GENETIC IMMUNE MODULATION OF Ran GTPase AGAINST DIFFERENT MICROBIAL PATHOGENS

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1. ABSTRACT

Septic shock characterized by pro-inflammatory cytokine storm can be induced by a variety of microbial infections. Typical pro-inflammatory cytokines include TNFα, IL1 and IL6. Although one or more of them is often expressed in any given microbial infection, usually it is the elevation of one cytokine that becomes predominant at a particular time in a given infection. Here we showed that administration of adenoviral antigens alone led to a predominant elevation of serum IL6 but not TNFα. Administration of endotoxin together with adenoviral antigens led to elevation of both serum IL6 and TNFα. In vivo expression of RanC/d, but not RanT/n or LacZ into peritoneal macrophages rapidly down-modulated the levels of these cytokines in both experimental situations. It also correlated with reduced liver inflammatory damage and increased resistance to septic shock. We conclude that RanC/d can be applied to down-modulating production of cytokines induced by microbial products other than endotoxin and to render resistance to mice against septic shock induced by one or more microbial pathogens. The ability of using RanC/d to down-modulate and RanT/n to up regulate host innate immune response induced by multiple microbial pathogens is illustrated in this study. Incorporation of either or both RanC/d and RanT/n alleles into appropriate vectors will produce genetics vaccines valuable for biodefense and medically important illness in which host immune system against invading agents is severely burdened.

2. INTRODUCTION

Innate immune response towards a variety of microbial pathogenic infections is an important first line of defense from the host. One of the hallmarks is an excessive cascade of events in inflammatory immune response, which is characterized by elevation of one or more pro-inflammatory cytokines, produced mainly by macrophages or dendritic cells. We have previously shown that Ran GTPase is involved in host innate immune response (1). Comparative functional studies on two alleles of Ran demonstrated that expression of RanC/d in mice confers resistance towards lethal endotoxin challenge; expression of RanT/n sensitizes them (2). Several studies have shown that one of the main causes for fatal shock induced by endotoxin is due to elevation of serum TNFα, IL1 or both – hallmark pro-inflammatory cytokines for septic shock (3-10). Indeed, further studies on resistance to endotoxic shock induced by RanC/d are consistent with the important role of these cytokines in endotoxic shock. Expression of RanC/d in peritoneal macrophages in vitro led to a down-modulation of endotoxin-induced TNFα production, whereas expression of RanT/n did not (11).

Mechanistic studies on the two Ran alleles also reveal important insights on the importance of mRNA structure affecting biological functions. RanC/d differs from RanT/n by only a single nucleotide, located at position 870 of its 3'-UTR (12). This leads to alterations in RNA structure. There is no significant change in RNA

stability between the two mRNAs. Ran proteins synthesized from RanC/d mRNA rapidly translocate into the nucleus, whereas RanT/n proteins linger in the cytoplasm. These findings link to the unique properties of Ran GTPase, which is a very unusual small GTPase that is nuclear at steady state and is involved in multiple biological phenomena, including nucleocytoplasmic transport, nuclear envelope formation, mitotic spindle assembly and cell cycle (13-18). The differential intracellular localization of RanC/d and RanT/n proteins are also associated with the uniqueness of Ran's different forms and their corresponding associated partners. Nuclear Ran is exclusively RanGTP, whereas cytoplasmic Ran is exclusively RanGDP. One associated partner of RanGTP is RCC1, which is exclusively nuclear; likewise, an associated partner for RanGDP is RanGAP, also exclusively cytoplasmic. Functionalities corresponding to these unique associations with exclusive intracellular compartmentalization have been suspected for quite sometimes. Along this line, expression of RanC/d with rapid nuclear localization and reduced innate immune response, as opposed to expression of RanT/n with cytoplasmic distribution and augmented response, fits perfectly well with recent findings that nuclear export, but not import, of NF-κB is specifically RanGTP-dependent (19-23). Increased export of NF-κB would obviously reduce transcriptional activation of genes involved in host immune response, including those encoding for pro-inflammatory cytokines. These work therefore lend further support on studies of RanC/d and RanT/n alleles, hence Ran's involvement in host innate immune response and septic shock (24).

The involvement of NF-κB in innate and chronic immune response is beyond doubt. Ran's linkage to NF-κB therefore presents to us a whole new way in our studies. We have already obtained proof-of-concept data regarding the ability of RanC/d to protect endotoxic shock in mice (2,25). Septic shock induced by many microbial pathogens or their products largely resemble that of endotoxic shock. The details of each one of them, however, is controversial and can be quite different. For example, endotoxic shock induced by *E. coli* LPS typically induce elevation of predominantly serum TNFα (4,6-10), septic shock induced by *B. anthracis* has been found associating predominantly with elevated serum IL1 (26,27), and fatal shock induced by high dose of adenovirus administration has prominent elevated serum IL6 (28-31). Since NF-κB is central in the transcriptional activation of each or all of these proinflammatory cytokines, we investigated in this study whether or not RanC/d down-modulation still applies in mice challenged with more than one pathogenic product, i.e. endotoxin and adenoviral antigens together. If nuclear Ran were important in controlling the activity of NF-κB, expression of RanC/d in vivo would result in reduced cytokine production across the board and confer more resistance against shock induced by both pathogenic products, even though activation of these cytokines might have achieved via different signal transduction pathways. If these were true, then these pathways must have ultimately merged towards NF-κB, which is expected based on a large volume of studies on NF-κB.

3. MATERIALS AND METHODS

3.1. Animals

We purchased CD-1 mice from Charles River Laboratories, and C3H/HeOuJ, C3H/HeJ and Balb/c inbred mice from Jackson laboratories. Typically, we used mice that were 6-8 weeks old after their acclimatization for at least 2 weeks.

