

Retinoic acid receptor antagonism *in vivo* expands the numbers of precursor cells during granulopoiesis

CR Walkley^{1,3}, Y-D Yuan⁵, RAS Chandraratna^{4,5} and GA McArthur^{1,2,3}

¹Molecular Oncology Laboratory, Peter MacCallum Cancer Institute, East Melbourne, Australia; Department of Haematology and Medical Oncology, Peter MacCallum Cancer Institute, East Melbourne, Australia; and ³Department of Medicine, St Vincent's Hospital, University of Melbourne, Fitzroy, Australia; ⁴Retinoid Research, Department of Chemistry, Allergan Inc., Irvine, CA, USA; and ⁵Retinoid Research, Department of Biology, Allergan Inc., Irvine, CA, USA

The role/s of retinoids in granulopoiesis has been recognised for many years, being powerful differentiation inducers. The physiological role/s of retinoic acid receptor (RAR)-mediated signalling during adult haemopoiesis has by contrast proved more elusive. The recent generation of highly specific pan-RAR antagonists has now made possible an assessment of the specific physiological role/s of RAR signalling, allowing the separation for the first time of the RAR and RXR pathways. Mice were treated with AGN194310, a synthetic retinoid that antagonises the physiological function of the three RAR isotypes (α , β , γ) but does not interact with RXRs. Analyses of the granulocytic lineage using Gr-1, c-Kit and CD11b antibodies, demonstrated that granulocyte numbers were strikingly increased across haemopoietic compartments in all AGN194310-treated mice. A significant increase in the frequency of progenitor cells containing granulocytes was observed in the bone marrow of mice following treatment with AGN194310. In contrast we were not able to detect any differences in cell death of either mature granulocytes or granulocytic progenitors from AGN194310-treated mice compared with control animals. These data demonstrate an essential role for RAR signalling in regulating the numbers of granulocytic precursors *in vivo*.

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Introduction

Retinoic acid and related molecules are the physiologically active derivatives of vitamin A that are important in a wide range of cell types for controlling differentiation, proliferation and apoptosis. These molecules are essential for normal development and embryogenesis in mammals.¹

The physiological effects of retinoids are mediated through direct binding to the ligand binding domain of retinoic acid receptors (RARs) or retinoid X receptors (RXRs), members of the nuclear hormone receptor superfamily.² In the physiological setting RARs heterodimerize with RXRs, and in turn this heterodimer binds DNA at retinoic acid response elements (RARE) in the regulatory sequences of target genes. In the unliganded state, RAR/RXR heterodimers bind to RARE and form repressive complexes through binding SMRT and NCo-R co-repressor complexes.³ On ligand binding the co-repressor complexes are released and co-activator complexes associate with the RAR/RXR dimer to activate gene transcription.^{4–6} Three isotypes of RARs and RXRs have each been identified: α , β and γ . Each RAR isotype is expressed as a number of isoforms.²

Currently several methods are available to study RAR func-

tion *in vivo*: gene targeting to generate null alleles of RARs, dietary deprivation models and the use of receptor specific ligands to antagonise the physiological function of RARs. Gene targeting studies have revealed a vital role for RAR α and RAR γ in granulopoiesis.⁷ Analysis of the myeloid cells derived from the bone marrow of mice null for both RAR α 1 and RAR γ revealed a block to differentiation at the colony forming stage of granulopoiesis. This result confirms the critical role for RARs in the development of granulocytes and demonstrates that loss of expression of RARs leads to a block in myeloid differentiation. A block to granulopoiesis at the promyelocytic stage was also observed when a carboxyl terminal truncation of RAR α or the PML/RAR α fusion gene, characteristic of acute promyelocytic leukaemia, is expressed in myeloid cells.^{8–10} This block to differentiation was shown to be due to the truncated RAR α or PML/RAR α functioning in a dominant negative manner, the effects of which can be overcome with supra-physiological levels of retinoic acid. Retinoic acid has also been demonstrated to shift the differentiation of progenitor cells away from the erythroid/monocytic lineages and toward the granulocytic lineage, suggesting a critical role for RAR signalling at multiple levels of granulocytic differentiation.^{11,12}

While informative, gene targeting models in mice are limited due to the presence of numerous RAR isotypes and isoforms resulting in the potential for functional redundancy.² In addition the use of RAR null models is further complicated by embryonic/juvenile lethality, restricting analysis of the effects of RARs to early developmental settings.^{13–16} A further limitation of receptor null studies is the resulting non-physiological transcriptional state of the RAR/RXR complexes with the absence of a receptor and transcriptional complexes being neither repressive nor activating failing to replicate the absence of the effects of retinoic acid.

To overcome some of these limitations, dietary deprivation models have been developed and these provide insight into the effects of retinoids in both development settings and in the adult animal.^{17,18} Vitamin A deficiency models have demonstrated a role of retinoids in controlling granulopoiesis *in vivo*, with loss of vitamin A resulting in a myeloid expansion across haemopoietic compartments while no other lineages are affected.¹⁷ However, dietary deprivation models are unable to distinguish between the physiological functions of RARs and RXRs. Unfortunately to date neither gene-targeting nor vitamin A deficiency models have allowed for the study of the specific role/s of the RARs in adult differentiation and homeostasis. As a consequence the specific role/s of the RARs in adult differentiation are currently unknown.

