2 was observed among the patients with ACS and CSA compared with healthy subjects ($p = 0.017$). In group 1, ACS patients' expression of this receptor occurred mostly in the absence of inhibitory homologues ($p = 0.04$). This was more common in patients with ACS, suggesting that in our study population, coronary disease activity correlated with an increased prevalence of $CD4^+CD28^-2DS2^+$ cells.

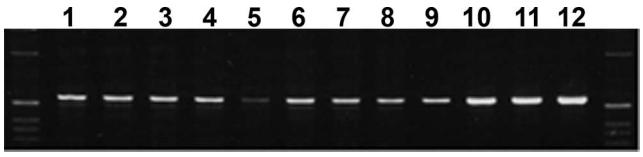

FIGURE 3. Ag-specific KIR mediated perforin response of CD4$^+$ CD28$^-$ T cells. KIR2DS2-mediated perforin transcription in response to exposure to hHSP60-pulsed autologous cells is demonstrated. The effect of MHC class I blocking (10 μg/ml) is presented. CD4$^+$CD28$^-$ cell clones expressing 2DS2 receptor alone or in the presence of its inhibitory homologues 2DL2 and 2DL3 were established from ACS patients and exposed to hHSP60- or PHA-pulsed cells, followed by the assessment of perforin transcription. *Panels 1*, *5*, and *9* represent hHSP60-exposed 2DS2$^+$2DL2$^+$ 2DL3$^+$ and 2DS2$^+$2DL2$^-$2DL3$^-$ clones and total CD4$^+$CD28$^-$ fraction, respectively. The interaction between KIR and MHC class I is blocked by anti-class I Abs. *Panels 2*, *6*, and *10* replica clones exposed to hHSP60 in the absence of blocking. Identical clones were exposed to PHA with class I blocking in *panels 3*, *7*, and *11* and without blocking in *panels 4*, *8*, and *12*. It can be seen from *panels 5* and *9* that blocking of the MHC class I molecules notably reduced the levels of perforin transcription in 2DS2$^+$ 2DL2$^-$2DL3$^-$ clones and total CD4$^+$CD28$^-$ fraction exposed to hHSP60. This effect is not observed where the inhibitory receptors are coexpressed regardless of the presence or absence of anti-MHC class I Abs (*panels 1* and *2*).

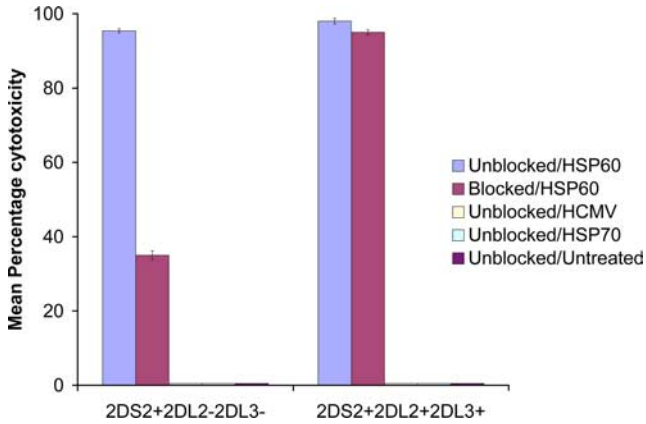

FIGURE 4. KIR2DS2-mediated effector function of CD4$^+$CD28$^-$ cells. Assessment of the effector function of 2DS2$^+$2DL2$^-$2DL3$^-$ and 2DS2$^+$2DL2$^+$2DL3$^+$ clones was conducted in the presence or absence of MHC class I blocking using the CytoTox96 assay kit. Target cells pulsed with 5 μg/ml hHSP60 or control Ags were exposed to T cell clones at an E:T ratio of 30:1, and the release of LDH was measured as a mean of assessing target cell lysis. The results are shown as $\pm$SD of mpc for each clone. The cytotoxic responses of 2DS2$^+$2DL2$^-$2DL3$^-$ and 2DS2$^+$ 2DL2$^+$2DL3$^+$ clones were notably different in that anti-human MHC class I Abs reduced cytotoxic effect of 2DS2$^+$2DL2$^-$2DL3$^-$ clones (Kruskal-Wallis, two groups: $p = 0.002$), but had no effect on 2DS2$^+$2DL2$^+$2DL3$^+$. None of the clones lysed untreated target cells or cells pulsed with hHSP70 or HCMV.

In 180 T cell clones from group 1 ACS, KIR2DS2 was expressed by 72%, and 30% expressed this receptor in the presence of one or both of the inhibitory 2DL2 and 2DL3 homologues. Analyzing 152 clones from group 2 ACS, 57% expressed KIR2DS2, which coincided with a higher occurrence of the inhibitory receptors (36%). Patients with CSA had a lower percentage of 2DS2$^+$ clones (25%), with coexpression of inhibitory proteins remaining low (13%). The prevalence of 2DS2$^+$ clones in healthy individuals was similar to CSA (24%), but its expression largely coincided with the presence of KIR2DL2 and KIR2DL3 (92%). The expression of other activating KIRs (2DS1, 2DS3, 2DS4, and 3DS1) was also analyzed, and the clones expressing these receptors were investigated for their effector responses (Fig. 2).

