RelA regulates the survival of activated effector CD8 T cells

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Received 04.10.04; revised 14.3.05; accepted 11.4.05; published online 27.5.05
Edited by JA Trapani

Abstract

Nuclear factor of kappa B (NF- κ B) transcription factors are critical regulators of T-cell activation and survival. The relative contribution of individual NF- κ B members to these processes remains elusive. We investigated the role of RelA in the regulation of CD8 T-cell activation. We overexpressed, in mature CD8 T cells, a transactivation domain-deficient RelA molecule (p65TAD). We show that p65TAD forms homo- and heterodimers with p50 that bind κ B sites and selectively inhibit RelA-dependent transactivation. Expression of p65TAD does not affect initial activation or cell cycle progression but induces the death of activated CD8 T cells *in vitro* and *in vivo*. However, the long-term survival of resting effector CD8 T cells seems not to be affected by p65TAD expression. Collectively, our results indicate that RelA is a critical regulator of survival of proliferating CD8 T cells but may be dispensable for the survival of resting effector T cells. *Cell Death and Differentiation* (2005) 12, 1398–1406.

doi:10.1038/sj.cdd.4401673; published online 27 May 2005

Keywords: T lymphocytes; transcription factors; cellular activation; cellular proliferation; memory

Abbreviations: Ag, antigen; NF- κ B, nuclear factor of kappa B; p65FL, p65 full length; p65TAD, p65 transactivation domain deficient; PKA, protein kinase A; RHD, Rel homology domain; TAD, transactivation domain; TCR, T-cell receptor

Introduction

The nuclear factor of kappa B (NF- κ B) family of transcription factors is composed of NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), cRel and RelB that bind to κ B motifs upon homo- or heterodimerization.¹ Under normal conditions, NF- κ B dimers are retained in the cytoplasm by the I κ B inhibitory protein that following activation is phosphorylated and degraded, thus releasing the NF- κ B complexes that can translocate to the nucleus.¹ In addition, post-translational

modifications contribute to transcriptional activity of NF- κ B complexes.^{2–4}

NF- κ B-binding sites are present in many genes involved in T-cell activation and survival, in cell cycle control and T-cell effector function. As a consequence, NF- κ B regulates multiple aspects of T-cell activation. Indeed, mature CD4⁺ or CD8⁺ T cells isolated from mice transgenic for a degradation-resistant I κ B α , which completely blocks the nuclear translocation of p65/p50 and cRel complexes, present a proliferative defect and an increased susceptibility to activation-induced apoptosis that is independent of IL-2/IL-2R α gene regulation.^{5,6} IL-2 production was also impaired in T cells expressing such an inhibitor of I κ B α . Furthermore, activation and cell cycle progression were not affected by adenoviral expression of the same constitutive inhibitor of I κ B α in mature T cells, but T-cell death occurred following S phase entry.⁷ NF- κ B may also regulate the survival of mature resting T cells.^{5,8–10}

Despite the critical role of NF- κ B in T-cell activation and survival, the relative contribution of individual NF- κ B members in these processes has remained elusive. In mature T cells, T-cell receptor (TCR) and CD28 stimulation primarily induce nuclear translocation of p65/p50 heterodimers while homo- and heterodimers of cRel with p65 and p50 are less abundant and their appearance may be delayed.¹¹ Despite this predominance, loss of RelA has only a moderate effect on T-cell activation as does cRel deficiency. Thus, RelA^{−/−} or p50^{−/−} T cells present a moderate proliferative defect, without increased apoptosis or impairment in IL-2 production.^{12,13} On the other hand, cRel-deficient T cells do not produce IL-2 following activation and, as a consequence, present a significant proliferative defect *in vitro*.^{13–16} However, CD4 T cells deficient for both cRel and p50 show a major impairment of T-cell activation and cell cycle entry, reduced IL-2 and CD25 expression and increased apoptosis.¹⁷ The mild phenotype of individual knock out mice as compared to that of mice deficient for both p50, cRel and p65 containing complexes suggested that deficiency in one subunit might be compensated by other NF- κ B complexes. In addition, the observed phenotype of NF- κ B-deficient T cells may in part result from perturbed T-cell development.^{5,18,19}

In this study, we re-evaluated the role of RelA in CD8 T-cell activation. To overcome side effects that may occur during T-cell development and the problem of possible compensation by other NF- κ B dimers, we overexpressed in mature CD8 T cells, a truncated RelA molecule (p65TAD, p65 transactivation domain deficient) that lacks the two transactivation domains (TAD) normally present in RelA but retains the Rel homology domain (RHD). We show here that TAD-deficient RelA selectively inhibits RelA-dependent transactivation. When overexpressed in CD8 T cells, TAD-deficient RelA induces premature cell death of activated CD8 T cells both *in vitro* and *in vivo* but does not impair initial T-cell activation. Collectively, our results show that RelA is a critical regulator of survival of activated CD8 T cells but may be dispensable for the survival of resting effector CD8 T cells.

