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Cutting Edge: CD40-Induced Expression of Recombination Activating Gene (RAG) 1 and RAG2: A Mechanism for the Generation of Autoaggressive T Cells in the Periphery

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It has been speculated that autoimmune diseases are caused by failure of central tolerance. However, this remains controversial. We have suggested that CD40 expression identifies autoaggressive T cells in the periphery of autoimmune prone mice. In this study, we report that CD40 was cloned from autoaggressive T cells and that engagement induces expression and nuclear translocation of the recombinases, recombination activating gene (RAG) 1 and RAG2 in the autoaggressive, but not in the nonautoaggressive, peripheral T cell population. Furthermore, we demonstrate that CD40 engagement induces altered TCR Vα, but not VB, expression in these cells. Therefore, CD40-regulated expression of RAG1 and RAG2 in peripheral T cells may constitute a novel pathway for the generation of autoaggressive T cells. The Journal of Immunology, 2003, 170: 3455–3459.

During a normal immune response, CD4+ T cells recognize foreign Ags through expression of Ag-specific TCR molecules. The generation of a functional TCR repertoire, that is T cells able to respond to diverse Ags, involves rearrangement of the TCR genes. This occurs through activation of recombination activating gene (RAG) 1 and 2 (1) early in T cell development. RAG1 and RAG2 translocate to the nucleus and form a protein complex that induces rearrangements of the V, D, and J regions of the TCR genes. This is a site-specific recombination process resulting in the diversification of Ag receptors (2).

Immunological dogma holds that RAGs are permanently inactivated once T cells exit the thymus. However, RAG expression has been reported in peripheral T cells (3–5). For example, peripheral NOD CD8+ T cells stimulated by repeated Ag exposure in vivo and in vitro induced RAG expression and subsequent TCR rearrangement (5). Peripheral RAG activation leading to altered TCR expression would necessarily result in nonthymically selected, potentially autoaggressive T cells.

We recently identified a highly autoaggressive peripheral CD4+ T cell subset, characterized by the expression of CD40 (6). In diabetic nonobese diabetic (NOD) mice, CD4+ CD40+ T cells represent 60% of the CD4+ compartment, but in age-matched BALB/c mice these cells represent ~5% of the CD4+ population (6). Furthermore, as NOD mice develop toward diabetes, the CD4+CD40+ T cell population expands (D. M. Waid, G. Vaitaitis, and D. H. Wagner Jr., manuscript in preparation). Earlier studies demonstrated that peripheral CD4+CD40+ T cells can alter TCR Vα surface expression when CD40 is engaged (7). Because these events occur in the periphery, this suggests a mechanism by which autoaggressive T cells can be generated.

CD40 was first described on APC (8). CD40 signals B cells to activate RAG1 and RAG2 in a RAG-dependent manner, in primary peripheral T cells (7). Importantly, change in Vα expression was induced by CD40 cross-linking of the 3A9 T cell hybridoma demonstrating that CD40 signals induced altered TCR expression and not clonal expansion of specific T cells (7). Peripheral CD40+ T cells occur mainly in the CD4low subset of CD4+ T cells in NOD mice (6) and CD4low, but not CD4high, peripheral NOD T cells readily transfer insulitis and eventually diabetes when injected into NOD.scid recipients (6). Our findings, and the recent reports that RAGs can be reactivated in the periphery (3–5), suggested that CD40 signals may induce RAG expression, leading to altered TCR expression, in peripheral CD4+CD40+ T cells.

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3 Abbreviations used in this paper: RAG, recombination activating gene; NOD, nonobese diabetic; CF, cytoplasmic fraction; NE, nuclear extract; RT, reverse transcription.
This ultimately may be responsible for the generation of autoaggressive T cells in the periphery.

In this study, we report that CD40 was cloned from an autoaggressive T cell clone and from purified, primary NOD splenic T cells. We demonstrate that these cells can be induced through CD40 engagement to alter surface expression of TCR Vβ. Significantly, expression and nuclear translocation of RAG1 and RAG2 is regulated by CD40 signals. Therefore, CD40+ T cells constitute a population of T cells capable of reactivating RAGs peripherally, potentially allowing the generation of autoaggressive T cells.