KIR interaction with hHSP60/MHC-I complex was selective and specific

KIR2D subfamily of NK cells is known to bind ligands encoded by HLA-C locus (20). Because KIR2DS2 was the predominant receptor expressed by CD4$^+$CD28$^-$ cells, we initially chose to investigate its potential interaction with class I/hHSP60 complex. We analyzed both 2DS2$^+$2DL2$^-$2DL3$^-$ and 2DS2$^+$2DL2$^+$2DL3$^+$ clones in functional studies using autologous hHSP60-pulsed monocyte-derived cells as target for cytotoxicity. The use of autologous cells ensured the expression of the same complement of KIRs on both the effector and APCs. HHSP70 and whole HCMV proteins were used as control Ags, and target cells untreated with any Ag were used as negative control. Presentation of exogenous Ags, namely hHSP60 and hHSP70 on MHC class I molecules by these cells, is well documented (29–31).

Initially, perforin transcription levels were used as a means of assessing T cell response. Fifteen 2DS2$^+$2DL2$^-$2DL3$^-$ clones from nine ACS patients were exposed to hHSP60-pulsed cells, and an elevation of perforin mRNA was noted. Pretreatment of target cells with anti-MHC class I Abs significantly reduced perforin levels, suggesting a KIR class I-mediated response (Fig. 3). Investigations of 15 2DS2$^+$2DL2$^+$2DL3$^+$ clones, however, proved unusual. Perforin mRNA up-regulation occurred upon hHSP60 exposure, suggesting that, unlike NK cells, in which the inhibitory KIRs have a surveillance role and protect cells presenting autologous peptides (32, 33), 2DL2 and 2DL3 receptors on CD4$^+$ CD28$^-$ cells may exhibit a compromised function. Although a previous report has described this phenomenon in redirected cytotoxicity assays (9), this is the first substantive evidence that cells presenting a specific autologous Ag are targeted by these 2DS2$^+$ 2DL2$^+$2DL3$^+$ T lymphocytes. When the cells were treated with anti-class I Abs, perforin response was not down-regulated by 2DS2$^+$ 2DL2$^+$2DL3$^+$ clones (Fig. 3). In NK cells, failure of interaction between the inhibitory KIRs and class I inhibits phosphorylation of cytoplasmic immunoreceptor tyrosine-based inhibitory motif, which is central in cellular activation (32, 33). This may explain the perforin mRNA up-regulation in these CD4$^+$CD28$^-$ clones upon MHC-I blocking. None of the replica 2DS2$^+$ clones reacted with the control Ags or unpulsed target cells. Isotype control Abs (7) showed no blocking effect on the effector responses of either set of clones. We further investigated the functionality of CD4$^+$ CD28$^-$ clones expressing other activating KIRs. Analysis of 24 clones expressing KIR2DS1, 2DS3, 2DS4, and 3DS1 (6 of each clone) revealed that none were responsive to hHSP60 or other Ags (Fig. 2).

CD4$^+$CD28$^-$2DS2$^+$ clones selectively lyse hHSP60-pulsed autologous cells

We then conducted functional studies and assessed the cytolytic capability of the hHSP60-reactive clones using CytoTox96 assay (Promega), which quantitatively measures LDH released upon cell lysis (34–36). As before, 2DS2$^+$2DL2$^-$2DL3$^-$ and 2DS2$^+$2DL2$^+$2DL3$^+$ clones were investigated using hHSP60-pulsed autologous cells in the presence or absence of anti-class I Abs (Fig. 4). Ten 2DS2$^+$2DL2$^-$2DL3$^-$ clones from 10 ACS patients lysed the target cells in the absence of blocking. The mean percentage cytotoxicity (mpc) was 94.6%. Duplicate clones tested in the presence of anti-class I Abs induced much lower levels of killing with the mpc at 36%. Control Ags prompted no cytotoxicity in the absence of blocking. These results support our earlier data and demonstrate a specific KIR-mediated effector response, which

