teraction. Surprisingly, and consistent with the data from the periphery, a fraction of the Foxp3^{gfp+} cells represented CD8$^+$ thymocytes dependent on expression of MHC class I molecules.

Overall, this study has established the validity of Foxp3 as a specific marker for regulatory T cells and has reported a novel mouse line of tremendous potential value in studies on immunoregulation. It has also raised some intriguing questions. Can the newly discovered CD8$^+$Foxp3^{gfp+} T cells exert regulatory function comparable with that of CD4$^+$CD25$^+$ T_R cells? If so, in what context(s) do they emerge as important control elements? Foxp3^{gfp+} T_R cells isolated from diverse sites showed some striking phenotypic differences; for example, peripheral organs were enriched in the CD25$^{lo/neg}$Foxp3^{gfp+} population, which had an activated phenotype and included proliferating cells. Might these cells be the key to self-tolerance within tissues? Unlike CD4$^+$CD25$^+$ T_R cells, other immune cells with regulatory potential, including NKT cells and Tr1 cells, express no or low levels of Foxp3; thus, it is unlikely that this transcription factor and the gene-expression program it specifies is the only means of establishing tolerance dominantly. What is the master regulator of these cell-types and when do they come into play?

The powerful in vivo model introduced by Fontenot et al. (2005) opens the door for new insights into T_R cell biology. There is sure to be an onslaught of studies on antigen-specific systems, as well as adaptations to a diversity of pathological situations, including autoimmunity, chronic infection, transplantation, and tumorigenesis.

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Do Cytotoxic Lymphocytes Kill via Reactive Oxygen Species?

A paper by Martinvalet et al. (2005) in this issue of *Immunity* examines the mechanisms used by granzyme A to kill target cells after its cytoplasmic injection by cytotoxic lymphocytes. They show that this protease induces mitochondrial damage and generation of reactive oxygen species that are necessary for cell death.

The current mechanistic paradigm for lymphocyte-mediated cytotoxicity is that perforin permeabilizes target cells to allow granzyme entry, and these proteases actually kill the target cell. For some years there was intense focus on granzyme B, because its highly unusual specificity for substrate cleavage at aspartic acid residues is similar to that of caspases, the endogenous suicide mediators. Granzyme B can directly trigger a caspase cascade by cleaving endogenous procaspases in target cells, and it can also act indirectly via two pathways of mitochondrial outer membrane damage: (1) cleavage of the Bcl-2 family protein Bid to a truncated form that potently attacks the outer mitochondrial membrane, and (2) cleavage of Mcl-1, which normally binds to and neutralizes Bim, another Bcl2 family protein that attacks the mitochondrial outer membrane. Damaging the mitochondrial outer membrane causes the release of caspase pathway activators like cytochrome c from the intramembrane space, so that granzyme B is clearly very effective in activating a target cell caspase cascade and subsequent death (Adrain et al., 2005).

Although textbook cartoons tend to make caspase activation by granzyme B look like the dominant death pathway, several lines of evidence suggest that this is only one of multiple pathways used by cytotoxic lym-

phocytes to kill target cells. For one thing, why have multiple granzymes of which only B has the critical "aspase" activity required for potent caspase activation? Human blood NK cells are good killers but lack granzyme B (Sayers et al., 2001). Knocking out granzyme B in CTL and activated NK cells slows development of target apoptotic features, but target lysis is only slightly inhibited (Shresta et al., 1995). Furthermore, caspase inhibitors shut down many of the apoptotic features of target death mediated by CTL and NK cells, but the targets still lyse on schedule (Sarin et al., 1998). Thus, one or more alternative, caspase-independent death pathways must also operate. Although one could say they must be necrotic, this merely reignites the debate about what apoptosis and necrosis really mean and points out the need for molecular death pathways.

The paper from Judy Lieberman's lab (Martinvalet et al., 2005) in this issue provides good evidence that reactive oxygen species (ROS) may be part of a caspase-independent death pathway triggered by cytotoxic lymphocytes. ROS can be generated after damage to the mitochondrial inner membrane accompanying the permeability transition, which seems to be one route for releasing caspase activators from the intramembrane space (Green and Kroemer, 2004). But it is generally not clear if this type of mitochondrial damage in itself can lead to the rapid death triggered by cytotoxic lymphocytes or whether caspases really do the job and the crippled mitochondrial function and ROS generation are an epiphenomenon. Thus it was hard to interpret evidence for damage to the mitochondrial inner membrane induced by granzymes B (Alimonti et al., 2001; Thomas et al., 2001) and C (Johnson et al., 2003).

Martinvalet et al. (2005) extend these observations to the other major granule protease, granzyme A. Like granzyme B, introduction of granzyme A into the cytoplasm induced rapid production of ROS and loss of mitochondrial membrane potential that was insensitive to caspase inhibitors. But granzyme A did not induce permeabilization of the mitochondrial outer membrane, so apoptotic mediators from the intramembrane space were not released, nor was the effect sensitive to bcl-2 expression. These results suggested that granzyme A could induce cell death via a novel pathway pathway involving ROS. Indeed, antioxidants and inhibitors of mitochondrial permeability transition gave striking inhibition of cell lysis, not only by granzyme A but also by CTL. Here is evidence that ROS are not just an epiphenomenon.

Both the hydroethidium signal and the ROS inhibitors used make a case for superoxide anion involvement, but superoxide reacts with a limited spectrum of biological molecules (Halliwell and Gutteridge, 1999), arguing against a widespread oxidation of the cell interior. Follow-up studies could test whether superoxide reacts with mitochondrial Fe-S proteins or nitric oxide, but at this point the downstream components of this death pathway remain undefined. ROS may also play critical role(s) in the ultimate disposition of the dying cell. ROS can activate pathways leading to degradation of target cell DNA and promote phosphatidylserine flip (Kagan et al., 2002), ensuring recognition and phagocytic cleanup of target cells. Although some cell death pathways in other cells show ROS production and attenuation by antioxidants, their relevance is unclear because cytotoxic lymphocytes kill so much more rapidly.

Martinvalet et al.'s results also suggest that ROS production is required for nuclear localization of the SET complex, components of which mediate granzyme A-induced DNA fragmentation (Martinvalet et al., 2005). The authors suggest that SET complex translocation is an oxidative stress response and ROS might promote nuclear colocalization of granzyme A and relevant substrates. Generally, it would seem unlikely that nuclear events would be relevant to rapid cell lysis, and CTL effectors can readily kill enucleated targets. Nevertheless, ROS production in CTL targets may serve to ensure irreversible cell death accompanied by DNA degradation.

One potential link between granzyme A-induced ROS and cell death is a redox active protein in the SET complex, Ape1/ref-1. Lieberman's lab has previously shown that Ape/ref-1 is a substrate for granzyme A, but not granzyme B, and noncleavable forms protect from granzyme A-induced cell death (Fan et al., 2003). A primary function of ref-1 is maintaining reduced thiols and DNA binding of transcription factors, such as AP-1, and ref-1 can inhibit intracellular ROS production. Thus, cleavage of Ape/ref-1 by granzyme A may promote oxidative stress and prevent repair of oxidative protein damage. How this relates to cell death remains to be determined.

The Martinvalet et al. paper is significant because it provides clear evidence that intracellular ROS production is part of a cytotoxic lymphocyte-induced, caspase-independent death pathway. More work is required before this is firmly established and the downstream players identified, but if one accepts the idea that heroic cytotoxic lymphocytes evolved mainly to fight off nasty intracellular pathogens, it is satisfying to think the good guys would evolve molecularly distinct, redundant death pathways that together would beat any single inhibitors the bad guys might evolve.

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