Retinoid receptor specific ligands, both agonists and antagonists, have recently been developed.^{19–22} These compounds are valuable because they allow the effects of the RARs to be studied using either selective antagonism or activation whilst maintaining receptor complexes, expanding on insights gained from receptor knock-out studies. The use of high affini-

Correspondence: G McArthur, Molecular Oncology Laboratory, Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, St Andrew's Place, East Melbourne, Victoria, 3002, Australia; Fax: (61) 3 9656 1411

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ity pan-RAR antagonists allows for the first time the separation of the RAR and RXR pathways *in vivo* in an adult context and thus allows the delineation of the role/s of this receptor family to normal differentiation. One such ligand is AGN194310, a pan-RAR antagonist.²³ Previous studies utilising pan-RAR antagonists have revealed that pharmacological RAR antagonism induces developmental defects reminiscent of vitamin A deficiency syndrome when administered during pregnancy and prevents the retinoic acid induced differentiation of keratinocytes.^{19,22} Pan-RAR antagonists have been shown to significantly affect haemopoietic differentiation, accelerating the differentiation of long- and short-term repopulating haemopoietic stem cells during *in vitro* culture.²⁴

In this study we have sought to identify the physiological contribution of RAR signalling as a whole to normal adult mouse haemopoiesis. Through use of a pan-RAR antagonist we were able to assess the specific contribution of RAR signalling to adult haemopoiesis, whilst not affecting RXR-specific pathways. We have observed a significant granulocytic expansion across haemopoietic compartments of all mice treated with AGN194310. We describe a strategy that utilises flow cytometry to examine the differentiation and proliferative potential of granulocyte precursors. Using a combination of this flow cytometry-based strategy and progenitor assays, we have carried out a detailed examination of haemopoiesis that reveals that the effects of RAR antagonism were associated with a significant expansion of the granulocytic precursor population, and that changes in the differentiation of these precursors most likely accounts for the granulocytic expansion observed in the mice. We also demonstrate that culture of primary granulocytic precursors *ex vivo* with AGN194310 delays the differentiation of these cells. These data demonstrate the critical role of RAR signalling in regulating normal precursor differentiation during adult granulopoiesis *in vivo*.

Materials and methods

Dosing of mice with AGN194310

Five-week-old (34–37 days) female C57Bl/6j mice (four generations backcrossed on to C57Bl/6j background from C57Bl/6j 129/SV hybrid, Peter MacCallum Cancer Institute Animal Facility) were dosed for 10 days with the pan-RAR antagonist AGN194310 (Allergan, Irvine, CA, USA) at a dose of 0.5 mg/kg/day. Analysis was undertaken on the 11th day. Drug was administered via oral gavage, dissolved in DMSO and diluted through peanut oil as vehicle (dosed at 5 ml/kg); control animals were dosed with DMSO and vehicle alone.

Flow cytometry analysis

Bone marrow, spleen and thymus were collected and a single cell suspension was prepared for flow cytometric (FACS) analysis. Cells (5×10^5) were incubated with combinations of fluorescein isothiocyanate (FITC)-conjugated anti-CD8, anti-Gr-1 (Ly-6G) or anti-IgM antibodies, with phycoerythrin (PE)-conjugated anti-CD4, anti-CD11b (Mac-1), anti-F4/80 or with biotin conjugated anti-c-Kit, anti-Ter-119 or anti-B220 (antibodies from PharMingen, San Diego, CA, USA; except anti-F4/80 from Caltag Laboratories, Burlingame, CA, USA). After staining for at least 15 min on ice, cells were washed twice and streptavidin conjugated reagents applied where required (Streptavidin-FITC or -PE; PharMingen). Following

staining cells were washed, resuspended in PBS/2% fetal bovine serum containing 1 μ g/ml 7-Amino Actinomycin-D (7-AAD; Molecular Probes, Eugene, OR, USA) and analysed on a FACSCalibur interfaced with the Cellquest software program (Becton Dickinson, San Jose, CA, USA).