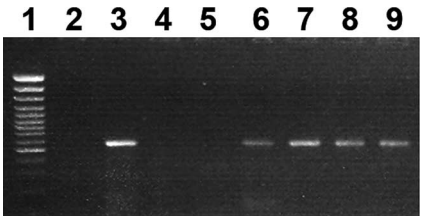

FIGURE 5. Perforin response of effector cells against hHSP60 peptides. The specificity of $CD4^+CD28^-2DS2^+$ cells for hHSP60 was further investigated by using synthetic peptides. The representative data show perforin mRNA levels to stimulation by four peptides. Cells were pulsed with 10 μg/ml of each peptide resuspended in DMSO and exposed to effector cells. Perforin response of the T cells was determined by RT-PCR. The results show reactivity to two overlapping peptides corresponding to positions 240–259 and 255–276 of linear hHSP60 sequence (Table I) in *panels 6* and *7*. No reactivity was detected to peptides corresponding to positions 31 and 136 (*panels 4* and *5*). There was no response to unpulsed cells (*panel 2*), whereas perforin level was up-regulated in response to PHA stimulation (*panel 3*). *Panels 8* and *9* show reactivity of $CD4^+CD28^-2DS2^+$ and whole $CD4^+CD28^-$ cells to whole hHSP60 Ag stimulation.

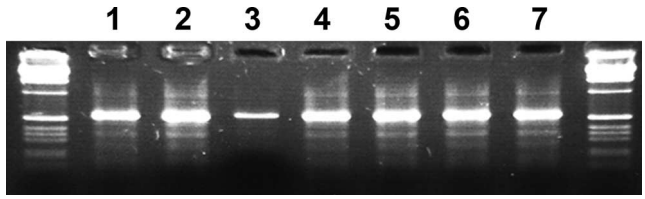

FIGURE 6. Effect of anti-HLA-A, B, and C blocking on effector function of $CD4^+CD28^-$ cells. The specificity of KIR2DS2 interaction with MHC class I molecules was investigated using 10 μg/ml of each anti-human HLA-A, B, and C Ab. The representative gel shows the effect of anti-HLA-C blocking in significantly reducing the perforin transcription level in $CD4^+CD28^-2DS2^+$ cells. Target cells were treated separately with each Ab for 1 h at room temperature before hHSP60 stimulation, followed by exposure to T cell clones (E:T ratio of 30:1). Perforin levels were measured to assess the effect of each Ab blocking. *Panels 1–3* represent blocking with HLA-A, B, and C, respectively. *Panels 4* and *5* represent untreated cells pulsed with hHSP60 and PHA as controls. *Panels 6* and *7* represent perforin levels after treatment with anti-human MHC class II and isotype control Abs. None of the Abs but anti-HLA-C Ab had a marked effect on the reduction of perforin responses.

is inhibited by class I blocking ($p = 0.002$). Functional analysis of nine $2DS2^+2DL2^+2DL3^+$ clones from nine ACS patients by the same procedure supported our earlier findings: cytolysis was high regardless of absence or presence of class I blocking (mpc of 97.5 and 95%, respectively). Our data suggest that KIR2DS2-driven autocytolysis of $CD4^+CD28^-$ cells may largely be unaffected by the presence of the inhibitory receptors. Activation of $2DS2^+2DL2^+2DL3^+$ clones in the presence of blocking, however, reflects the inability of the inhibitory receptors to interact with the class I ligand, thus inducing the activation cascade. Thus, we propose that 2DL2 and 2DL3 receptors are not functionally silent on $CD4^+CD28^-$ cells, but may have acquired a compromised protective role. Our results raise the question of differing degree of interaction and binding affinity of the KIRs with hHSP60/class I complex and how the inhibitory receptors may exhibit a compromised role in this regard. Replica clones did not lyse cells pulsed with HCMV or hHSP70, again confirming the specificity of response. As earlier, isotype control Abs (7) did not have any effect on the level of cytotoxicity.

$CD4^+CD28^-$ cell reactivity with peptides from hHSP60

The specificity of interaction of $CD4^+CD28^-2DS2^+$ clones with hHSP60 in the context of class I was further investigated using synthetic peptides. Because heat shock protein (HSP)60 is a chaperone protein, these experiments were designed to differentiate between T cell response against hHSP60 itself or any exogenous peptides that might have been chaperoned by it. Our results show significant up-regulation of perforin in response to target cells pulsed with two overlapping peptides spanning aa sequence 240–276 of hHSP60 sequence (Table I). Peptides from positions 31 band 136 as well as control Ags did not generate a cytotoxic response (Fig. 5).

Interaction of KIR2DS2 with class I/hHSP60 complex is HLA-C specific

The specificity of KIR2DS2 for MHC class I molecules was further analyzed in blocking experiments. Although human anti-HLA-C blocking significantly decreased perforin response by $CD4^+CD28^-2DS2^+$ cells to hHSP60-pulsed target cells, anti-HLA-A, anti-HLA-B, and isotype control Abs (7) showed no such effect (Fig. 6). These results agree with the current understanding that KIR2D subfamily expressed on NK cells binds HLA-C-encoded molecules, although with relatively low affinity (37). KIR2DS2 on $CD4^+CD28^-$ cells, however, appears to bind these complexes, presenting hHSP60 with a high degree of affinity required to initiate an effector response. Future studies are needed to further increase our understanding of these mechanisms.