Results

Expression of transactivation domain-deficient RelA in CD8 T cells

To evaluate the role of RelA in CD8 T-cell activation and survival, we wished to generate a molecule that, when expressed in mature T cells, would selectively inhibit RelA-dependent transactivation. Previous studies have indicated that TAD-deficient RelA molecules may act as transdominant inhibitors of NF- κ B-dependent transactivation.^{20,21} We therefore engineered a similarly truncated RelA molecule (p65TAD) lacking the two transactivation domains normally present in RelA while maintaining the RHD, the nuclear localization sequence and the protein kinase A (PKA) phosphorylation site. Such truncated RelA molecules would thus maintain homo- and heterodimerization, interaction with histone deacetylases and CBP/p300 and DNA-binding activity, but should lack transactivation properties.^{2,3,22} To determine whether indeed p65TAD may act as an inhibitor of κ B-dependent transcription, we coexpressed in 293 T cells p65TAD or, as control, full-length RelA (p65FL) and an NF- κ B reporter construct in which expression of the firefly luciferase gene is under the control of NF- κ B sites from the Ig κ enhancer element. As expected, p65TAD did not show significant NF- κ B transcriptional activity in this assay (Figure 1). Furthermore, p65TAD inhibited in a dose-dependent fashion the transcriptional activity of cotransfected p65FL indicating that p65TAD inhibits RelA-dependent transactivation (Figure 1).

To study the role of RelA in T-cell responses to antigenic stimulation, we generated retroviruses that express either p65TAD or p65FL using a bicistronic retroviral vector containing an IRES-GFP that permits the isolation of infected cells by FACS sorting. The CD8 T cells were isolated from Désiré TCR transgenic mice (referred hereafter as CD8⁺Des⁺ T cells) and are specific for the alloantigen H-2K^b. The different retroviral constructs were used to infect 22 h *in vitro* activated CD8⁺Des⁺ T cells. Initial T-cell activation and G0 to G1

transition, therefore, proceed normally, in the absence of the transgene. Thus, p65TAD expression begins when nuclear translocation of the different NF- κ B complexes is still high (not shown). Truncated RelA therefore likely competes with all endogenous NF- κ B complexes induced by TCR and CD28 stimulation.

Infection of CD8⁺Des⁺ T cells with the different retroviral constructs led to overexpression of the corresponding molecules to a comparable level without significant change in the level of expression of endogenous p65, p50 and cRel (Figure 2a). We examined by EMSA whether p65TAD translocates to the nucleus of activated T cells and shows DNA-binding activity. Two complexes, N1 and N2, were detected in mock or p65FL-infected CD8⁺Des⁺ T cells (Figure 2b). Supershift experiments with extracts from mock-infected cells indicated that the N1 complexes mainly contained p65 homo- and heterodimers with p50, while the N2 complexes correspond to p50 homodimers (Figure 2b). In contrast to mock-infected cells, CD8⁺Des⁺ T cells overexpressing p65FL demonstrated increased nuclear representation of the p65-containing N1 complexes, as compared to the N2 complex (Figure 2b). Nuclear extracts of CD8⁺Des⁺ T cells overexpressing p65TAD contain low level of N1 and predominantly express a faster migrating N3 complex (Figure 2b). Despite numerous attempts, we did not succeed in separating the N2 and N3 complexes in p65TAD-infected cells. Expression of the N2 complex, although at low levels, is nevertheless suggested by the persistence of a complex with a mobility corresponding to the N2 complex in anti-p65 supershifts, which disappeared in anti-p50 supershifts (Figure 2b). Generation of the N1 complex present in p65TAD-transfected cells was inhibited by anti-p65 and p50 Ab and only weakly by cRel-specific Ab. The N1 complex detected in CD8⁺Des⁺ T cells overexpressing p65TAD, thus likely corresponds to p65 and cRel homo- and heterodimers with p50. The anti-p65 Ab completely inhibited the generation of the N3 complex, which was only partially inhibited by anti-p50 Ab, suggesting that the N3 complex found in p65TAD-expressing CD8⁺Des⁺ T cells contains homo- and heterodimers of RelA with p50. The faster migration of the N3 complexes as compared to conventional p65-containing complexes indicates that they are exclusively composed of TAD-deficient RelA.