Isolation and characterisation of immature granulocyte populations and culture

Bone marrow from an AGN194310-treated and a control-treated mouse was collected, and prepared as described above (*Flow cytometry analysis*). The collected samples were divided equally and one aliquot stained with anti-c-Kit and anti-Gr-1 (Ly-6G) and the second aliquot with anti-CD11b (Mac-1) and anti-Gr-1. Samples were sorted on a FACStar^{plus} flow cytometry system interfaced with Cellquest software (Becton Dickinson). Following sorting, 5×10^4 cells were cytocentrifuged for morphological assessment, the remaining cells (approximately equal numbers from each sorted region) were cultured in DMEM containing 20% foetal bovine serum (FBS, Trace Scientific, Sydney, Australia), 15% BHK-SCF cell conditioned media (source of SCF, a gift from S Collins, Divisions of Clinical Research and Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA, USA), 1000 U/ml rhG-CSF (Amgen, Thousand Oaks, CA, USA), 0.5% ciprofloxacin (2 mg/ml, Bayer, Sydney, Australia) and 10 μ g/ml 5-bromo-2-deoxyuridine (BrdU; Sigma, St Louis, MO, USA) for 3 days. Following culture, a sample of cells was prepared by cytocentrifugation and the cells reanalysed using FACS analysis (as described above). Approximately one-third of the cells were fixed in ethanol and BrdU analysis undertaken. Following fixation in 95% ethanol, cells were permeabilised and stained with 5 μ l of anti-BrdU antibody (Becton Dickinson). After staining for 30 min, cells were washed twice and secondary antibody applied (FITC-anti mouse IgG; Cappel, West Chester, PA, USA). Following staining cells were washed, resuspended in propidium iodide (10 μ g/ml (Sigma) in sodium citrate buffer) and analysed on a FACSCalibur interfaced with the Cellquest software program (Becton Dickinson).

For culture of primary granulocytic progenitors with AGN194310, cells were isolated as described and placed in culture with 10^{-7} M AGN194310. No BrdU was used in these culture experiments.

Progenitor cell assays

Colony-forming cell assays were performed using both 3% methylcellulose-based media and 0.3% agar-based media. For agar-based assays, bone marrow cells (5×10^4) were cultured for 7 days as 1 ml cultures. Agar (0.3% (Difco, Detroit, MI, USA) in Dulbecco's modified Eagle media (DME), Gibco, Grand Island, NY, USA) cultures were prepared using 20% FBS (Trace) and the following cytokines: rhG-CSF (1000 U/ml, Amgen), rmGM-CSF (1000 U/ml), rmlL-3 (1000 U/ml), and rmSCF (100 ng/ml, recombinant cytokines from PeproTech, Rocky Hill, NJ, USA). Agar cultures were scored on days 7 and 14. For progenitor survival assays cytokine (G-CSF and GM-CSF) was withheld for 24 or 48 h, then added in 200 μ l of DME containing 20% FCS.

For methylcellulose-based assays, methylcellulose (3% in Iscove's modified Dulbecco's media; Gibco) supplemented with 20% FBS (Trace), 2 U/ml erythropoietin (Janssen-Cilag,

Sydney, Australia), 10% BHK-SCF cell conditioned media (final concentration equivalent of 100 ng/ml purified recombinant SCF) and 1% X63Ag8-653-IL3 cell conditioned media (Ref. 25), source of IL3, 1000 U/ml). Erythroid colonies were scored on day 8.

Analysis of cell death of mature granulocytes

Analysis of apoptosis was carried out on spleen cells (2×10^7) using a modified method of that previously described.¹⁷ Briefly, spleen cells were cultured overnight for 16 h in DMEM containing 10% FBS (Trace) and 1% ciprofloxacin (2 mg/ml, Bayer). Cells were collected after incubation and stained with PE conjugated anti-CD4 or CD8 or CD11b or Gr-1 (PharMingen). After incubation for at least 15 min, cells were washed once in 2% FBS/PBS, then in PBS. Cells were resuspended in 0.2 ml of annexin binding solution (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) containing 0.02 µg of FITC conjugated Annexin-V (PharMingen) and 1 µg/ml 7-AAD (Molecular Probes). Binding of Annexin-V was determined by flow cytometry on a FACSCalibur interfaced with Cellquest software. Cultures were also undertaken in the presence of AGN194310 and cell death then determined as described.

Cell death was also assessed by quantitation of hypodiploid mature granulocyte populations following overnight culture of spleen cells as described. Cells were stained with FITC conjugated Gr-1, fixed in 95% ethanol and stained with propidium iodide/RNAase A (PI (Sigma), 10 µg/ml in sodium citrate buffer; RNAase A (Sigma), 0.25 mg/ml in PBS). Cells were incubated in darkness with the PI/RNAase stain for 30 min at 37°C, then stored on ice until analysed by flow cytometry.

Histological and haematological analysis

Blood smears of peripheral blood and cytospin preparations of bone marrow, spleen and thymus derived cells were prepared and stained with May-Grünwald-Giemsa solution for morphological assessment. Peripheral blood was analysed on a Sysmex K1000 machine and by manual counts.

Statistical analysis

Statistical analysis was performed using an unpaired Student's two-tailed *t*-test to assess differences between the means. The standard error of the mean was used to determine the deviation from the mean.