HLA-C typing of patients with ACS

Receptors of the KIR2DS subfamily recognize polymorphisms on HLA-C molecules (20). We therefore investigated the possibility of interaction of 2DS2 phenotype with a particular HLA-C polymorphism. HLA-C typing of the patients revealed polymorphic variants commonly recognized by KIR2D (HAL-C *01, *03, *07) (20), as well as HAL-C *04 and *05, but with no significant enrichment compared with HLA-C reference distribution in Caucasian population (38, 39).

Expansion of hHSP60-reactive $CD4^+CD28^-2DS2^+$ cells and disease status

We addressed the expansion and clinical relevance of perforin-responding hHSP60-specific $CD4^+CD28^-2DS2^+$ cells in our

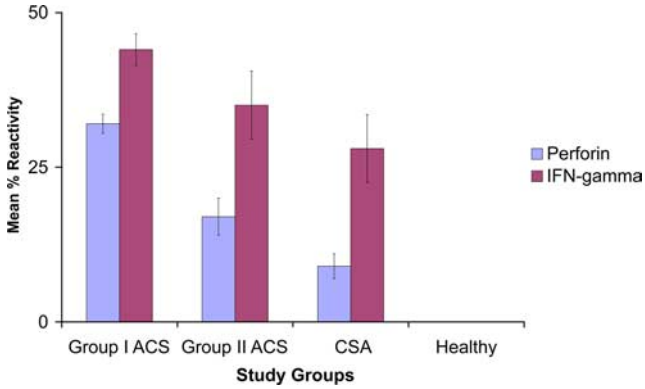

FIGURE 7. Prevalence of $CD4^+CD28^-2DS2^+$ T cell clones responding to hHSP60 by perforin and/or IFN-γ in patient and control groups. Assessments of T cell responses were conducted by measurements of perforin and IFN-γ transcription levels after exposure to hHSP60-pulsed target cells (E:T ratio, 30:1). The results are shown as mean percentage reactivity of clones responding by perforin and/or IFN-γ in each group. Larger number of clones from each patient group responded by IFN-γ compared with perforin ($p = 0.008$, Sign test), although the cytotoxic response always accompanied cytokine up-regulation. None of the clones from the healthy individuals was found to react to this Ag.

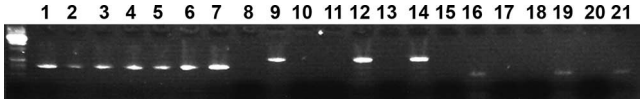

FIGURE 8. DAP-12 expression and cytotoxicity among cytokine-responding clones. Higher proportion of $CD4^+CD28^-2DS2^+$ clones reacted with hHSP60 by IFN-γ than perforin ($p = 0.008$, Sign test). The gel represents elevated perforin transcription among seven IFN-γ-responding clones and its relation with expression of DAP-12. Measurements of IFN-γ and perforin transcriptions were conducted by RT-PCR after exposure to Ag-pulsed target cells. Only three clones exhibited perforin response and were also positive for DAP-12 expression. *Panels 1–7* and *8–14* represent IFN-γ and perforin responses; *panels 15–21*, show the expression of DAP-12, respectively.

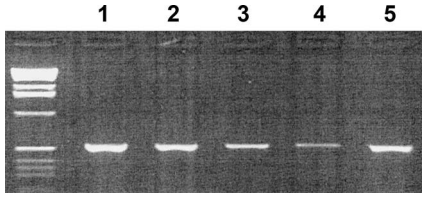

FIGURE 9. Anti-KIR2DS2 Ab blocks perforin transcription in $CD4^+CD28^-2DS2^+$ cells. The 2DS2-mediated perforin response of $CD4^+CD28^-2DS2^+$ clones against hHSP60-pulsed cells was reduced by blocking with anti-human KIR2DS2 Ab. The blocking effect was demonstrated by treating the clones with different concentrations of the Ab for 1 h at room temperature before target cell exposure (E:T ratio, 30:1). Ten clones were analyzed, and sufficient replicas of each were prepared for the experiment. *Panel 1* represents perforin response of an untreated clone. *Panels 2–4* (respectively) represent the effect of 2, 10, and 20 μg/ml of the Ab on the perforin response. *Panel 5* represents the effect of blocking (20 μg/ml) on a clone exposed to PHA-pulsed cells.

patient and control groups (Fig. 7). Assessment of perforin response confirmed varied presence of perforin-releasing $CD4^+CD28^-2DS2^+$ clones in patients with ACS and CSA, and healthy people (Kruskal-Wallis; $p = 0.017$). In 105 clones from five group 1 ACS patients, 32% responded to hHSP60. The remainder was nonreactive to hHSP60 or control Ags. Analyzing 34 clones from group 2 ACS and 23 clones from CSA patients, 17 and 9% were respectively responsive. $CD4^+CD28^-2DS2^+$ clones from healthy individuals were nonreactive. We conclude from our results that expansion of hHSP60-reactive $CD4^+CD28^-2DS2^+$ cells in ACS patients may correlate with disease development.