3.2. Adenovirus vector construction, propagation and titer determination

For Ad-RanC/d and Ad-RanT/n, RanC/d and RanT/n cDNAs were excised using BamH1to digest the plasmids pCD-Lps^n or pCD-Lps^d. The resultant 1.1kb fragments were separately inserted into the E1A region of adenovirus 5 (Ad), and expression of Ran genes were driven by the cytomegalovirus (CMV) promoter (2,11). For Ad-LacZ, LacZ gene was cloned into the same relative position, as did RanC/d and RanT/n cDNAs.

The adenovirus vectors Ad-LacZ, Ad-Ran C/d and Ad-Ran T/n were prepared using a large-scale stock preparation as described (2,11,32). The titer was determined using cytopathic effects assay, plaque assay and absorbance at 260nm (OD_{260}). The cytopathic effects assay was carried out as described by Nyberg-Hoffman et al (33). Briefly the assay involves seeding 293 human kidney cells in a 96 well tissue culture plate at a concentration of $1x10^4$ cells/well then infecting the cells with various dilutions of a particular virus stock at concentrations ranging from 10^{-9} to 10^{-14}. The plates are incubated at 37°C in 5% CO2 for 7-10 days. We then calculated the titer by multiplying the number of wells with obvious cytopathic effects at the highest dilution and dividing it by the total volume of viral supernatant used for testing. Each titration is done in triplicate. Plaque assay was performed as described by the same group with slight modifications. The OD_{260} was determined by making a 1:10 dilution of 50ul of purified virus stock in 0.1% SDS/PBS, heated at 56ºC for 10 min. and then measured the absorbance at 260nm. Particle number was calculated by using the equation: OD_{260} x dilution factor x $1.1x10^{12}$ particles = OPU/ml

3.3. Polymerase chain reaction (PCR)

PCR was used for the detection of viral vectors and their messages in both animal tissues and cell lines. The following is a summary of the reaction conditions utilized: For Ad-Ran C/d or Ad-Ran T/n: The PCR was conducted using primers homologues to regions on the 1.1kb Ran C/d or Ran T/n insert that includes a 39bp HA tagging sequence resulting in a 158bp product. The sequence for the sense primer, S21G, is 5'-TTGTTGCCATGCCTGCTCTT g-3' and the anti-sense primer, A21A, 5'-GGTCATCATCCTCATCTGGGA-3'. The PCR reaction conditions for 30 cycles are as follows: denaturation 95°C for 1min; annealing 60°C for 30s; extension 72°C for 30s. The sample was then analyzed on 1.5- 2% agarose gel. The PCR reaction yielded two products: a 119bp band, represented the endogenous Ran; and a second 158bp band, represents the exogenous or viral Ran. These two bands were subjected to densitometry analysis (UVP gel analysis software) and the percentage of
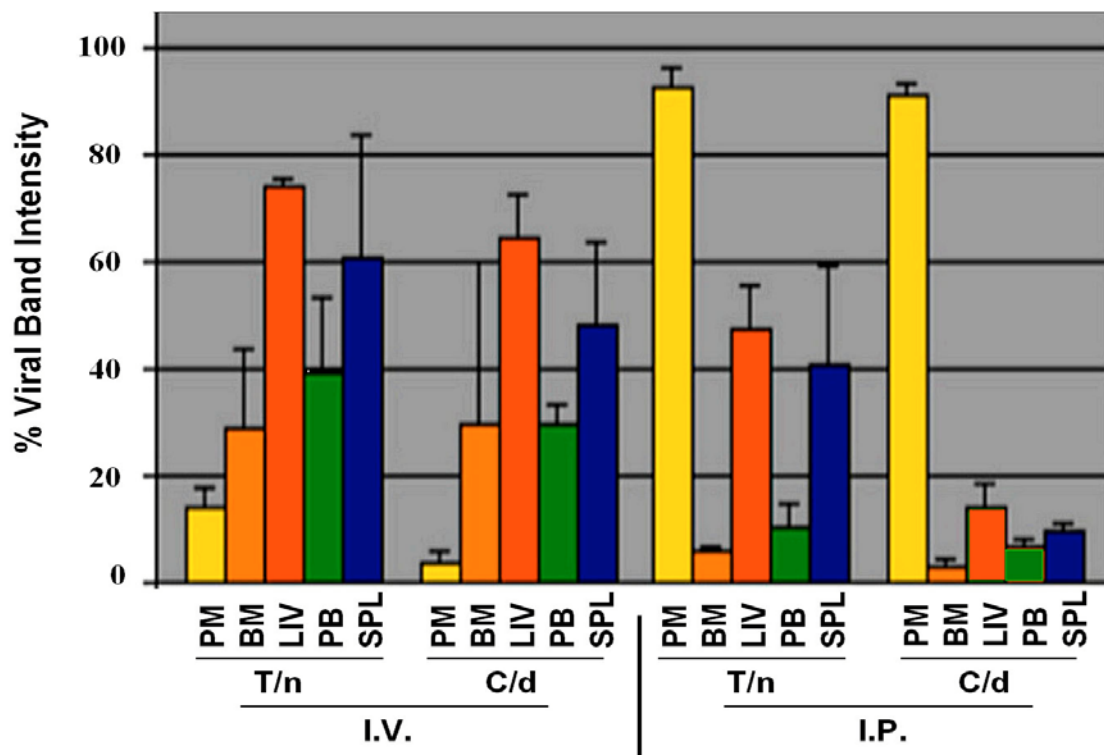
Figure 1. Ad-RanT/n and Ad-RanC/d vector distribution and routes of inoculation. The vectors were inoculated into mice, i.v. or i.p., with a titer of $1x10^{11}$ pfu per mouse. Two hours after vector administration, we extracted DNA from peritoneal cells (PM), bone marrow (BM), liver (LIV), peripheral blood (PB) and spleen (SPL) and performed PCR amplification using Ran-specific primers as described in Materials and Methods. The upper band, with a size of 158bp, indicates the viral Ran transgene (with 39nt HA epitope sequence inserted within either T/n or C/d sequence); the lower band, with a size of 119bp, shows the endogenous Ran sequence. The chart represents cumulative data from 6 mice and % viral band intensity is the intensity of the viral band versus intensity of both viral and endogenous bands in each lane. Standard deviation of the mean is shown on top of each bar as well.