Materials and Methods

Mice

NOD mice were from The Jackson Laboratory (Bar Harbor, ME) or Taconic Farms (Germantown, NY) and were maintained under pathogen-free conditions at the Webb-Waring Institute for Cancer, Aging, and Antioxidant Research, Institutional Animal Care and Use Committee-approved facility (Health Sciences Center, University of Colorado, Denver, CO).

Antibodies

CyChrome-conjugated anti-CD4 (clone H129.19) and anti-TCR-Vα Ab, as listed (see Table I), were from BD Biosciences (San Diego, CA). Anti-RAG1, sc-5599, and anti-RAG2, sc-7623, Ab as well as secondary HRP-conjugated Ab, were from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated anti-CD40 (clone 1C10; Ref. 10) and anti-Fcγ Ab (clone 2.4G2; Ref. 11) were produced in-house. Anti-actin Ab, A2066, was from Sigma-Aldrich (St. Louis, MO).

Cell culture

BDC-2.5 cells were cultured as described (12). NOD splenic T cells were purified over nylon wool columns then, in some experiments, sorted on CD4low/hi using a MoFlo flow cytometer (Fort Collins, CO). Purified T cells were cultured in RPMI 1640 medium with 10% FCS.

cDNA cloning and sequencing

NOD T cells were cultured as above. BDC-2.5 T cells were cultured as described (12), then allowed to come to rest for 2 wk. Total RNA was extracted using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA) then was reverse-transcribed. Using intron-spanning primers: CD40 sense, 5'-CTCACGGAAGCTGTTCTCC-3'; RAG1 antisense, 5'-CGATAGGGTCTCGGATGATCC-3'. The PCR product was sequenced, and found to have complete homology to the CD40 cDNA cloned from BDC-2.5 and NOD splenic T cell mRNA.

Flow cytometry

Spleenic T cells were isolated from female, NOD mice (12–16 wk of age) as above, then CD40 cross-linked, or not, for 48 h. BDC-2.5 T cells were allowed to come to rest for 2 wk then were CD40 cross-linked every 4 days for 2 wk in the absence of APC. After cross-linking, cells were treated with anti-FcγIII/IIIR Ab then stained with CyChrome-conjugated anti-CD4, biotinylated anti-CD40 (followed by washes then PE-streptavidin) and FITC-conjugated Ab to TCR Vα2, Vα3.2, Vα8, Vβ4, or Vβ8. Flow cytometry was done on a BD Biosciences FACSCalibur (San Jose, CA).

Western blot

Cells from 6- to 9-wk-old NOD splenens were purified as above. T cells were determined by flow cytometry to be >97% CD3+ and then cross-linked with biotinylated Anti-CD40 Ab, or not, followed by streptavidin. Controls received isotype control Ab. Protein was extracted as described below then 20 μg of cytoplasmic (CF) or 10 μg of nuclear (NE) protein were electrophoresed according to Laemmli (13). Protein was transferred to a polyvinylidene difluoride membrane and RAG1 levels were analyzed. The blots were stripped and reprobed for RAG2. Blots of CF were further stripped and probed for actin.

Cytoplasmic and nuclear cell extract preparations

Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Igepal CA630, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.2 mM PMSF) then homogenized. Nuclei were pelleted at 16,000 × g and the supernatant (CF) was frozen. Nuclei were washed once in hypotonic buffer then resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 25% glycerol, 1.5 mM MgCl₂, 340 mM KCl, 1 mM DTT, protease inhibitors as above) and ultraconcentrated for 10 s. Insoluble matter was pelleted at 20,000 × g and the supernatant (NE) was frozen.

Semiquantitative RT-PCR

Cultured cells were cross-linked, or not, with CD40 Ab as described earlier. RNA was prepared and 0.1–1 μg were reverse transcribed as above. For PCR, one-hundredth to one-twentieth of the resulting cDNA was PCR-amplified using RAG1- or RAG2-specific primers. RAG1 sense, 5’-TCACTCGAGA CAGTCCCTCC-3’; RAG1 antisense, 5’-CGATAGGGTCTCGGATGATCC-3’. RAG2 sense, 5’-CGAGGGGAAAAACGCCAAATGC-3’; RAG2 antisense, 5’-CCTGGCGGAGAAGTGGTGGTCC-3’. PCR product was cloned, sequenced, and found to have complete homology to the RAG1 and RAG2 sequences at the beginning of CD4low T cell subpopulation, we cloned CD40 mRNA using RT-PCR.