We further addressed the question of noncytotoxic $CD4^+CD28^-2DS2^+$ cells in our study groups by analyzing the expression of the adaptor protein DAP-12. Redirected cytotoxicity work has previously shown that DAP-12 is an essential component of 2DS2-mediated cytotoxicity in $CD4^+CD28^-$ cells (9). This phenomenon has also been demonstrated in NK cells, whereby DAP-12 directs the cells to the cytotoxic process by activating the ZAP70/Syk pathway and phospholipase Cγ phosphorylation (40).

Investigation of 73 $2DS2^+$ clones from ACS and CSA patients by RT-PCR (41) revealed that perforin response to hHSP60 always coincided with expression of DAP-12 (ANOVA, $p = 0.20$; Fig. 8). In addition, all 24 cytotoxic $2DS2^+2DL2^+2DL3^+$ clones analyzed expressed DAP-12. The $2DS2^+$ clones from healthy subjects were also negative for DAP-12 expression. Expression of DAP-12^+ cells and induction of hHSP60 reactivity by $CD4^+CD28^-2DS2^+$ cells were disease related and higher in patients with ACS ($p = 0.017$). This may suggest that DAP-12 expression and transition of $2DS2^+$DAP-12^- to $2DS2^+$DAP-12^+ may occur as a result of mechanisms induced by chronic Ag exposure, which are enhanced with disease development.

Higher proportion of $CD4^+CD28^-2DS2^+$ cells in ACS and CSA responds to hHSP60 by IFN-γ release

To further decipher the activation pathways of $CD4^+CD28^-2DS2^+$ cells, we investigated the prevalence of TCR-induced cytokine response (Fig. 7). Assessment of 162 clones, analyzed earlier for cytotoxicity, revealed that a higher proportion of these clones responded to hHSP60 by IFN-γ compared with perforin ($p = 0.008$, Sign test). The percentages of IFN-γ-positive clones were 43% for group 1 ACS, 34% for group 2 ACS, and 27% for patients with CSA (ANOVA, three groups, $p = 0.068$). The $2DS2^+$ clones from healthy controls and clones expressing other activating KIRs ($n = 24$) were nonreactive. The presence of smaller proportion of hHSP60-reactive T cells in group 2 ACS and CSA compared with group 1 ACS clarifies the reason for these groups' total $CD4^+CD28^-$ cells giving undetectable levels of perforin and IFN-γ mRNA up-regulation upon hHSP60 stimulation, as observed in this study and previously (7). Perforin response always coincided with IFN-γ up-regulation, although in some clones the latter response was present in the absence of the former (Fig. 8). Analysis of DAP-12 expression showed that $2DS2^+$ clones responding by IFN-γ alone lacked this adaptor protein (Fig. 8). This novel observation suggests that cytokine activation occurs via interaction of TCR with hHSP60 in the context of MHC involvement.

Independent pathways drive cytokine and cytotoxic responses of $CD4^+CD28^-2DS2^+$ cells to hHSP60

Ab blocking work further underlined the nature of perforin and IFN-γ responses in $2DS2^+$DAP-12^+ cells. Anti-human MHC class II Abs significantly reduced IFN-γ response of the five clones tested to hHSP60 compared with replicas tested in the absence of blocking. Inhibition of class II, however, did not affect perforin response. This situation was reversed with MHC class I blocking, which radically reduced perforin transcription in similar clones ($n = 5$), although no detectable effect was noted on IFN-γ mRNA production. To support this observation and our earlier data, we conducted KIR-specific blocking. Using 10 hHSP60-reactive $2DS2^+$ clones, we demonstrated that perforin response against target cells was markedly reduced in the presence of anti-KIR2DS2 Ab. Titration experiments showed that the inhibition of 2DS2 function was dose dependent (Fig. 9). KIR blocking had no effect on IFN-γ response in replica clones. Finally, when we analyzed the simultaneous effect of class I and II blocking on $2DS2^+$ clones ($n = 5$), both IFN-γ and perforin responses were notably declined. Isotype-matched control Abs used in each set of experiments did not affect the IFN-γ and perforin responses of clones. Taken together, these observations suggest independent cytokine and cytotoxic response mechanisms in $CD4^+CD28^-$ cells following hHSP60 exposure.