the viral band was calculated as a percentage of the combined density of both the exogenous and endogenous bands for each organ sample.

3.4. Administration route of adenovirus

C3H/HeOuJ mice were infected using either an inter-peritoneal (IP) or an inter-venous injection (IV) with $0.5-1.0x10^{11}$ pfu/mouse of either Ad-Ran C/d, Ad-Ran T/n virus or Ad-LacZ. At 2 hrs post-infection the animals were sacrificed and the peritoneal cells, liver, lung, heart spleen, peripheral blood and kidneys were harvested from each animal. The DNA was extracted from these tissues using DNAzol reagent (GibcoBRL) and PCR analysis was conducted as described above.

3.5. Cytokine and endotoxin assay

Periphery blood samples were collected at various time points and cytokine levels were determined by ELISA techniques using commercially available antibodies and recombinant cytokines (R & D Systems; BD/Pharmingen). Endotoxin level was determined for random blood samples from new shipments of mice using Limulus Amebocyte Lysate method (LAL assay, BioWhittaker, MD) following recommended protocol. Results were analyzed using Prism software.

4. RESULTS

4.1. Early difference in vector distribution after intraperitoneal administration

We separately cloned RanT/n and RanC/d cDNAs into a first generation Ad vector, which is known to produce adenoviral antigens and can induce host innate immune response. As Ad-RanT/n and Ad-RanC/d are identical viral genomes, with the exception of the single base difference in the 3'-UTR of the two Ran alleles, the study of the two vectors in parallel should serve as powerful controls for each other (2). We also compared the routes of vector administration. Each mouse received a vector with a titer of $1x10^{11}$pfu/ml, i.v. or i.p. Two hours after vector administration, DNA extracted from various organs were subjected to PCR amplification as described before (12). We observed no significant difference in vector distribution between the two vectors when they were administered i.v. (Figure 1). Multiple tissues expressed vector signals in livers, spleens, peripheral blood, bone marrow and peritoneal macrophages, in descending order of intensity. By contrast, a striking difference in vector distribution among various tissues was apparent with i.p. administration. Signals in peritoneal macrophages were highest with both vectors; they were also strong in livers
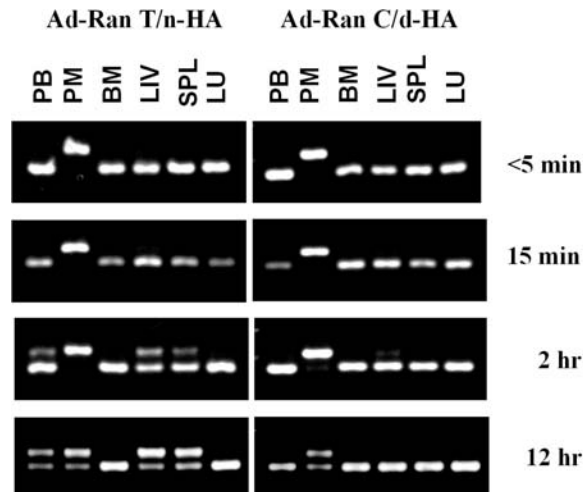
Figure 2. Early difference in tissue distribution of vectors after intraperitoneal inoculation.   Ad-RanT/n and Ad-RanC/d were injected i.p. into each mouse in groups of 4 mice, at 10^{11} pfu/mouse. Genomice DNA from Peripheral blood (PB), peritoneal macrophages (PM), bone marrow (BM), livers (LIV) and spleens (SPL) were extracted at various indicated time after virus inoculation. About 0.2ug of extracted genomic DNA were subjected to competitive PCR analysis as previously described (11). Ten microliters of each PCR product was loaded onto 2% agarose gel. The upper band represents amplified viral Ran-HA DNA fragment with a size of 158bp; the lower band represents amplified endogenous Ran DNA fragment with a size of 119bp.

and spleens in mice transduced with Ad-RanT/n but not in mice with Ad-RanC/d.

Time course analysis indicated that the difference occurred within 2 hours. At various time points after Ad administration, a competitive PCR analysis was done on DNA extracted from various organs of each mouse in groups of six. In RanT/n- transduced mice, the viral band was present in multiple organs, whereas in RanC/d-transduced mice, it was present predominantly in peritoneal macrophages (Figure 2). The intensity of the viral band in RanC/d-transduced mice became apparent in livers only 12 hours later, but the intensity of the viral band in all other organs at early time points, except peritoneal macrophages, was minimal when compared to RanT/n-transduced mice. These data suggest the existence of a biological difference between RanT/n- and RanC/d-transduced mice early on with time.