TAD-deficient RelA inhibits transactivation from some κ B sites

The different NF- κ B complexes have distinct DNA-binding specificity and affinities *in vitro*.²³ Some κ B sequences within a defined promoter may thus be selective for some subunits *in vivo*. We therefore determined whether TAD-deficient RelA may inhibit selectively RelA-dependent transcription in CD8 T cells. We examined the regulation of 16 genes known to contain κ B sites in their promoter sequences that bind RelA-containing complexes *in vitro* and are expressed in CD8 T cells.^{24–35} Further analysis of T cells with impaired NF- κ B transactivation suggests, however, that these genes exhibit distinct κ B-dependent expression. Based on these studies, we delineated five groups of genes that may be regulated by

Figure 1 TAD-deficient RelA inhibits RelA-dependent transactivation. 293 T cells were transfected with the indicated amount (μg) of NF- κ B-Luc plasmid together with a combination of empty vector (Mock), vector encoding for full length (p65FL) or TAD-deficient RelA (p65TAD), as indicated. Luciferase activity was read 24 h after transfection. The mean relative luciferase activity of three independent transfections performed in one representative experiment out of three performed is presented

Figure 2 Expression of TAD-deficient RelA in CD8 T cells. CD8⁺Des⁺ T cells were stimulated *in vitro* for 22 h and then infected with either empty retrovirus (mock) or retrovirus encoding full-length (p65FL) or TAD-deficient (p65TAD) RelA. Infected cells were FACS sorted based on GFP expression and cytoplasmic and nuclear extracts prepared 3 (b) or 4 (a) days after retroviral infection. (a) Cytoplasmic extracts were analyzed by Western blotting using Ab specific for the amino-terminal domain of p65, cRel, IκBα, IκBβ, p50 or actin, as indicated. The different blots were quantified and the relative value, corrected for loading differences based on the actin signal, is expressed as ratio of the mock control. FL = 65 kDa form of p65 and TAD = 45 kDa truncated form of p65. (b) Nuclear extracts were analyzed by EMSA with an NF-κB-binding probe in the absence or presence of p50, p65 amino-terminal domain and cRel-specific Ab, as indicated. The different complexes N1, N2 and N3 are indicated by an arrowhead. Two different exposures of the same EMSA are shown to highlight the weakly expressed complexes. One representative experiment out of three performed is shown

different NF-κB complexes *in vivo*. Group I genes p65, cRel and Bcl-2 are not affected in T cells expressing a degradation-resistant IκBα or deficient for both p50 and cRel and are thus likely not regulated by NF-κB in this cell type.^{5,17,19} Expression of these genes is not downmodulated in CD8⁺Des⁺ T cells overexpressing p65TAD (Table 1). Likewise, expression of cRel or Bcl-2 is not altered by p65FL overexpression (Table 1). The increased level of p65 protein observed in p65FL-transfected cells reflects both endogenous and overexpressed transgenic RelA, which cannot be distinguished in this experimental system. The involvement of NF-κB in the regulation of group II genes is still unknown since expression of these genes in T cells with deregulated NF-κB transcriptional activity has not yet been evaluated. Within that group of molecules, only FasL expression is slightly downmodulated by p65TAD overexpression though p65FL has no effect on the expression of this molecule (Table 1). This slight effect of p65TAD suggests that RelA, although not essential, may potentiate either directly or indirectly the transcriptional activity of the complexes that bind to the FasL promoter. Among genes that are extinguished in T cells with impaired NF-κB activity, we found that expression of Bcl-x_L is significantly reduced following p65TAD overexpression (Table 1, group III). Genes that are repressed in cRel but not in RelA-deficient T cells were not significantly affected by p65TAD or p65FL overexpression (Table 1, group IV). Finally, expression of IκBα and CD69, two genes that are tightly regulated by RelA, is significantly downmodulated in CD8⁺Des⁺ T cells transduced with p65TAD and upregulated in cells transduced with p65FL (Table 1, group V and Figure 2a). We confirmed that the

effect of p65TAD on Bcl-x_L, CD69 and IκBα expression was at the transcriptional level by showing that expression of the corresponding mRNA was reduced in CD8⁺Des⁺ T cells transduced with p65TAD as compared to that of mock or p65FL-expressing cells (Table 1).

The contrasting effect of p65TAD on the different genes analyzed suggests that transactivation domain-deficient RelA may selectively inhibit transcription from RelA-regulated κB sites.

Activated CD8⁺Des⁺ T cells overexpressing TAD-deficient RelA are short lived

We further evaluated the impact of p65TAD overexpression on T-cell responses *in vitro*. Thus, 22 h-activated CD8⁺Des⁺ T cells were infected with retroviral particles expressing truncated or full-length p65 or were mock infected, as controls. The GFP⁺ cells were FACS sorted 3 days postinfection and clonal expansion and cell death were evaluated, after an additional 48 h culture in IL-2. As shown before, expression of CD25 is comparable for the different infected populations (Table 1). Overexpression of p65FL had no effect on the expansion or survival of the CD8⁺Des⁺ T cells (Figure 3a and b). In contrast, expression of p65TAD led to a global reduction of the number of CD8⁺Des⁺ T cells recovered at the end of the 48 h-culture period (Figure 3a). This reduction in cell recovery was not due to cell cycle arrest but rather to increased cell death. Indeed, cell death was augmented for p65TAD-expressing CD8 T cells, which showed normal cell cycle entry or cell cycle progression (Figure 3b and c).