A PCR product of the predicted CD40 cDNA size, 952 bp, was produced in both the T cell clone and the purified, primary CD4low T cells (Fig. 1, arrow). This product was cloned, sequenced, and found to have complete homology to the CD40 sequence reported for B cells (GenBank accession no. M83312). To ensure that the product in the BDC-2.5 sample did not stem from irradiated APC used in the culturing of the BDC-2.5 T cells, irradiated APC were maintained in culture alone but otherwise under the same conditions as when culturing BDC-2.5. As expected, only degraded RNA could be prepared and no RT-PCR product was obtained, after irradiation, from the APC.

Results and Discussion

CD40 cDNA cloned from BDC-2.5 and NOD splenic T cell mRNA

We previously demonstrated the presence of CD40 on the surface of CD4lowCD3+ T cells, autoaggressive, peripheral T cells and on several diabetogenic T cell clones including the NOD CD4+/CD8− Th1 clone, BDC-2.5 (6). To confirm the presence of CD40 on the T cell clone and the CD4low T cell subpopulation, we cloned CD40 mRNA using RT-PCR.

A PCR product of the predicted CD40 cDNA size, 952 bp, was produced in both the T cell clone and the purified, primary CD4low T cells (Fig. 1, arrow). This product was cloned, sequenced, and found to have complete homology to the CD40 sequence reported for B cells (GenBank accession no. M83312). To ensure that the product in the BDC-2.5 sample did not stem from irradiated APC used in the culturing of the BDC-2.5 T cells, irradiated APC were maintained in culture alone but otherwise under the same conditions as when culturing BDC-2.5. As expected, only degraded RNA could be prepared and no RT-PCR product was obtained, after irradiation, from the APC.

CD40 induces altered TCR Vβ, but not Vβ8, surface expression in NOD spleen and BDC-2.5 T cells

We have shown that peripheral T cells of several autoimmune-prone strains of mice, e.g., SWR/J, MRL, and NZB × NZW, and cells of the 3A9 T cell hybridoma alter their TCR Vα surface expression in response to CD40 cross-linking (7). We have also shown that the CD40+ T cell population occurs almost exclusively in the CD4low portion of peripheral T cells in diabetes-prone NOD mice (6). In this study, we show that CD40 cross-linking caused a 10-fold increase in Vα3.2 and a 2.5-fold increase in Vα8 expression within the CD40+CD4low population compared with untreated cells (Table I). Vα8 expression was not significantly affected by CD40 cross-linking.

FIGURE 1. RT-PCR of CD40 expression in BDC-2.5 and NOD T cells. RT-PCR was performed on RNA extracted from BDC-2.5 or NOD T cells using CD40-specific, intron-spanning primers. A band of the expected size, 952 bp, was detected. As controls, nonirradiated (Non-irr.) and irradiated (Irr.) APC RNA was included.
on CD40−CD4low T cells was not affected. As predicted, CD40−CD4high T cells did not change their Vα expression in response to CD40 cross-linking. Interestingly, the Vβ molecules examined did not change in either the CD4low or the CD4high cells. The numbers reported are within the gated CD4high T cells. The numbers reported are within each gated population, that is CD40/− versus CD40/+. An important question is whether CD40 engagement induced selective clonal expansions or resulted in altered Tα expression. Clonal expansions are not likely because substantial expression of both RAG proteins simultaneously as well as their populations and therefore do not reflect changes in total T cells. Therefore, T cells were gated by exclusion of low forward scatter. Experiments were done three times; n.d., not done.

CD40 cross-linking of primary NOD T cells and the BDC-2.5 diabetogenic T cell clone induces alteration in Vα expression