Liver biopsy examination supports that this early difference is the result of differences in biological response between mice transduced with RanC/d- and those with RanT/n. Ad vectors or PBS as control were given to mice one day prior to liver sampling. The results showed that while both livers of RanT/n- and RanC/d mice exhibited significant inflammation, livers of T/n-mice had more pronounced infiltration of inflammatory cells, especially in the area around the central vein in the livers (Figure 3). The

centrilobular area showed dropout of hepatocytes and replacement by a mixed inflammatory infiltrate consisting of lymphocytes, neutrophils and macrophages. There was also congestion of the central veins and some veins showed damage to the endothelial cells. This centrilobular or Zone 3 area being farthest from the blood supply is also most sensitive to ischemic or anoxic injury. Zone 3 hepatocytes are also often more sensitive to drug or endotoxin induced liver damage than the other hepatocytes.

4.2. Down-modulation of different cytokine profiles separately induced by endotoxin and adenoviral antigens by RanC/d but not RanT/n

Another difference could be due to differences in the levels of various serum pro-inflammatory cytokines that are produced in RanT/n- and RanC/d-transduced mice. Groups of six mice were inoculated intraperitoneally with Ad-RanT/n, Ad-RanC/d, Ad-LacZ or PBS and blood were collected either 1 hour or 8 hours afterwards (Figure 4). By ELISA, we observed no significant difference in terms of serum TNFα levels. On the other hand, there was a striking elevation of serum IL6 in both LacZ- and RanT/n-transduced mice, whereas serum IL6 levels in RanC/d- and control mice remained low at both 1 hours and 8 hours after virus inoculation. The levels of serum IL1β were also significantly higher in LacZ- and RanT/n- but not RanC/d-transduced and control mice. These data corroborated with the competitive PCR result and histological examination, showing a more severe inflammatory condition in RanT/n-compared to RanC/d-mice.