Results

Granulocytic expansion following retinoic acid receptor antagonism *in vivo*

To evaluate the role/s of RARs in normal adult cellular differentiation we utilised the pan-RAR antagonist AGN194310 to pharmacologically antagonise physiological RAR function. AGN194310 displays higher affinity for all three isoforms of the RARs than the endogenous ligand ATRA (ATRA *K_d*: RARα 15 nM, RARβ 13 nM and RARγ 18 nM; AGN194310 *K_d*: RARα 3nM, RARβ 2 nM and RARγ 5 nM,²³ AGN194310 does not show affinity for RXRα, β or γ). This compound does not

interact with or antagonise RXRs, providing a means of specifically determining the contribution of signalling through the RAR isoforms to haemopoiesis. Based on highly reproducible pharmacokinetic data of AGN194310 in mice, we estimate the serum concentration of drug to be in the range of 100 to 200 ng/ml (0.2–0.4 µM). After 10 days of continuous treatment with AGN194310 all mice appeared outwardly healthy and no significant pathology was observed on autopsy.

Analysis of the bone marrow and spleen cells revealed that 100% of mice treated with AGN194310 had an increase in the number and proportion of granulocytes by approximately two-fold (Figure 1a and b). The total number of Gr-1 (Ly-6G) positive cells per femur was increased from a mean of 5.2×10^6 for control treated mice (*n* = 7) to a mean of 1.0×10^7 for AGN194310 treated mice (*n* = 8). This increase was significant (*P* ≤ 0.05) and observed in all mice exposed to AGN194310 (Figure 1a). In contrast to the granulocyte populations, overall cellularity of the bone marrow was not significantly altered following AGN194310 treatment ($2.1 \pm 0.4 \times 10^7$ cells/femur in control mice compared with

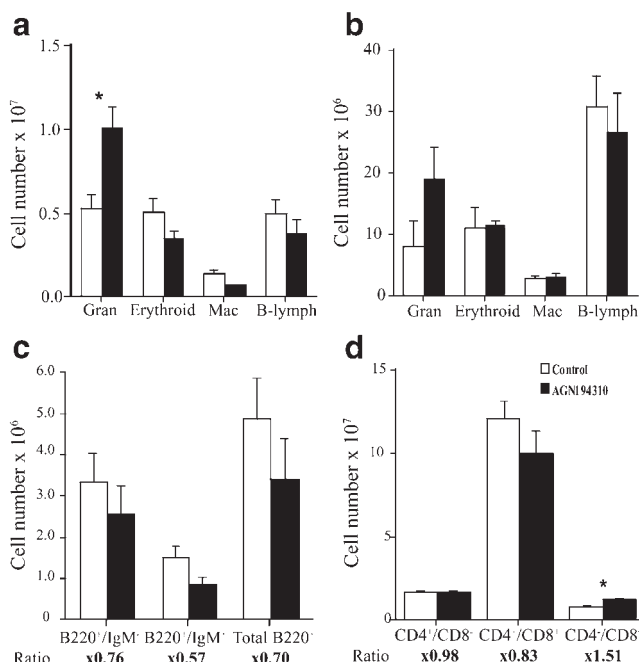


Figure 1 RAR antagonism results in an expansion of the granulocyte populations in the bone marrow and spleen. (a) Number of cells/femur of each lineage (Gran, mature granulocytes (Gr-1⁺), erythroid cells (Ter119⁺), Mac, macrophages (F4/80⁺), B lymph, B lymphocytes (total B220⁺)) in the bone marrow following treatment with AGN194310 (*n* = 8) or control (*n* = 7) as determined by FACS analysis. (b) Number of cells of each lineage in the spleen following treatment with AGN194310 (*n* = 8) or control (*n* = 7). (c) Analysis of the absolute number of B lymphocyte populations in the bone marrow (B220⁺/IgM⁻, pre-B cells; B220⁺/IgM⁺, immature B cell; total B220⁺, sum of all B220 expressing cells) following treatment with AGN194310 (*n* = 8) or control (*n* = 7) as determined by FACS analysis. Ratio, the number of cells in AGN194310 treated/control mice. (d) Number of cells of each lineage (CD4⁺/CD8⁻, CD4 single positive thymocyte; CD4⁺/CD8⁺, double positive; CD4⁻/CD8⁺, CD8 single positive) in the thymus following treatment with AGN194310 (*n* = 8) or control (*n* = 7) as determined by FACS analysis. Ratio, the number of cells obtained from AGN194310 treated/control mice. Percentage of cells follows similar trend to absolute number data. Data expressed as the mean ± s.e.m. (**P* ≤ 0.05). All *in vivo* data are pooled from multiple experiments.

$2.5 \pm 0.3 \times 10^7$ cells/femur in AGN194310 treated mice, $P=0.42$).

AGN194310 treatment did not result in changes in the absolute numbers of macrophages, erythroid cells and total B lymphocyte populations in the bone marrow, demonstrating that RAR antagonism selectively affects the granulocyte lineage in the bone marrow (Figure 1a). Further analysis of the B lymphocyte population did not show a change in the relative proportion of pre-B cells compared with immature B cells, suggesting that RAR antagonism does not affect the differentiation of B lymphocytes (Figure 1c).

Expansion of the granulocyte population occurred in the spleen, with a mean of 8.2×10^6 Gr-1 positive cells/spleen in control mice ($n=7$) compared with a mean of 1.9×10^7 Gr-1 positive cells in AGN194310 treated mice ($n=8$). No significant change in lineages other than granulocytes was observed in the spleen (Figure 1b).