Table 1 TAD-deficient RelA inhibits transactivation from some κB sites

	Ratio of mock (mean ($\pm$ S.D.))			
	Protein		mRNA ^a	
	p65FL	p65TAD	p65FL	p65TAD
Group I: NF-κB independent				
p65 ^b	7.65 ($\pm$ 1.59)	0.93 ($\pm$ 0.07)		
cRel ^b	1.3 ($\pm$ 0.25)	0.8 ($\pm$ 0.21)		
Bcl-2 ^b	1.28 ($\pm$ 0.71)	0.77 ($\pm$ 0.07)		
Group II: unknown				
I $\kappa B\beta$ ^b	3.2 ($\pm$ 0.19)	0.9 ($\pm$ 0.11)		
ICAM1 ^c	1.8 ($\pm$ 0.3)	1.3 ($\pm$ 0.16)		
Fas ^c	1.75 ($\pm$ 0.22)	1.44 ($\pm$ 0.17)		
FasL ^c	1.03 ($\pm$ 0.35)	0.78 ($\pm$ 0.07)		
Group III: NF-κB dependent				
Bcl-xL ^b	0.95 ($\pm$ 0.05)	0.58 ($\pm$ 0.08)	1.93 ($\pm$ 0.53)	0.57 ($\pm$ 0.14)
TAp73			1.02 ($\pm$ 0.03)	1.24 ($\pm$ 0.35)
Bcl-3 ^b	1.41 ($\pm$ 0.52)	1 ($\pm$ 0.47)		
IFN- γ ^d	1.13 ($\pm$ 0.16)	0.96 ($\pm$ 0.04)		
Group IV: cRel dependent				
CD25 ^c	1.15 ($\pm$ 0.14)	1.09 ($\pm$ 0.09)		
IL-2 ^d	1.31 ($\pm$ 0.28)	0.83 ($\pm$ 0.04)		
Bfl-1			2.34 ($\pm$ 0.03)	1.57 ($\pm$ 0.45)
Group V: RelA dependent				
I $\kappa B\alpha$ ^b	9.3 ($\pm$ 3.43)	0.38 ($\pm$ 0.08)	5.14 ($\pm$ 2.07)	0.57 ($\pm$ 0.1)
CD69 ^d	1.3	0.59	1.69 ($\pm$ 0.14)	0.64 ($\pm$ 0.09)

CD8⁺Des⁺ T cells were transduced with mock, p65FL or p65TAD retroviral particles as described in Figure 2. Unsorted cells were analyzed by FACS staining on day = 3 or 4 postinfection. Alternatively, GFP⁺ T cells were FACS sorted on day = 3 postinfection and total RNA or cytoplasmic protein extracts prepared. Relative values are expressed as ratio of the mock control. The mean ratio ($\pm$ standard deviation (S.D.)) of at least three measurements is shown except for Bcl-2 and Bcl-xL protein level which was analyzed twice. Values that are significantly different from the mock control ($P < 0.05$) are underlined. ^amRNA expression levels were measured by real-time PCR as described in the Material and Method section. ^bProtein expression level was determined by Western blotting as described in the Material and Methods section. ^cProtein expression level was determined by FACS staining, which showed a single homogeneous peak. The mean fluorescence intensity of mock and experimental samples was, therefore, measured and used to calculate the relative values. ^dProtein expression was determined by FACS staining, which showed a bimodal distribution of positive and negative cells. The percentage of positive cells in mock or experimental samples was, therefore, used to calculate the relative value

Collectively, these results show that expression of p65TAD did not affect T-cell cycle progression but induced the death of activated CD8 T cells.