A large volume of investigations has shown that endotoxin-induced shock results in elevation of serum TNFα (4-6,8-10). Elevation of serum IL-6 but not TNFα induced by high titer of adenoviral vectors in these mice reminded us of a clinical picture seen in the death of a patient that unfortunately died of high dose Ad gene therapy, also with elevation of IL6 but not TNFα (31). These data therefore also suggested that RanC/d might be effective also on Ad vector-induced toxicities. Therefore, we next investigated if the profile of these serum pro-inflammatory cytokines would change when mice were stimulated with both endotoxin and adenoviral antigens. Groups of 20-25 mice were inoculated with recombinant Ad-RanT/n, Ad-RanC/d ($5x10^{10}$ pfu/mouse) or PBS i.p. Four days later, 450ug of *E coli* LPS were given to each mouse intraperitoneally. Percent of survival was observed over time. Typical death occurred within 2 days after endotoxin treatment (Figure 5). Control mice (with endotoxin alone) had a survival rate of 40%. As expected, more of Ad-RanT/n-mice died, with a rate of 22%, whereas Ad-RanC/d-mice were best protected, with a rate of 67%. When serum cytokine profiles of groups of six mice under identical experimental protocol were examined, we obtained more informative data (Figure 6). First, unlike mice treated with adenovirus alone (above), these mice exhibited elevation of both serum TNFα and serum IL6. Second, serum levels of both TNFα and IL6 were reduced in Ad-RanC/d-mice compared to Ad-RanT/n-mice. These
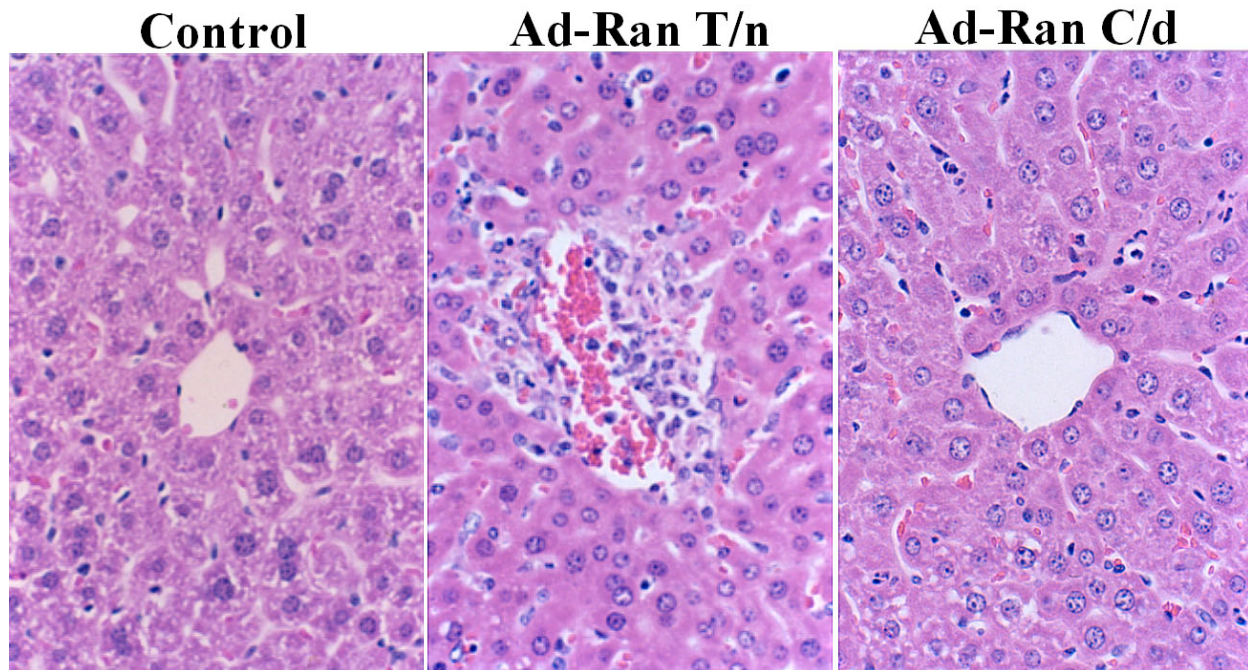
Figure 3. Histochemical analysis on liver biopsy. The hepatocytes around this central vein (Zone 3) show dropout with replacement by a mixed inflammatory cell infiltrate. There is congestion of the central vein and some damage to the endothelial cells. Different panels represent histological slides of livers of (a) untreated mice, (b) Ad-RanT/n-treated mice, (c) Ad-RanC/d-treated mice. Hematoxylin and eosin stain, magnification: 200x for all slides.
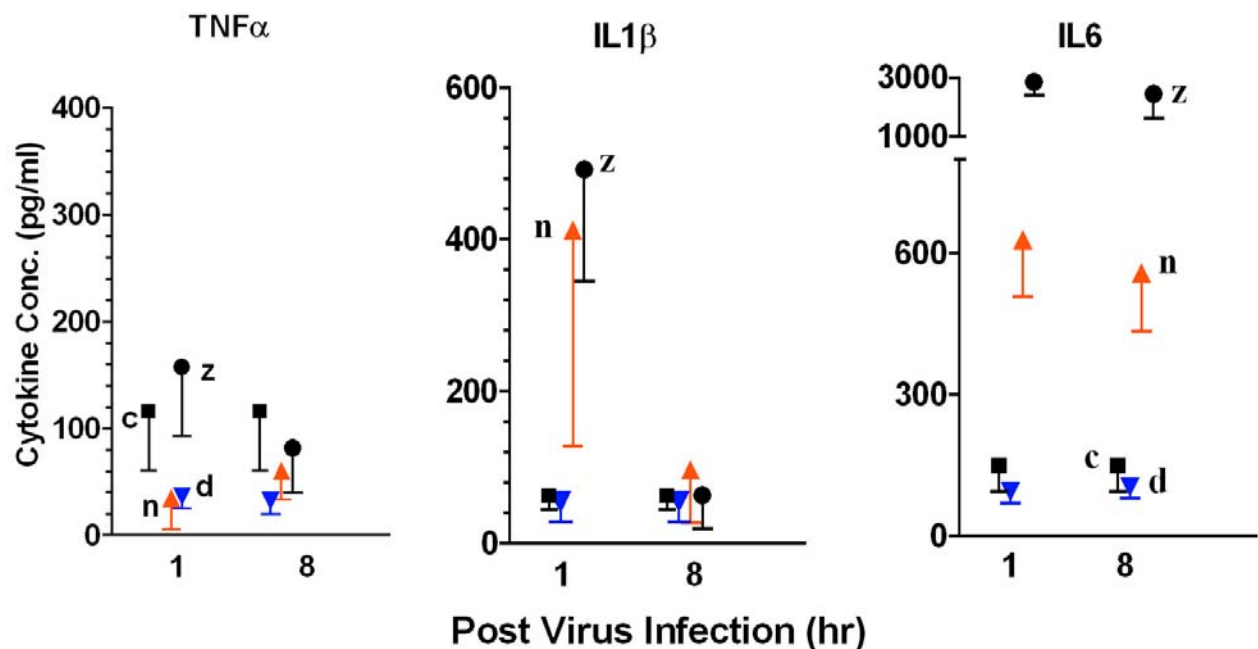
Figure 4. Plasma cytokine levels after vector administration. Groups of 6 mice were inoculated i.p. with PBS, Ad-LacZ, Ad-RanT/n or Ad-RanC/d at 1011 pfu/mouse. Peripheral blood was collected 1 and 8 hours after inoculation. Various cytokines were measured by standard ELISA assay as we described previously (11). Black squares = PBS controls; Black circles = Ad-LacZ; red triangles = Ad-RanT/n; blue triangles = Ad-RanC/d.