Analysis of thymic T lymphocytes showed an increase in the number and proportion of CD8 single positive cells in mice treated with AGN194310 ($P=0.01$, Figure 1d). It has been previously reported that retinoic acid inhibits thymocyte maturation *in vitro*,²⁶ a result consistent with the effects observed following *in vivo* RAR antagonism. The results obtained with AGN194310 reveal roles for RAR signalling in the development of thymic CD8 single positive T lymphocytes *in vivo*. The effect is specific to this lineage as no change in the thymic CD4 single positive T lymphocytes was observed, nor could changes in cell death be detected.

Analysis of peripheral blood isolated from the mice showed an increase in mature granulocytes and a decrease in platelet and lymphocyte counts following treatment with AGN194310 (Table 1). Only mature granulocytes were observed in the peripheral blood.

Expansion of granulocyte precursor populations by retinoic acid receptor antagonism

The increased numbers of granulocytes in the bone marrow, spleen and peripheral blood may have been due to an increase in the size of the pool of granulocyte precursors, increased proliferation of granulocyte precursors or reduced cell death of mature and/or immature granulocytic cells. To distinguish between the possibilities, granulopoiesis was

Table 1 Analysis of peripheral blood from AGN194310 and control treated mice

Peripheral blood counts	Control treated mice ($n=7$)	AGN194310-treated mice ($n=8$)
White blood cells/ μl ($\times 10^3$)	5.5 ± 1.2	5.0 ± 0.9
Haematocrit (%)	38.9 ± 1.2	39.9 ± 0.6
Erythrocytes/ μl ($\times 10^6$)	7.2 ± 1.2	8.2 ± 0.3
Platelets/ μl ($\times 10^3$)	938 ± 35.3	$749 \pm 76.5^*$
Lymphocytes/ μl ($\times 10^3$)	4.8 ± 0.2	$3.7 \pm 0.1^{\dagger}$
Granulocytes/ μl ($\times 10^3$)	1.0 ± 0.2	$1.5 \pm 0.1^{\dagger}$

Results expressed as mean \pm SEM of the indicated number of mice. Percentages of cells as determined by Sysmex K1000 count; * $P \leq 0.05$, $\dagger P \leq 0.01$. Lymphocyte and granulocyte counts confirmed by differential counts of blood films. Monocytes constitute 3% or less of overall peripheral blood WBC in control or AGN194310-treated mice.

evaluated utilising a flow cytometry-based isolation strategy with c-Kit/Gr-1 and CD11b/Gr-1 labelled bone marrow cells.

Granulocyte populations were first divided based on the level of Gr-1 expression into Gr-1^{bright} and Gr-1^{dim} populations, with the Gr-1^{dim} population further divided into c-Kit/Gr-1^{dim} and c-Kit^{bright}/Gr-1^{dim} (Figures 2 and 3). Following isolation the fractions were cultured for 3 days in SCF and G-CSF containing BrdU as a measure of proliferative potential. After 3 days, the immature CD11b/Gr-1^{dim} cells became morphologically mature granulocytes, as assessed by morphology and Gr-1 expression. 53.6% of the CD11b/Gr-1^{dim} cells incor-