RelA controls the homeostatic proliferation of activated CD8 T cells *in vivo*

We further examined whether p65TAD may also affect T-cell expansion and/or survival *in vivo*. For this experiment, we analyzed the homeostatic proliferation of activated T cells *in vivo*. We adoptively transferred into irradiated hosts *in vitro* activated effector CD8⁺Des⁺ T cells that were infected with either one of the three different retroviral preparations 5 days before. We transferred the entire culture without sorting the GFP⁺ population. Expression of GFP permits direct analysis of the infected population, while the GFP negative population serves as an internal control for experimental variation. Since over 90% of *in vitro* activated CD8 T cells have lost expression of the lymph node homing receptor CD62L, they home to and remain in the spleen while fewer than 10% access the lymph node (not shown). We therefore only present the number of CD8⁺Des⁺ T cells detected in the spleen. In agreement with published observations, activated CD8⁺Des⁺ T cells proliferate when transferred into irradiated 'empty' hosts

(Figure 4). This proliferation was comparable for the non-infected CD8⁺Des⁺GFP⁻ T cells present in the three different retroviral infections. Expression of p65FL or p65TAD did not alter homing of the transferred T cells, since similar numbers of CD8⁺Des⁺GFP⁺ were detected in the three different conditions at day 3 post transfer (Figure 4b). Proliferation and survival of the mock-infected CD8⁺Des⁺ T cells were comparable to those of noninfected GFP⁻ T cells indicating that retroviral infection did not alter the homing, proliferation and survival of *in vitro* activated CD8⁺Des⁺ T cells (Figure 4). Overexpression of p65FL slightly increased the survival and proliferation of the transferred T cells. In sharp contrast, the number of CD8⁺Des⁺GFP⁺ T cells expressing p65TAD remained constant over the 21 days of analysis (Figure 4b). These results suggest that p65TAD may inhibit the homeostatic proliferation and/or the survival of activated T cells *in vivo*. The homeostatic proliferation of uninfected CD8⁺Des⁺ T cells was important between day 7 and 13 but stopped almost completely thereafter, as suggested by the limited increase in the number of CD8⁺Des⁺ T cells between day 13 and 21 (Figure 4a). The increase in the number of splenocytes and host CD8 T cells followed the same kinetics, indicating that by day 13 after transfer, the lymphoid compartment had repopulated and the homeostatic

Figure 3 CD8⁺Des⁺ T cells overexpressing TAD-deficient RelA are short lived. The 22 h-activated CD8⁺Des⁺ T cells were infected with either mock, p65FL or p65TAD encoding retrovirus as described in Figure 2. (a, b) Infected cells were FACS sorted based on GFP expression 3 days after retroviral infection. 1×10^6 sorted cells were maintained for an additional 48 h in IL-2-containing medium and the number of living cells, expressed as ratio of the mock control (a) and percentage of dead cells (b), were determined by trypan blue exclusion. The mean value and standard deviation of four experiments are shown. (c) Cell cycle analysis was performed at day 3 postinfection on unsorted populations. The profile of GFP⁺ or GFP⁻ cells is shown

Figure 4 Impaired homeostatic proliferation of p65TAD-expressing CD8 T cells. 22 h-activated CD8⁺Des⁺ T cells were infected with mock, p65FL or p65TAD-encoding retrovirus as described in Figure 2. At day 5 postactivation, the infected CD8⁺Des⁺ T cells were collected and the total, unsorted population was adoptively transferred into irradiated syngeneic B10.BR mice. The percentage of CD8⁺Des⁺GFP⁻ (a) and CD8⁺Des⁺GFP⁺ (b) cells present in the spleen of the different mice was determined at the indicated days post-transfer by FACS staining and the corresponding number of cells present in the spleen is shown. One experiment out of three with mean value and standard deviation of three mice is shown

proliferation was ending. Collectively these observations suggested that, by this time point, transferred effector cells likely became resting cells. Importantly, the number of

p65TAD-expressing CD8 T cells did not decline between day 14 and day 21, when homeostatic proliferation had ceased, suggesting that p65TAD has no effect on long-term survival of resting effector cells (Figure 4b).

RelA controls the survival but not the early proliferation of *in vivo* activated CD8 T cells

We further examined the response of p65TAD-expressing CD8 T cells to antigen (Ag) stimulation *in vivo*. In contrast to homeostatic proliferation, Ag challenge induces a rapid proliferation of Ag-specific T cells. We could thus more clearly determine whether p65TAD may affect the proliferation or the survival of CD8 T cells *in vivo*.

We performed the same adoptive transfer experiment as described above, but in this case immunized some of the mice by intravenous injection of H-2K^b-expressing LPS blasts, a condition that permits full activation of naive CD8⁺Des⁺ T cells *in vivo* (not shown). The number of CD8⁺Des⁺ expressing or not the relevant construct was evaluated by FACS analysis of GFP expression 3 days postimmunization. Importantly, in this experimental set-up, p65TAD is expressed at the time of TCR re-engagement allowing us to directly examine the effect of p65TAD on initial re-activation of Ag experienced T cells. As expected, *in vivo* immunization induced the proliferation of uninfected H-2K^b-specific T cells as shown by the increased number of CD8⁺Des⁺GFP⁻ T cells in the spleen of immunized mice as compared to unimmunized animals (Figure 5b). Likewise, infected T cells proliferated following *in vivo* challenge (Figure 5b). The proliferation of T cells expressing the Mock or p65TAD constructs was comparable to that of the corresponding GFP⁻ population, while expression of p65FL slightly increased cell proliferation (Figure 5b). This conclusion is further evident when comparing the percentage of GFP⁺ cells detected in immunized and unimmunized mice, which is similar for the mock- or p65TAD-infected population and significantly increased for p65FL-infected cells (Figure 5a). In addition, cell cycle progression was comparable for the three different populations (Figure 5c). Therefore, as observed *in vitro*, RelA is not a critical regulator of the initial proliferation of Ag-stimulated T cells *in vivo*.