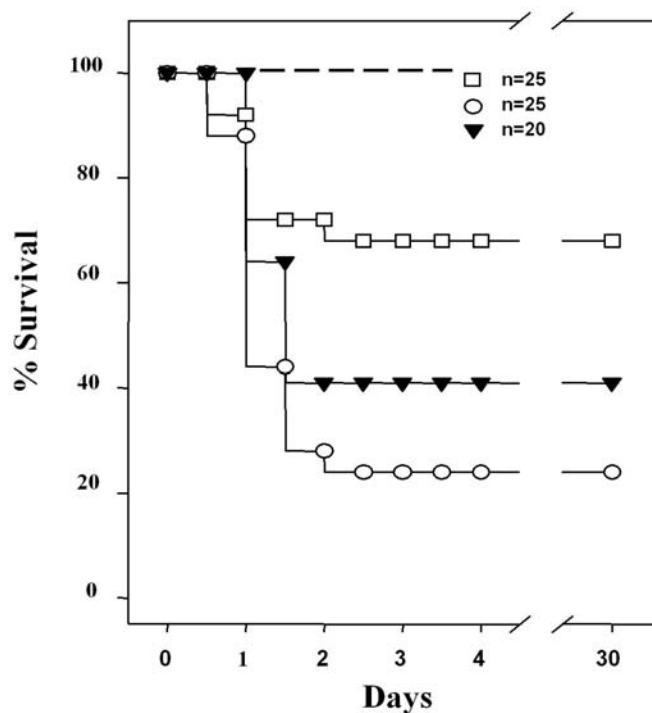
Figure 5. Prophylaxis against adenoviral inflammation and endotoxin challenge. Acclimatized mice were inoculated i.p. with Ad-RanT/n (open circles), Ad-RanC/d (open squares), or with PBS alone (closed triangles). Each vector was given at a titer of 5×10^{10} pfu/mouse. Four days later, each mouse was challenged with 450ug of *E. coli* LPS (Sigma). The survival rate was monitored daily and experiment was terminated after 30 days. The number of mice in each experimental group (T/n and C/d mice) was n= 25; for control mice (PBS+LPS), n= 20. The dotted line at 100%-mark represents groups of 10 mice inoculated with Ad-RanT/n alone or Ad-RanC/d alone. None died.
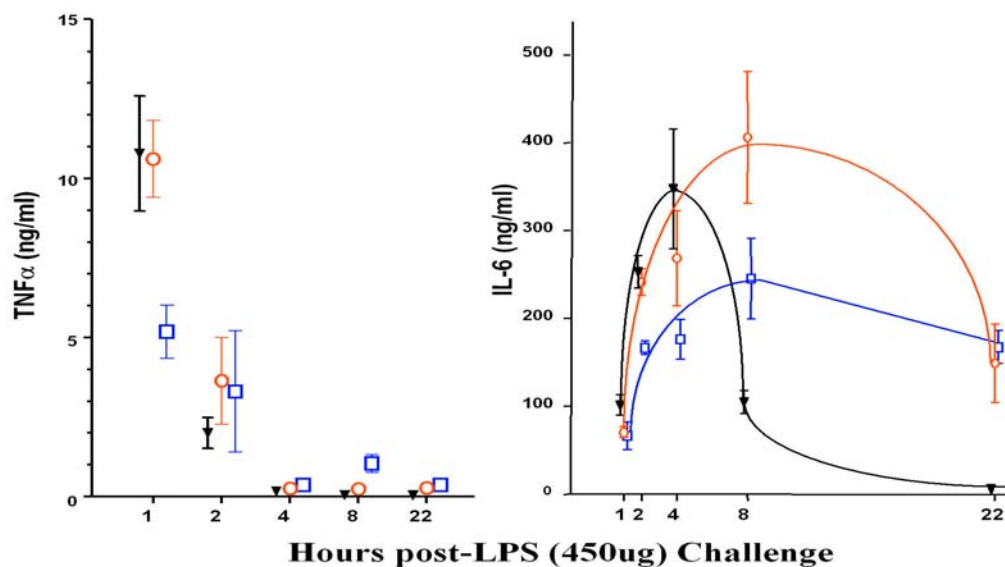
Figure 6. Level of plasma pro-inflammatory cytokines. Groups of 20 mice were each given either Ad-RanT/n, Ad-RanC/d, i.p., at 5×10^{10} pfu/mouse, or with PBS. Four days later, all mice were challenged with 450ug of *E coli* LPS. At various time points afterwards, peripheral blood were collected from 4 mice per group and level of TNFα and IL6 in each mouse was measured by ELISA. Solid black triangles are values from mice with no vector but with LPS; open red circles, mice with Ad-RanT/n and LPS; open blue squares, mice with Ad-RanC/d and LPS. For plasma TNFα level at one-hour time point, the difference between Ad-RanT/n mice and Ad-RanC/d mice has a P value of <0.0016. For serum IL-6 at 2-hr time point, the difference between Ad-RanT/n mice and Ad-RanC/d mice has a P value of <0.008, and at 8-hr time point, the difference between mice with no virus and T/n mice has a P value of <0.0016.
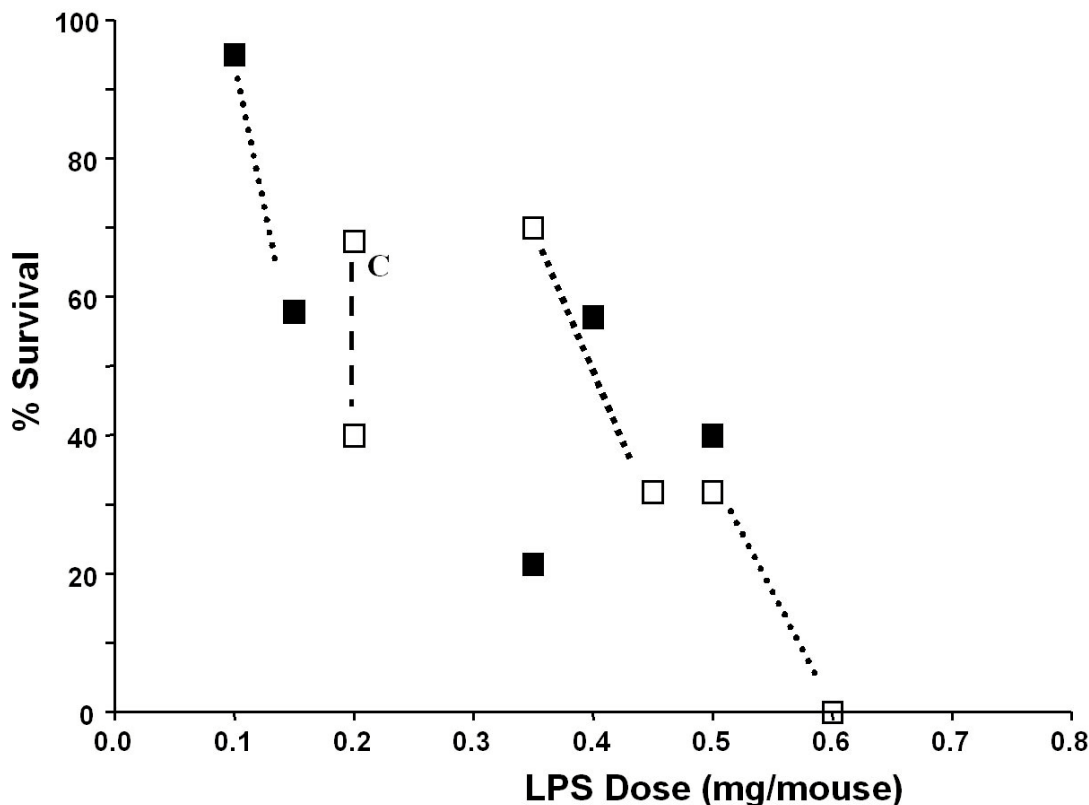
Figure 7. Survival of mice after endotoxin challenge. Different shipments of CD-1 (filled square) and Balb/c mice (empty square) from two different vendors (Jackson Laboratory, Charles River Laboratory: labeled as C) were challenged with different doses of LPS. Survival rates were monitored daily for a month. Each point represented at least 20 mice (ranged 20-25). Experimental points connected with dotted lines represented mice from the same shipment but tested with different doses of endotoxin in different experiments done within 10 days.