Discussion

In chronic inflammatory conditions, expanded $CD4^+CD28^-$ cells exhibit aberrant expression of the activating KIR2DS2 usually in the absence of the corresponding inhibitory KIR2DL2 and KIR2DL3 (9, 23). Predominant expression of this receptor unopposed by the inhibitory mechanisms is believed to be the drive behind the cytotoxic potentials of these T cells (23). In our previous work, we reported on the Ag specificity of $CD4^+CD28^-$ cells and showed that they reacted with hHSP60 in 54% of the patients with ACS (7). We demonstrated that upon specific Ag stimulation, the cells responded by producing both IFN-γ and perforin. Moreover, we observed that blocking the CD4 receptor or the MHC class II pathway significantly reduced the cytokine response (7). In the present study, we further investigated the role of KIRs and

class I pathway in the activation of $CD4^+CD28^-$ cells using hHSP60-presenting target cells. For the purpose of the study, we cloned $CD4^+CD28^-$ cells from patients with ACS and CSA and healthy individuals and determined the pattern of KIR expression on each T cell clone. Different patterns of KIR expression by these cells were observed in our subjects. KIR2DS2 was preferentially expressed by patients' T cells ($p = 0.017$), largely in the absence of KIR2DL2 and KIR2DL3 ($p = 0.04$). In line with previous work (9), this was more common in patients with ACS, suggesting that coronary disease activity correlates with an increased prevalence of $CD4^+CD28^-2DS2^+$ cells.

To investigate the interaction of KIRs with hHSP60 in the context of MHC class I, we used autologous monocyte-derived cells. Presentation of exogenous Ags, in particular hHSP60 and hHSP70, on MHC class I molecules by these cells is well documented (29–31). These Ags are internalized via receptor-mediated endocytosis and reach endosomal compartments for processing and loading onto HLA class I and class II molecules (30, 31). Therefore, these target cells may be used to study the Ag presentation and recognition pathways via MHC class I or II pathways in vitro.

Analysis of the effector function in T cells from ACS patients exposed to hHSP60-pulsed target cells showed up-regulation of perforin mRNA. To determine whether this response was mediated via interaction of 2DS2 receptor with class I on the target cells, we performed Ab-blocking experiments. Blocking of the class I on the target cells with anti-human MHC class I Ab or of KIR2DS2 on the effector clones with anti-2DS2 Ab significantly reduced perforin response to hHSP60 exposure. Our data conclusively and for the first time demonstrate the interaction of KIR2DS2 with class I/hHSP60 peptides, leading to generation of activation signals and perforin release. Investigation of the role of the inhibitory KIR2DL2 and KIR2DL3 in opposing this activation pathway proved unusual. The $2DS2^+2DL2^+2DL3^+$ clones were activated upon Ag exposure both in the absence and presence of class I blocking. We can conclude that unlike NK cells (32, 33), the inhibitory KIRs on $CD4^+CD28^-2DS2^+$ cells may exhibit a compromised ability to counter the activation signals generated by the 2DS2 receptor upon encounter with class I/hHSP60 complex. This may suggest that the generation of the inhibitory cascade may be different from what is known for NK cells (32, 33) or that it is somehow overridden by the interaction of 2DS2 ligand. A previous study has shown that KIR2DS2 was able to activate NK cells in the presence of the inhibitory 2DL2 and 2DL3 receptors using a low concentration of plastic-immobilized CD158b mAb (42). Higher concentrations, however, triggered the inhibitory KIRs to counter the 2DS2-induced activation (42). These results together with our findings may suggest that the activating and inhibitory KIRs on $CD4^+CD28^-$ cells have different threshold levels of occupancy for stimulation or alternatively exhibit varying degree of affinity for interaction with the ligand.

Activation of $2DS2^+2DL2^+2DL3^+$ clones in the presence of class I blocking unveiled a novel finding. Interaction of inhibitory receptors and class I is essential for generation of inhibitory signals (14–16), and blocking of this pathway would result in effector responses (19). Our results in this study were quite interesting. Although the inhibitory 2DL2 and 2DL3 receptors on $CD4^+CD28^-$ cells appeared unable to oppose the effector function of 2DS2 receptor upon encounter with class-I/hHSP60 complex, blocking of the class I 2DL2 and 2DL3 interaction directed the cells to increase perforin transcription. If these inhibitory receptors were redundant, class I blocking should have inhibited the 2DS2 receptor interaction and reduced perforin mRNA. This did not, however, occur, and we therefore propose that 2DL2 and 2DL3 receptors on $CD4^+CD28^-$ cells may still be functional in their surveillance role, but hindered in their capacity to protect hHSP60-presenting cells.

One limitation to our experiments in this study is the lack of ability to demonstrate KIR protein expression on $CD4^+CD28^-$ cells. There is currently no Ab commercially available distinguishing KIR2DS2 from the KIR2DL2 and KIR2DL3.