We next determined whether, as suggested by the previous observation (Figures 3 and 4), RelA might regulate long-term survival of Ag-activated T cells *in vivo*. We performed the same experiment as described above, but analyzed the mice 7 days postimmunization. For both mock- and p65FL-infected populations, the percentage of GFP⁺CD8⁺Des⁺ T cells was comparable in unimmunized and immunized animals, indicating that the Ag-induced proliferation of CD8⁺Des⁺ T cells was not affected by expression of either of these transgenes (Figure 6). In contrast, the percentage of p65TAD-expressing CD8⁺Des⁺ T cells was significantly reduced in mice immunized with H-2K^b-expressing APC as compared to nonimmunized controls, suggesting that when activated p65TAD-expressing CD8 T cells died (Figure 6).

Expression of transactivation-deficient RelA thus has no significant effect on the *in vivo* proliferation but increases the

Figure 5 RelA does not control the early proliferation of *in vivo* activated CD8 T cells. 22 h-activated CD8⁺Des⁺ T cells were infected with mock, p65FL or p65TAD encoding retrovirus as described in Figure 2. At day 5 postactivation, the infected CD8⁺Des⁺ T cells were collected and the total, unsorted population was adoptively transferred into irradiated syngeneic B10.BR mice. Adoptively transferred mice were immunized with H-2K^b-expressing B-cell blasts (immunized) or injected with PBS (unimmunized). The percentage of CD8⁺Des⁺GFP⁻ and CD8⁺Des⁺GFP⁺ cells present in the spleen of the different mice was determined 3 days postimmunization and the corresponding number of cells was calculated. The mean percentage of GFP⁺ T cells (a) or the number of CD8⁺Des⁺GFP⁻ and CD8⁺Des⁺GFP⁺ cells (b) and standard deviation detected in four unimmunized and six immunized mice analyzed are shown. Numbers above bars are fold increase in immunized versus unimmunized mice. (c) Cell cycle analysis was performed at day 3 immunization and the profile of GFP⁺ T cells is shown. One representative experiment out of two performed is shown

Figure 6 RelA controls the survival of *in vivo* activated CD8 T cells. 22 h-activated CD8⁺Des⁺ T cells were infected with mock, p65FL or p65TAD encoding retrovirus as described in Figure 2. At day 5 postactivation, the infected CD8⁺Des⁺ T cells were collected and the total, unsorted population was adoptively transferred into irradiated syngeneic B10.BR mice. Adoptively transferred mice were immunized with H-2K^b-expressing B-cell blasts (immunized) or injected with PBS (unimmunized). The percentage of CD8⁺Des⁺GFP⁺ cells present in the spleen of the different mice was determined 7 days postimmunization with PBS (dashed bars) or H-2K^b-expressing LPS blasts (filled bars) by FACS staining. The numbers above bars indicate the decreased percentage of CD8⁺Des⁺GFP⁺ cells as compared to the input. One experiment out of two performed with the mean value and standard deviation of four individual mice is shown

death of Ag-stimulated CD8⁺Des⁺ T cells. Collectively, these data strongly suggest that RelA critically regulates the survival of activated effector CD8 T cells.

Discussion

In this study, we evaluated the role of RelA in the regulation of CD8 T-cell activation. To overcome the possibility that other NF- κ B complexes may compensate for RelA deficiency, we used a TAD-deficient RelA molecule. When overexpressed in T cells, this truncated RelA molecule forms homo- and heterodimers with p50 that bind to κ B motifs. We found that TAD-deficient RelA has no intrinsic transcriptional activity and inhibited p65FL-dependent transcription in 293 T cells. In addition, we evaluated the regulation of the expression level, by p65TAD, of 16 genes that contain NF- κ B-binding sites in their promoter region.^{24–35} Among the different genes tested, the expression level of genes known to be regulated by p65 such as I κ B α and CD69 was significantly downmodulated by expression of transactivation domain-deficient RelA, while expression of genes regulated by cRel (IL-2, CD25 and Bfl1), or not affected in NF- κ B-deficient T cells (cRel, p50 and Bcl-2), remained unaltered.^{5,6,8,14,16,17,29,36} In all cases analyzed, the decrease in protein expression was comparable to that of mRNA. Collectively, these results suggest that, *in vivo*, TAD-deficient RelA binds to and selectively inhibits transcription from RelA-binding sites.