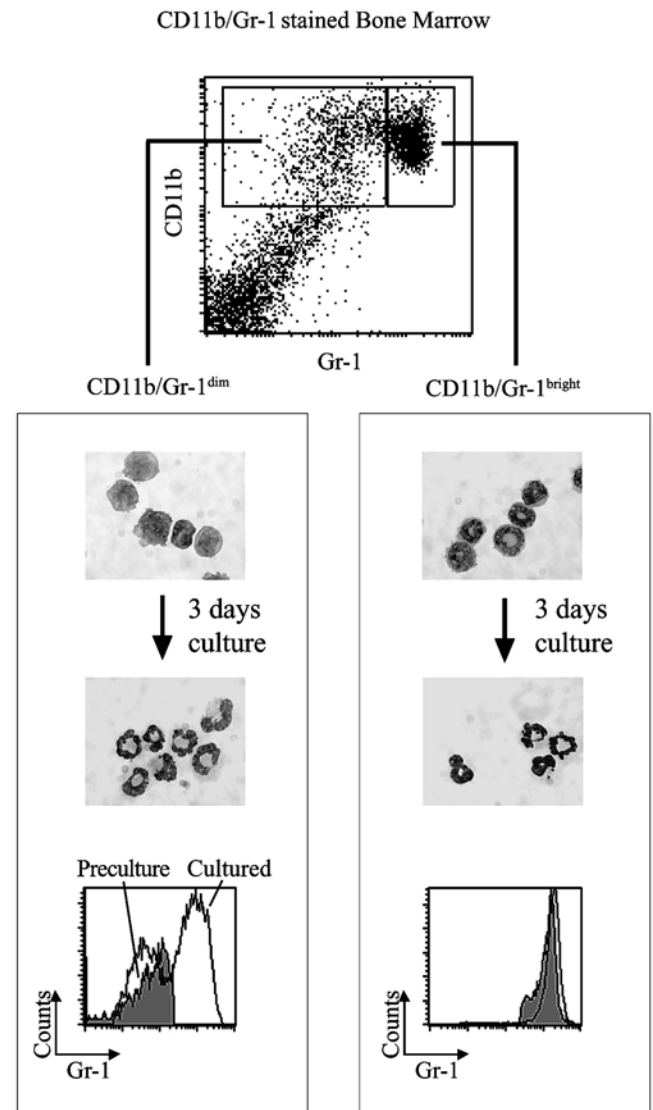


Figure 2 Characterisation of granulocyte populations using CD11b/Gr-1 and *ex vivo* culture. Representative FACS dot plot of bone marrow stained with CD11b/Gr-1 with immature cell (CD11b/Gr-1^{dim}) and mature cell (CD11b/Gr-1^{bright}) fractions isolated. Morphology of the isolated fractions is shown following staining with May-Grünwald-Giemsa. Following 3 days in culture with 20% FBS, 15% SCF conditioned media and 1000 U/ml rhG-CSF the morphological maturation of the CD11b/Gr-1^{dim} cells can be seen when compared with the isolated population. Histograms demonstrate the up-regulation of Gr-1 in the CD11b/Gr-1^{dim} population following 3 days in culture (Gr-1 expression of *ex vivo* cultured cells is shown in black lines overlaid on the Gr-1 expression histogram of the precultured cells in grey). Cells isolated from AGN194310 treated and control mice display similar maturation *ex vivo*.