In the same context and in line with our observations, a recent study has shown that KIRs on T cells function fundamentally differently from the regulatory KIRs on NK cells (43). Investigations of KIR2DL2 regulation of $CD4^+CD28^-$ cell function showed that its engagement by HLA-cw3 had no effect on conjugate formation between T cells and target cells, leading to formation of activating immune synapses and induction of cytotoxicity. KIR2DL2 did, however, inhibit transcriptional profile of TCR-dependent genes, thus diminishing IFN-γ production and cell proliferation (43). The reason for this response pattern is believed to be due to the differences in the kinetics of KIR2DL2 translocation in the cell membrane of $CD4^+CD28^-$ cells and NK cells. In $CD4^+CD28^-$ cells, KIR2DL2 was first recruited to the peripheral supramolecular activation cluster and subsequently to central supramolecular activation cluster, which resulted in failing to inhibit phosphorylation of early signaling proteins and induction of cytotoxicity (43). Different dynamics of KIR translocation in $CD4^+CD28^-$ cells may explain the inability of 2DL2/2DL3 to inhibit cytotoxicity following KIR2DS2 engagement with MHC I/hHSP60 complex, as observed in the present work. The mechanisms mediating this process are currently unresolved and require future investigations.

In parallel control experiments, HCMV proteins and hHSP70 did not prompt perforin response from any of the clones; however, total $CD4^+CD28^+$ fraction from the same patients reacted, confirming previous exposure to the Ags (Fig. 2). These findings suggest that $CD4^+CD28^-2DS2^+$ cells may exhibit restricted class I/peptide effector response, and the nondiscriminate killing potential of these cells, as previously proposed (23), may not in fact be the case. Further investigations showed that hHSP60 activation of $CD4^+CD28^-$ cells was receptor specific and skewed toward KIR2DS2 and not other KIRs.

To assess the killing potential of the $CD4^+CD28^-2DS2^+$ cells upon encounter with autologous HSP60, we performed functional assays using the CytoTox96 cytotoxicity assay kit. Although the use of monocytic cells instead of endothelial cells as targets for cytotoxicity does not entirely reflect the vascular events, our prime aim was to investigate the potential interaction of various KIRs expressed by $CD4^+CD28^-$ cells with hHSP60/MHC I complex and to describe the mechanism and specificity of activation. The data obtained in this study complemented our earlier findings. Effector $2DS2^+2DL2^-2DL3^-$ clones specifically recognized and lysed target cells presenting hHSP60 in the context of class I, and blocking of this pathway using anti-class I Ab notably protected the target cells. The $2DS2^+2DL2^+2DL3^+$ clones, in contrast, lysed the hHSP60-pulsed target cells both in the absence and presence of the class I blocking. Our data encourage further investigations into the regulatory mechanisms governing KIR function in $CD4^+CD28^-$ cells. Understanding of these mechanisms could significantly boost our approaches to molecular therapeutic interventions for ACS.

HSPs, including HSP60 and 70, are among the most important molecular chaperones. To validate the up-regulation of perforin transcription in $CD4^+CD28^-2DS2^+$ cells as a result of hHSP60 stimulation as opposed to possible chaperoned peptides, we tested synthetic peptides from hHSP60 in the cytotoxicity assays. Our results showed specific responses to overlapping peptides spanning positions 255–274 of the linear hHSP60 amino acid sequence. Future work using overlapping peptides covering the entire length of

this protein would be significant in identification of other regions that could potentially generate an effector response by $CD4^+CD28^-2DS2^+$ engagement.

The receptor- and Ag-specific cytolytic response of $CD4^+CD28^-$ cells prompted us to investigate the interaction of 2DS2 receptor with class I allotypes. The blocking experiments showed the specificity of binding of KIR2DS2 with HLA-C-encoded molecules. We further studied the possibility of an association between expression of a particular variant of HLA-C and an increased risk of ACS. HLA-C typing of our patients showed no significant enrichment of a HLA-C variant. Commonly KIR2D-recognized variants (*01, *03, *07) (20), as well as *04 and *05, were identified. Enrichment of HLA-C ^{*05} has been reported in patients with RV (23) who also exhibit expanded levels of circulatory $CD4^+CD28^-2DS2^+$ cells. It has also been shown that HLA-C recognition may not follow a set pattern, and various peptides bound in the groove of different alleles could modify the recognition (44).

We further studied the correlation between the expansion of the autoreactive $CD4^+CD28^-2DS2^+$ cells and disease status. We investigated the entire panel of $CD4^+CD28^-2DS2^+$ clones from our patients and healthy groups for reactivity to hHSP60. Expanded numbers of perforin-responding $CD4^+CD28^-2DS2^+$ cells were observed in patients with ACS compared with CSA and healthy groups (Kruskal-Wallis; $p = 0.017$). Our results clearly demonstrated an association between increased presence of the hHSP60-reactive T cells and disease status. We propose that the autoreactivity to hHSP60 by these T cells may begin during early stages of the disease and gradually increase during the chronic phase and contribute to disease progression and ACS.