Our study also reveals novel targets of RelA regulation. Thus, we found that TCR-mediated induction of FasL expression is slightly decreased in CD8⁺Des⁺ T cells overexpressing p65TAD. This suggests that RelA-containing complexes, although not essential, may either directly or indirectly optimize FasL transcription. In agreement with our observation, two p50/p65-binding sites are found in the

promoter of FasL. Although not essential, one of these κ B sites contributes to maximal FasL expression in Jurkat cells.³³ We also show that Bcl_{xL} expression is substantially down-regulated in T cells transduced with p65TAD. The regulation of Bcl_{xL} expression in activated T cells is clearly dependent on NF- κ B.^{8,17} Our results show that RelA, directly or indirectly, has a critical role in this process.

In this study, we evaluated the role of RelA in sustaining the activation of naive CD8 T cells, during restimulation of effector cells and for homeostasis of Ag-experienced T cells. We found that overexpression of TAD-deficient RelA in 22-h-activated CD8 T cells did not significantly block cell cycle progression during a primary *in vitro* response (Figure 3c). In addition, p65TAD expression did not impair cell cycle progression or initial proliferation induced by restimulation of effector cells (Figure 5). However, we found that overexpression of TAD-deficient RelA led to increased death of CD8 T cells during primary responses *in vitro* (Figure 3b). These observations suggest that RelA regulates a critical survival step during cell cycle progression or cell cycle exit of Ag-stimulated T cells. Such a role of RelA in the survival of cycling cells is supported by our *in vivo* studies that show that the clonal expansion of preactivated CD8 T cells overexpressing TAD-deficient RelA either during homeostatic proliferation or at day 7 postimmunization was significantly impaired (Figures 4 and 6). TAD-deficient RelA did not, however, affect the long-term survival of Ag experienced T cells, suggesting that RelA may be dispensable for resting effector T-cell homeostasis (Figure 5). The phenotype of CD8 T cells overexpressing TAD-deficient RelA is very similar to that of mature T cells expressing a degradation-resistant I κ B α , further suggesting that RelA-containing complexes are critical regulators of T-cell survival.^{5,7,8} Interestingly however, in addition to increased apoptosis, cell cycle entry of p50^{-/-}-cRel^{-/-} T cells is significantly reduced.¹⁷ None of the conventional NF- κ B complexes (RelA, cRel and p50 homodimers) were detected in nuclear extracts from p50^{-/-}-cRel^{-/-} T cells that predominantly express p52-containing complexes.¹⁷ Since cell cycle entry is not affected by overexpression of TAD-deficient RelA or degradation-resistant I κ B α , inhibition of this process in p50^{-/-}-cRel^{-/-} T cells is likely due to expression of p52-containing complexes.

The mechanism(s) by which RelA may regulate the fate of activated T cells is still unknown. Fas is an important inducer of activated T-cell death both *in vitro* and *in vivo*.^{37,38} Susceptibility to Fas-mediated death is, however, not increased by overexpression of TAD-deficient RelA (not shown). In addition to Fas-mediated apoptosis, several additional TCR-induced death pathways that are regulated by different antiapoptotic molecules have been described. Wan and DeGregori⁷ recently showed that p73, a p53 family member whose expression is regulated by NF- κ B, promotes apoptosis of cycling T cells. We found normal expression of p73 in CD8 T cells transduced with TAD-deficient RelA, suggesting that this pathway may not be involved in our experimental system. Previous studies^{7,8,19,29,39} have suggested that NF- κ B regulates the expression of the antiapoptotic molecules Bcl-2, Bcl-x_L or Bfl1. We found a significant reduction in Bcl-x_L expression, while expression of Bcl-2 or Bfl1 was not significantly diminished in T cells overexpressing

TAD-deficient RelA. Several studies suggest that the prosurvival Bcl-2 family members Bcl-2 and Bcl-x_L are critical regulators of activated T-cell survival during the contraction phase of an immune response *in vivo*.^{40,41} Further experiments are needed to determine whether death of p65TAD-expressing CD8⁺ Des⁺ T cells results from decreased Bcl-x_L expression or other mechanisms.