data strongly suggest that RanC/d can down modulate different cytokine profiles, separately induced by endotoxin and adenoviral antigens, across-the-board.

4.3. Pre-existing innate immune status of recipient mice

Endotoxin is notoriously widespread in the environment. For identical experimental design and reagents used, we had in the past experienced very significant experimental variations among different shipments of mice, even from reputable inbred mouse suppliers such as Jackson Laboratory or Charles River. One way we found out this problem was that we did several endotoxin dose survival curves using mice from different shipments (Figure 7). From these endotoxin dose experiments, it was clear that the sensitivity of mice varied from shipment to shipment. Since we used groups of 20 mice for each experimental point, and the survival rate declined with increased dosage used within each shipment of mice (except the one shipment from Charles River, labeled C), the data appeared to be quite reliable. Local environmental influence did exert some effects, as the lines connecting two points for any pair were not parallel in all experiments (only two out of three); this was particularly true for mice from Charles River where treated mice given with the same dose of endotoxin (same type of experiment but was done three days apart) had

very different survival rate (70% versus 40%). No conclusion should be made on the quality of mice from Charles River though because only one shipment was analyzed. Unconnected single points are results on mice from different shipments.

With these variations, we concluded that supplier and local environment for housing mice are important factors that might significantly influence the experimental outcome (especially for our type of experiments where the status of pre-existing host innate immune response is a crucial influence factor in retrospect). Endotoxin is the obvious one to check. We sought to determine the plasma endotoxin level of different strains and different shipments of mice (Figure 8). Blood samples were taken randomly from 10 mice per shipment shortly (except one experiment) after arrival from vendors. Plasma samples were collected and the endotoxin level of each sample was measured using LAL assay according to manufacturer's instructions. Indeed, variations could be observed from shipment to shipment. Not only the average value of plasma endotoxin varied significantly, the distribution of individual value also varied tremendously. Shipments CD1-1 and Balb/c-2 had individual values mostly clustered together and were under 2Eu/ml, whereas shipments Balb/c-1 and CD1-2 had individual values that varied greatly, especially with CD1-
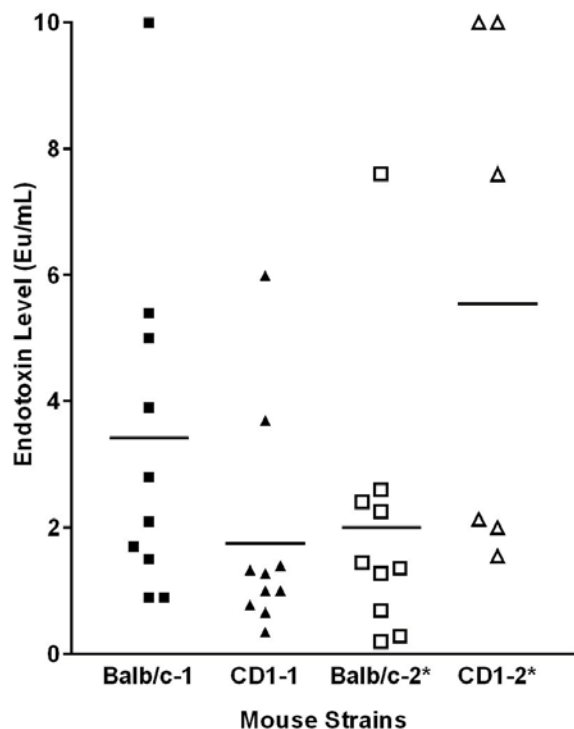
Figure 8. Plasma endotoxin levels in two different strains of mice from different shipments. Shortly upon arrival, blood samples were taken randomly from 10 mice (except for CD1-2, only 6 mice were tested). Endotoxin level was determined using LAL assay following manufacturer's protocol. Each data point represented one mouse entry shown with average in each group (horizontal line).

2, in which individual plasma endotoxin levels polarized towards either end. Indeed, experiments on shipments of mice with uniformly low plasma endotoxin levels produced the more consistent data.

DISCUSSION

In this study, we showed that administration of high titer of adenovirus alone resulted in elevation of IL6 but not TNFα, and that this IL6 elevation was reduced in Ad-RanC/d-treated mice. When both adenoviral antigens and endotoxin were administered, mice expressed high levels of both serum IL6 and TNFα, and again, the levels of both of these cytokines were reduced in Ad-RanC/d-treated mice and improved survival was observed in these mice compared to Ad-RanT/n-treated mice. It is well documented that mice treated with endotoxin alone exhibit predominantly high levels of TNFα (5-10). Therefore, the ability of RanC/d to reduce serum levels of IL6 and TNFα, either separately in different mice or simultaneously in the same mice treated with two pathogen products, suggests that RanC/d can be used to down modulate production of pro-inflammatory cytokines across the board, regardless whether elevation of these serum cytokines were induced by one or more microbial pathogens. These findings are significant for us to consider applying RanC/d technology for septic shock in the future, where patients in intensive care units are often infected with multiple microbial pathogens.