| NOD Primary Untreated CD4low | NOD Primary Anti-CD40- Treated CD4high | NOD Primary Untreated CD4high | NOD Primary Anti-CD40- Treated CD4high | BDC2.5 Untreated | BDC2.5 Anti-CD40-Treated |
|-----------------------------|----------------------------------------|-----------------------------|----------------------------------------|-----------------|--------------------------|
| Vβ4 1.35 ± (1.12)           | 1.4 ± (0.17)                           | 2.16 ± (0.25)               | 2.2 ± (0.61)                           | n.d.            | n.d.                     |
| Vβ8 7.6 ± (0.32)            | 7.1 ± (0.53)                           | 3.46 ± (0.24)               | 3.4 ± (0.37)                           | n.d.            | n.d.                     |
| Vα2 0.93 ± (0.2)            | 0.6 ± (0.4)                            | 2.8 ± (0.4)                 | 2.6 ± (0.5)                            | 1.2 ± (0.2)     | 30.3 ± (2.1)              |
| Vα3.2 1.85 ± (0.25)        | 16± ± (1.7)                           | 4.9 ± (0.12)                | 5.06 ± (0.15)                          | 1.2 ± (0.5)     | 6.3 ± (3.1)               |
| Vα8.3 3.0 ± (0.4)          | 7.9 ± (0.3)                            | 6.5 ± (0.3)                 | 6.5 ± (0.045)                          | 0.9 ± (0.3)     | 8.7 ± (5.01)              |

*R Splenic T cells from three NOD mice, age 12 wk, were purified over nylon wool. BDC-2.5 T cells were maintained as described (12). Primary T cells were >96% TCR+, T cells were treated with biotin-anti-CD40 followed by streptavidin to cross-link. Primary T cells were treated for 48 h and BDC-2.5 every 4 days for 2 wk. T cells were stained with anti-CD4 (CyChrome), biotin-anti-CD40 (then PE-streptavidin), and Vα or Vβ Ab (FITC) as listed in the table. Cells were analyzed by flow cytometry. Numbers are percentage Vα+ or Vβ+ T cells. Bold numbers in anti-CD40-treated columns are significantly different (*p < 0.01, by χ² test) compared to untreated T cells. In primary cells, numbers compare the effect of anti-CD40 treatment on CD4low versus CD4high T cells and percentages are within each gated population.

Possible mechanisms for alteration of TCR Vα expression include the re-expression of the RAG products in the periphery as demonstrated by McManus et al. (3, 4) and Serra et al. (5). Because of our observations that engagement of CD40 on splenic T cells alters surface expression of TCR Vα, and that Vα changes could not be induced in TCR transgenic mice on a RAG−/− background (7), we examined whether CD40 engagement affects RAG1 and RAG2 protein levels in peripheral T cells.

Expression of a product of the reported RAG1 size, 119 kDa, was not detected in the cytoplasmic fraction of NOD splenic T cells (Fig. 2A). However, upon CD40 cross-linking for 6 h, RAG1 was detected in the nuclear fraction (Fig. 2A, arrow). When RAG2 was examined, a band of the reported size, 59 kDa, was present in the cytoplasmic fraction of all samples suggesting constitutive expression of RAG2 (Fig. 2B, arrow). However, CD40 cross-linking increased RAG2 levels in the cytoplasm at 6 h. Nuclear localization of RAG2 was detected only after CD40 cross-linking for 6 h in NOD T cells (Fig. 2B). Recombination activity requires that RAG1 and RAG2 form a protein complex (16) and, importantly, RAG1 and RAG2 must translocate to the nucleus to access the TCR genes. These data clearly demonstrate that CD40 cross-linking provides signals that cause the expression of both RAG proteins simultaneously as well as their translocation into the nucleus fulfilling these criteria.

**RAG1 and RAG2 mRNA expression is regulated by CD40 signals**

We performed RAG1- and RAG2-specific RT-PCR on RNA prepared from NOD splenic T cells. RAG1 mRNA was present in untreated NOD peripheral T cells and expression was slightly increased in response to CD40 cross-linking (Fig. 3A, B).
duced RAG-expression in CD4low vs CD4high primary T cells. We speculated that this could lead to the generation of autoaggressive T cells in the periphery, that is, nonthyroid-bearing T cells, as opposed to CD4highCD40+T cells, transfers diabetes to NOD.scid animals (6), we compared CD40-induced RAG-expression in CD4low vs CD4high primary T cells. NOD splenic T cells were sorted into CD4low vs CD4high populations. CD4low T cells, the predominant CD40+T cell subpopulation (6), were induced to express RAG1 and RAG2 mRNA in response to CD40 cross-linking while RAG expression for either mRNA. It has been reported that engagement of TCR prevents RAG activation in thymocytes (18). In addition, presence of RAG1 and 2 mRNA, but untreated cells in unsorted experiments showed the presence of RAG1 and RAG2 mRNAs, not detectable after CD40 cross-linking of resting cells from this highly diabetogenic T cell clone (Fig. 4, B). Taken together, these data demonstrate that CD40 cross-linking on T cells affects not only RAG1 and RAG2 protein translation and translocation, but also affects mRNA transcription as well. By contrast, CD4lowCD40+T cell subpopulation of NOD splenic T cells, as opposed to CD4highCD40+T cell transfers diabetes to NOD.scid animals (6), we compared CD40-induced RAG-expression in CD4low vs CD4high primary T cells. NOD splenic T cells were sorted into CD4low vs CD4high populations. CD4low T cells, the predominant CD40+T cell subpopulation (6), were induced to express RAG1 and RAG2 mRNA in response to CD40 cross-linking while RAG expression was not detected in CD4high T cells (Fig. 4, A and B). As a control, CD3 was cross-linked but showed no induction of expression for either mRNA. It has been reported that engagement of TCR prevents RAG activation in thymocytes (18). In the absence of RT, no product was obtained (data not shown).