Previous studies have highlighted the role of NF- κ B in protecting T cells from activation-induced cell death. Our study extends these observations and shows that NF- κ B regulates survival of T cells during initial activation but also at later phases of the programmed proliferation. Interestingly however, NF- κ B may be dispensable for survival of resting naive, resting effector and memory cells, suggesting that NF- κ B controls survival of cycling T cells (^{7,8} and this study). Finally, our results suggest that, as observed for other cell types, among the different NF- κ B family members, RelA has a critical role in the regulation of activated T cells survival.^{42–46}

Materials and Methods

Mice and adoptive transfer

The Désiré (Des,⁴⁷) transgenic mice were maintained on a B10.BR background. For adoptive transfer, B10.BR mice were γ -irradiated at 7 Gy and injected i.v. with 5×10^6 T cells 3 days later. When indicated, mice were immunized by intravenous injection of 5×10^6 24 h-activated LPS blasts derived from C57Bl/6 mice.

Retroviral vectors, transfection and retroviral infections

Full length (p65FL) and a fragment corresponding to amino acid 1–310 (p65TAD) of human RelA were subcloned into the bicistronic pMX-EGFP retroviral vector (generous gift from Arai⁴⁸) that contains an IRES-EGFP. To generate retroviral particles, the PlatE packaging cell line (generous gift from Kitamura,⁴⁹) was transfected with the different constructs. CD8⁺ T cells were purified from Désiré transgenic mice by negative selection as previously described.⁵⁰ 5×10^5 T cells were stimulated for 22 h with 1×10^6 T-cell-depleted splenocytes from B6 mice as APC, then infected with retrovirus-containing supernatants as previously described.⁵¹ Infected cells were maintained in IL-2 (50 U/ml)-containing medium.

For transient transfection, 293 T cells were transfected using Lipofectamine PLUS (Life Technology, Grand Island, NY, USA) with either of the three different retroviral constructs described above and an NF- κ B reporter plasmid corresponding to three κ B sites from the Ig κ promoter regulating the expression of the luciferase gene. Luciferase activity was read 24 h after transfection as previously described.⁵⁰

Antibodies and FACS staining

Allophycocyanin-conjugated anti-CD8 Ab and PE-conjugated streptavidin were purchased from Pharmingen (Pharmingen, LaJolla, CA, USA). The Désiré anti-clonotype (Des) was prepared and biotinylated at the Centre d'Immunologie de Marseille Luminy. For cell cycle analysis, cells were fixed with 2% paraformaldehyde, then incubated with 200 μ g/ml RNase A (USB, Cleveland, OH, USA) and 20 μ g/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). Cell cycle analysis was performed using FlowJo software and the two-population algorithm.

Protein extract, EMSA and Western blot

Cytoplasmic and nuclear extracts were prepared as previously described.⁵⁰ EMSA were performed using double-stranded NF- κ B oligonucleotides derived from the mouse CD25 promoter (5'-GGAACGG-CAGGGGAATTCCTCCTT-3') in the presence or absence of blocking Ab as previously described.⁵⁰ In total, 8–10 μ g of cytoplasmic extracts were analyzed by Western blotting as previously described.⁵⁰

Ab used for supershifts or Western blots were sc-109 that is specific for the amino-terminal domain of NF- κ B RelA, the p50-specific Ab sc-1192, the cRel-specific Ab sc-71, the I κ B α -specific Ab sc371, the I κ B β -specific Ab sc945, the Bcl-2-specific Ab sc-492, Bcl-x_L-specific Ab sc-634 and Bcl-3-specific Ab sc-185 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and an anti-actin Ab (Sigma). The different blots were quantified with a luminescent image analyzer (LAS-1000 plus; Fuji). Measured values were corrected for loading differences based on the actin signal. Results are expressed as ratio of the relative quantitation value of the sample divided by the mock.

RNA preparation and quantitative RT-PCR

Total RNA was extracted using the High Pure RNA Isolation kit (Qiagen, Valencia, USA) and cDNA was synthesized as previously described.⁵² Real-time PCR was performed on cDNA samples using the QuantitectTM SYBR[®] Green system (Qiagen). The primer sequences and cycling conditions are available upon request. Analysis used the sequence detection software supplied with the instrument. The relative quantitation value is expressed as $2^{-\Delta C_T}$ where ΔC_T is the difference between the mean C_T value of duplicates of the sample and of the endogenous HPRT control. Results are expressed as ratio of the relative quantitation value of the sample divided by the mock.

Acknowledgements

We thank S Ghosh for the NF- κ B-luc reporter construct, N Arai for the PMX-IRES retroviral vector and T Kitamura for the PlatE cells. We thank C Boyer, M Buferne, B Malissen, L Leserman for helpful discussion and critical reading of the manuscript. This work was supported by institutional grants to SG and AMSV from 'Institut National de la Santé et de la Recherche Médicale' and 'Centre National de la Recherche Scientifique' and by grants from 'Association pour la Recherche sur le Cancer', 'Ligue Nationale contre le Cancer (LNCC)' and 'LNCC-Comité des Bouches du Rhône'.

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