The above findings are also consistent with our working model that Ran GTPase is linked to NF-κB, which in turn, to immune modulation, then to resistance or sensitivity to septic shock (12,24). Expression of RanC/d leads to rapid nuclear localization of the proteins, which encourage nuclear export of NF-κB, therefore reduced transcriptional activation of pro-inflammatory cytokine genes. By contrast, expression of RanT/n leads to a preferred cytoplasmic localization of newly synthesized Ran proteins, which discourage nuclear export of NF-κB, and therefore increased transcriptional activation of pro-inflammatory cytokine genes. The dictating factor therefore appears to be the ratio of nuclear and cytoplasmic Ran GTPase, rather than the absolute amount of Ran GTPase in a given cell. Fundamentally speaking, this concept is essentially the same as one recently proposed by Newport and associate, who reported another new role of Ran in DNA re-replication (34). A molecule called MCM normally participates in chromatin complex during DNA replication. During S phase, cyclinE-Cdk2 phosphorylates MCM, causing it to associate with RanGTP and Exportin1. This complex formation prevents MCM from binding to its normal complex for DNA re-replication. Interestingly, formation of RanGTP, exportin1 and MCM is also influenced by nuclear/ cytoplasmic ratio of Ran GTPase.

The results of our experiments on routes of vector inoculation indicate the importance of transducing the correct target cells. Intraperitoneal inoculation of adenoviral vectors indicated that peritoneal cells were effectively transduced. Cytoprep staining indicated that more than 80% of them are monocytic macrophages. More importantly, biological difference could be observed as early as two hours after vector administration. The difference must be biological for the following reasons. First, vector transduction efficiency must be very high: (a) adenoviral infection is known to be highly efficient, (b) we inoculated 5×10^{10} pfu virus particles per mouse, (c) we experimentally determined that up to 10 millions peritoneal cells per mouse were present in peritoneal cavity, (d) the competitive PCR showed that the viral band (upper) intensity of PM sample at <5 minute time point was extremely high as opposed to a virtual absence of endogenous Ran band (lower) and this was not seen in other organs at the same time point. Second, this transduction protocol allows for vector delivery into the correct target cells – tissue macrophages that are responsible for acute inflammatory responses to take place. This is consistent with the fact that over time, signals in the liver appears to be most dominant in RanT/n-mice at 2-hr time point and that tissue macrophages account for most of the Kupffer cells in the liver. Third, viral gene expression can occur as early as 1.5 hours (35). Rapid binding of adenovirus particles is through high affinity association between viral fibers and the Coxsackie's adenovirus receptor (CAR), as well as through binding of viral penton base proteins to a co-receptor of the αV integrin family (36). Adsorption and internalization of adenoviral particles can occur in less than 1 minute (37). Given the high

efficiency of vector transduction into the correct target tissue macrophages and early viral Ran gene expression, we observed rapid biological effects. The observed biological differences are also consistent with modulating effects of RanT/n and RanC/d.

Exhibition of rapid biological effects as a result of adenoviral transfer of RanC/d has attracted a lot of our attention as it may have great potential to treat septic shock, which is really an excessive host innate inflammatory response induced by one or more invading pathogens. Along this line, we have recently found out that such rapid response could be explained by the fact that pre-package viral mRNA is present in virions of DNA adenovirus (32). After internalization, together with viral genome uncoating and budding away from endosome into cytoplasm of the host cell, the presence of pre-packaged viral mRNA would certainly be effective in immediately offering itself as a template for protein synthesis. By RNA in-situ hybridization, we have also shown that, unlike RanT/n mRNA, RanC/d mRNA is predominantly perinuclear and nuclear (38); once Ran proteins are synthesized, majority of them translocate into the nucleus (12) and encourage nuclear export of NF-kB, enhancing immune down-modulation. From this molecular picture and others discussed above, the impetus is set to further develop RanC/d as a potential genetic vaccine for septic shock induced by a variety of emerging microbial infections.

Results of our studies on pre-existing host innate immune status explain and emphasize the importance of using large number of animals in each experimental point to increase the statistical power of each experimental result. Initiation of clinical trials is often based on important conclusions drawn from animal studies. When a particular clinical protocol works in animals but fails in humans, concluding remarks often point to substantial biological differences between humans and animals. While this must be true, a thorough examination in the literature concerning pre-clinical animal studies indicates that improvement in experimental design, such as inclusion of large numbers of animals per experimental point and consideration of pre-innate immune response status of individual recipients, especially in the areas of septic shock induced by multiple microbial pathogens, would undoubtedly strengthen the validity of certain drugs with potential clinical benefits. A case in point is the unfortunate death of an 18-year old patient with partial ornithine transcarbmaylase (OTC) deficiency in an adenovirus gene therapy clinical trial (31). This patient was one of three who received highest dose of adenoviral vector and was the only one who died. His clinical course was marked by systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulation, multiple organ failure and death at 98 hour after gene transfer. His serum contained marker elevation of IL6 but not TNFα. While the other two patients had similar host immune response to the adenovirus, the magnitude of the immune response was lower and was not sustained. Pre-existing innate immune response status of the patients such as serum endotoxin level was not checked. It is possible that the patient who died of SIRS was previously sensitized with agents similar to but distinct from endotoxin.

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