The fact that RAG expression occurred only in the CD4lowCD40+ population suggests that by re-expressing RAGs in CD4lowCD40+ T cells, autoimmune prone animals, such as NOD, have a high percentage of CD4lowCD40+ T cells that increases with age (D. M. Waid, G. Vaitaitis, and D. H. Wagner, Jr., manuscript in preparation). Therefore, the likelihood of generating autoaggressive T cells is high. Activation of RAGs in CD4lowCD40+ T cells of nonautoimmune prone animals, such as BALB/c, has not been ruled out but if it occurs, the likelihood of generating autoaggressive T cells would be substantially less because these animals have much fewer CD4lowCD40+ T cells. Additionally, the BALB/c CD4low CD40+ T cell population does not expand with age as in NOD (D. M. Waid, G. Vaitaitis, and D. H. Wagner, Jr., manuscript in preparation). As yet, no means of activating RAG in peripheral CD4high T cells has been reported.

The fact that untreated cells in unsorted experiments showed the presence of RAG1 and 2 mRNAs, but untreated cells in sorted cell experiments did not, may be explained by the fact that in the former all T cell populations were incubated together. This could enable the interaction between CD40 ligand-bearing T cells and CD40-bearing T cells that may be separated by compartments in vivo.

We confirmed the ability of CD40 to activate RAG using a T cell clone. RAG RT-PCR was performed on RNA from untreated or CD40 cross-linked BDC-2.5. RAG1 and RAG2 were only detectable after CD40 cross-linking of resting cells from this highly diabetogenic T cell clone (Fig. 4, C and D). Because this is a well-described T cell clone this shows that the results are T cell-specific and rules out any possible B cell contamination. Again, irradiated APC, used in the culturing of the BDC-2.5 T cells, when maintained in culture alone but otherwise under the same conditions as when culturing BDC-2.5, yielded only degraded RNA and no RT-PCR product (data not shown). In the absence of RT, no products could be detected in either the RAG1-specific or the RAG2-specific PCR experiments (data not shown).

The data presented in this report suggest a mechanism to generate autoimmune T cells post thymic selection. This means that in addition to breach of central tolerance, autoimmune T cells may be generated in the periphery and thus bypass thymic selection altogether. Diseases like type 1 diabetes have been closely linked to viral infections, e.g., coxsackie virus (19, 20). During infection, CD154 levels are even higher in autoimmune T cells (21). Furthermore, CD154 levels are even higher in autoimmunity (22), increasing the potential for interaction between CD40 and its ligand (CD154) during autoimmune disease. Therefore, these events may alter the specificity, even if slightly, of the original TCR. This would be advantageous if the results are a TCR with higher affinity for invading pathogen Ags, e.g.,
coxsackie virus, as has been suggested to occur in T cell epitope spreading (23, 24). In a nonautoimmune prone animal, CD40+ peripheral T cells may serve as a "safety" pool of T cells from which TCR molecules can be generated to respond to an Ag for which there was previously no, or only a low affinity, TCR present. In all probability some of these changes would result in a TCR with specificity for self-Ags, for example pancreatic islet β-cell Ags such as occur in type 1 diabetes. When the numbers of the CD40+ CD4+ T cells are increased, as in autoimmune prone animals (7), the risk of generating a self-reactive TCR is increased as well. In light of our findings and the recent report that peripheral CD8+ T cells can perform a similar feat (5) a new picture of how the peripheral T cell repertoire is shaped is beginning to emerge.

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