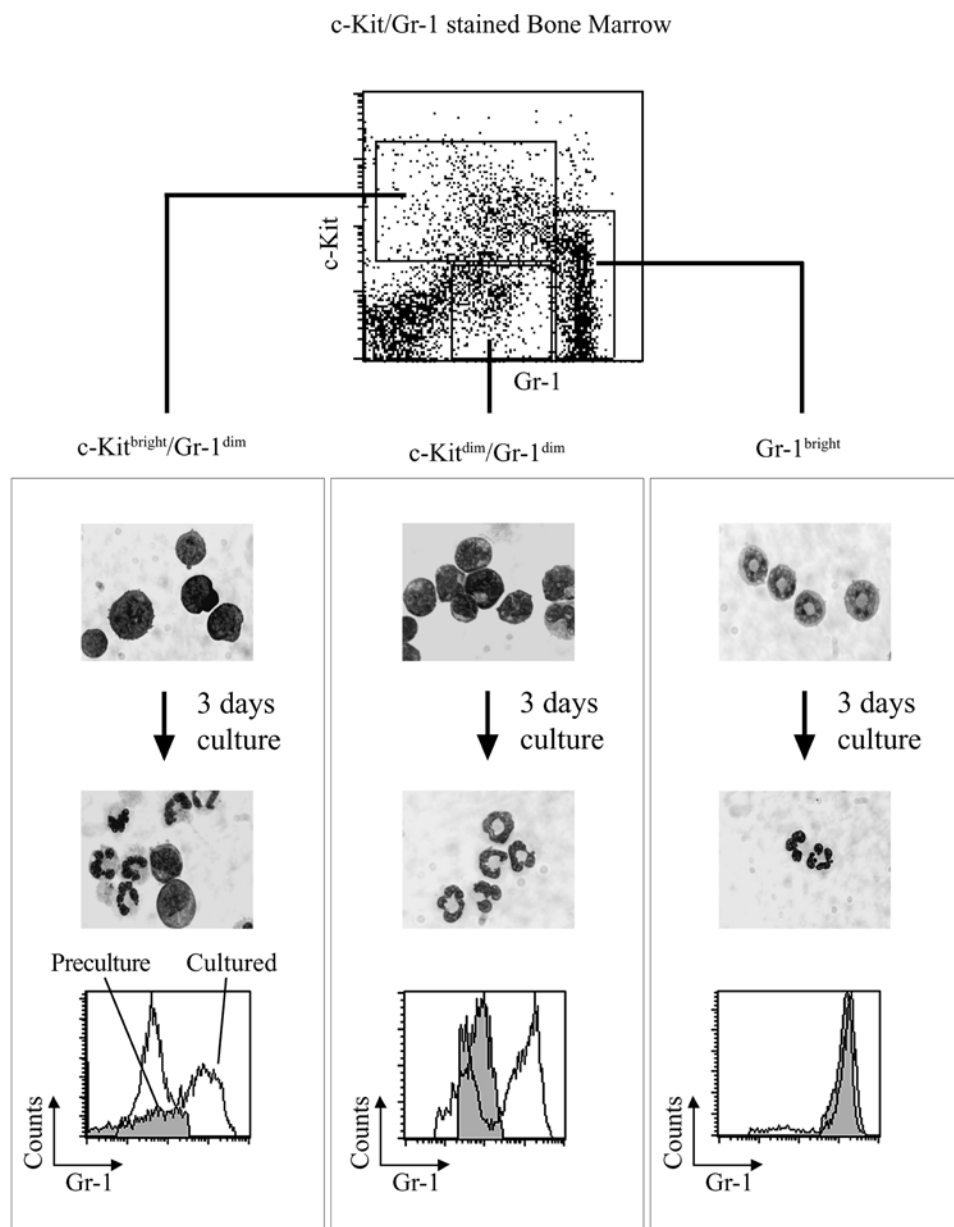


Figure 3 Characterisation of granulocyte populations using c-Kit/Gr-1 and *ex vivo* culture. Representative FACS dot plot of bone marrow stained with c-Kit/Gr-1 with the three isolated fractions as labelled. Morphology of the isolated fractions is shown following staining with May-Grünwald-Giemsa. Following 3 days in culture as described in Figure 2, the morphological maturation of the c-Kit/Gr-1^{dim} and the c-Kit^{dim}/Gr-1^{dim} cells can be seen when compared with the originally isolated population. Histograms demonstrate the up-regulation of Gr-1 on the c-Kit/Gr-1^{dim} and c-Kit^{dim}/Gr-1^{dim} populations following 3 days in culture (Gr-1 expression of *ex vivo* cultured cells is shown in black lines overlaid on the Gr-1 expression histogram of the precultured cells in grey). Cells isolated from AGN194310 treated and control mice display similar maturation *ex vivo*.

porated BrdU during culture compared with 2.1% of the CD11b/Gr-1^{bright} cells (Figures 2 and 4a).

Staining with c-Kit/Gr-1 revealed three populations of cells that display distinct maturation kinetics. 5.8% of the c-Kit^{dim}/Gr-1^{dim} cells incorporated BrdU, are initially morphologically and phenotypically immature, but are able to mature during the culture period. A second immature subset of cells was identified as c-Kit^{bright}/Gr-1^{dim}, 92.3% of these cells incorporated BrdU during culture and also matured during culture. The mature c-Kit^{dim}/Gr-1^{bright} cells failed to incorporate BrdU (3.7%) following culture (Figure 3). The use of c-Kit/Gr-1 staining allows the isolation of three distinct populations of cells, each displaying a distinct proliferative potential. The morpho-

logically immature c-Kit^{dim}/Gr-1^{dim} and the mature c-Kit^{dim}/Gr-1^{bright} cell populations display a poor proliferative capacity *ex vivo*, in contrast to the c-Kit^{bright}/Gr-1^{dim} population that has a very high *ex vivo* proliferative capacity. Granulocytes can therefore be divided into mature (CD11b/Gr-1^{bright} or c-Kit^{dim}/Gr-1^{bright}) and immature (CD11b/Gr-1^{dim} and c-Kit^{bright} or ^{dim}/Gr-1^{dim}) compartments (Figures 3 and 4b).

Strikingly, bone marrow cells from AGN194310 treated mice displayed increased numbers of the less mature CD11b/Gr-1^{dim} and c-Kit^{dim/bright}/Gr-1^{dim} cells, with no change in overall bone marrow cellularity, suggesting that an expanded pool of granulocytic precursor cells is contributing to the myeloid expansion observed in treated mice (Figure 5).