Investigation of the cytokine response by $CD4^+CD28^-2DS2^+$ clones gave interesting results, showing that higher proportions of these T cells responded by IFN-γ than perforin ($p = 0.008$). IFN-γ-responding clones were more prevalent among group 1 ACS patients and may correlate with disease presentation. Up-regulation of perforin mRNA to hHSP60 stimulation interestingly always coincided with IFN-γ response, whereas some clones responded to Ag exposure only by cytokine release. This is in agreement with a previous report that claims that in noncytotoxic $CD4^+CD28^-2DS2^+$ cells, KIR2DS2 costimulates IFN-γ production in conjunction with suboptimal TCR activation (41). Our findings may represent an important developmental pattern in $CD4^+CD28^-2DS2^+$ cell activation, which begins with cytokine response. To our knowledge, this is the first functional data suggesting that proinflammatory response by $CD4^+CD28^-2DS2^+$ cells may precede cytotoxicity through sequential TCR and KIR activation.

It is known that the function of stimulatory KIRs is determined by the expression of KAPA/DAP-12 adaptor molecule (40). Observation of non-hHSP60 cytotoxic $CD4^+CD28^-2DS2^+$ clones directed us to study the expression of this protein. The results obtained clearly showed that perforin response of $CD4^+CD28^-2DS2^+$ cells was dependent on the expression of DAP-12, although interaction with hHSP60/MHC I complex was a prerequisite for generation of the activation signals. The clones that were only responded by IFN-γ release did not express DAP-12. In support of our observations, the role of DAP-12 in directing 2DS2-mediated cytotoxicity in $CD4^+CD28^-$ cells has previously been reported using redirected cytotoxicity methods (9). This and the finding that 2DS2 receptor is expressed largely in the absence of its inhibitory receptors on $CD4^+CD28^-$ cells have led to the thinking that these T cells may be capable of nonspecific targeting and killing. In this study, we propose that although KIR2DS2 and DAP-12 are the main components of $CD4^+CD28^-$ cell cytotoxicity, interaction of KIR2DS2 with hHSP60/class I complex may be a key requirement for the in vivo generation of this response. Our results, based on Ag-mediated T cell responses that largely mimic the vascular events, have for the first time shown that despite the deficient expression of the protective inhibitory KIRs, $CD4^+CD28^-2DS2^+$ cell reactivity is specific and discriminate. Our data may also suggest that DAP-12 expression can be an important late stage of $CD4^+CD28^-$ cell development that begins during the chronic phase and is significantly up-regulated with disease progression and changes in vascular environment. Evaluation of circulating levels of hHSP60-reactive $CD4^+CD28^-2DS2^+DAP\text{-}12^+$ may prove to be a useful marker for monitoring disease status.

Investigations of the hHSP60 reactivity of $CD4^+CD28^-2DS2^+$ cells prompted us to further define the nature of their perforin and IFN-γ responses. Blocking of MHC class II receptor on the hHSP60-pulsed target cells using anti-class II Abs significantly reduced IFN-γ response by $CD4^+CD28^-2DS2^+DAP\text{-}12^+$ clones. Inhibition of class II, however, did not affect perforin mRNA levels in response to the Ag. Similarly, blocking of the class I on the target cells or of KIR2DS2 on the effector clones radically decreased perforin transcription, but with no detectable effect on the IFN-γ. In line with present observations, another study using immobilized Abs has shown that KIR2DS2 on $CD4^+CD28^-$ was only capable of initiating cytotoxicity through DAP-12 (41). $CD4^+CD28^-DAP12^+$ cells were shown to be capable of KIR2DS2-mediated cytotoxicity following 2DS2 receptor cross-linking without any requirement for TCR stimulation (41). This supports the present work in its novelty, which analyzes cytotoxicity in the setting of 2DS2 ligand. Stimulation of TCR by hHSP60 in the presence of class I or KIR blocking in $2DS2^+DAP12^+$ clones did not result in up-regulation of perforin message, although it led to IFN-γ response. We have also shown that stimulation of $2DS2^+DAP12^-$ clones with hHSP60-pulsed cells did not generate perforin, but IFN-γ response by some clones. Using this system, which mimics in vivo Ag-mediated T cell stimulation, we have demonstrated that KIR and TCR may function independently on $CD4^+CD28^-$ cells, and TCR recognition of hHSP60 may not on its own render lysis of the presenting cells. This may be due to different degree or affinity of interaction between the KIR or TCR and the presented hHSP60 peptides, or, equally, other signaling mechanisms may be involved.

To our knowledge, this is the first investigation of the cellular and molecular components responsible for Ag-specific $CD4^+CD28^-$ cell activation in CAD patients. Our findings shed light on the specific Ag-receptor interactions and pathways involved in the generation of proinflammatory and effector responses that may lead to ACS. Our findings may have implications for novel molecular and cellular therapeutic approaches to CAD, and may also help in developing new strategies of the monitoring of the CAD progression and plaque instability in patients with CAD.

Disclosures

The authors have no financial conflict of interest.

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