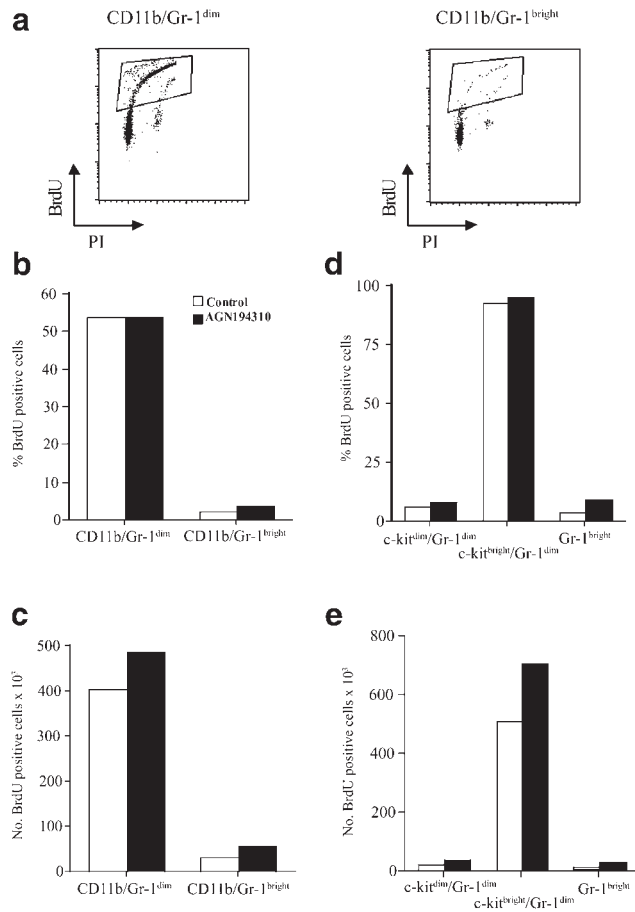


Figure 4 RAR antagonism does not alter the proliferation potential of granulocytic progenitors *ex vivo*, but expands the number of progenitors. The isolated cell populations described in Figures 2 and 3 were cultured as described with the addition of 10 μ g/ml BrdU for the 3-day culture period. Cells were harvested after 3 days and incorporation of BrdU was analysed using FACS. (a) Representative BrdU profiles from CD11b/Gr-1^{dim} and CD11b/Gr-1^{bright} populations. Cells that have incorporated BrdU during the culture period are contained within the marked region. (b) Percentage of cells that have incorporated BrdU in the CD11b/Gr-1 isolated fractions. Note no difference in the percentage of cells that have incorporated BrdU between the AGN194310 derived cells and the control cells. (c) Quantitation of the number of cells that have incorporated BrdU during culture. Whilst the percentage incorporation is similar, the number of CD11b/Gr-1^{dim} cells (immature fraction) capable of incorporating BrdU is increased following AGN194310 treatment. (d) Percentage of cells that have incorporated BrdU in the c-Kit/Gr-1 isolated fractions. (e) Quantitation of the number of cells capable of incorporating BrdU during culture of the c-Kit/Gr-1 fractions. Again, note that the percentages of cells that have incorporated BrdU is similar between AGN194310-treated and control, the absolute number of immature cells is expanded following AGN194310 treatment. Data are from a representative experiment.

Following treatment with AGN194310, there is a significant increase in the absolute numbers of immature cells capable of incorporating BrdU (Figure 4c and e). These results confirm that the expanded cell populations are granulocytic precursors, and that these populations are significantly increased following RAR antagonism. The data obtained from BrdU labelling of the cell fractions demonstrate that whilst the absolute numbers of immature granulocytic precursors is increased following RAR antagonism the proliferation capacity of these

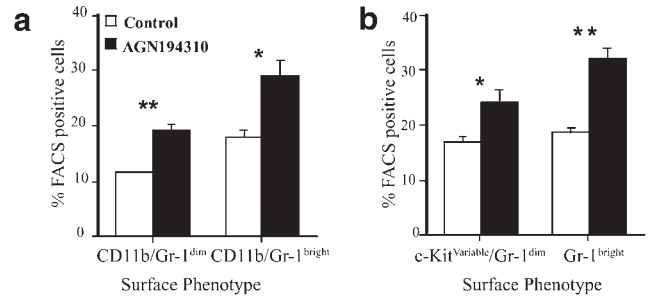


Figure 5 Expansion of immature and mature granulocytes following RAR antagonism *in vivo*. (a) Percentage of immature (CD11b/Gr-1^{dim}) and mature (CD11b/Gr-1^{bright}) cells from AGN194310 treated ($n=8$) and control treated mice ($n=7$). (b) Percentage of immature (c-kit/Gr-1^{dim}; c-Kit includes c-Kit/Gr-1^{dim} and c-Kit^{dim}/Gr-1^{dim}) and mature (Gr-1^{bright}) cells from AGN194310 treated control ($n=8$) and control treated mice ($n=7$). Data expressed as the mean \pm s.e.m. (* $P=0.01$, ** $P=0.05$). All *in vivo* data are pooled from multiple experiments. Note no change in bone marrow cellularity is observed between AGN194310-treated and control mice.

cells *ex vivo* is unchanged when compared with control treated cells.

Increased frequency of granulocytic progenitors following retinoic acid receptor antagonism

In conjunction with the FACS analysis described above, progenitor cell assays were performed on bone marrow cells to determine if AGN194310 treatment resulted in a change in the frequency of granulocytic precursors. Analysis of progenitor cell populations revealed that there was a significant increase in the number of progenitor cells following treatment with AGN194310 (Figure 5). There was an increase in the number of colonies grown in the presence of G-CSF and a significant increase in number grown in GM-CSF (61.0 ± 2.0 progenitors per 5×10^4 whole bone marrow cells from AGN194310 treated mice ($n=3$) and 37.0 ± 1.7 progenitors from control mice ($n=3$), $P=0.01$) from mice treated with AGN194310 (Figure 6a). In contrast, no significant increases were observed in the numbers of colonies of other lineages (data not shown). No differences were observed in erythroid colonies between control-derived and AGN194310-derived bone marrow cells (data not shown).

These data and the data obtained from isolation of immature granulocytic cell fractions by FACS demonstrates that there is a significant expansion of immature granulocytic cells following RAR antagonism *in vivo*, an effect not observed in Vitamin A deficiency models.¹⁷

Retinoic acid receptor antagonism does not alter granulocyte cell death

Although treatment with AGN194310 induced an expansion in the pool of granulocyte precursors it is also possible that RAR antagonism inhibited cell death of granulocytes and thereby increased the numbers of mature granulocytes.

To determine if the changes in cell death were associated with the increased number of mature granulocytes in mice treated with AGN194310, spleen-derived cells were cultured overnight in cytokine-deprived conditions and apoptotic cell death was assessed by annexin-V binding. No change in the

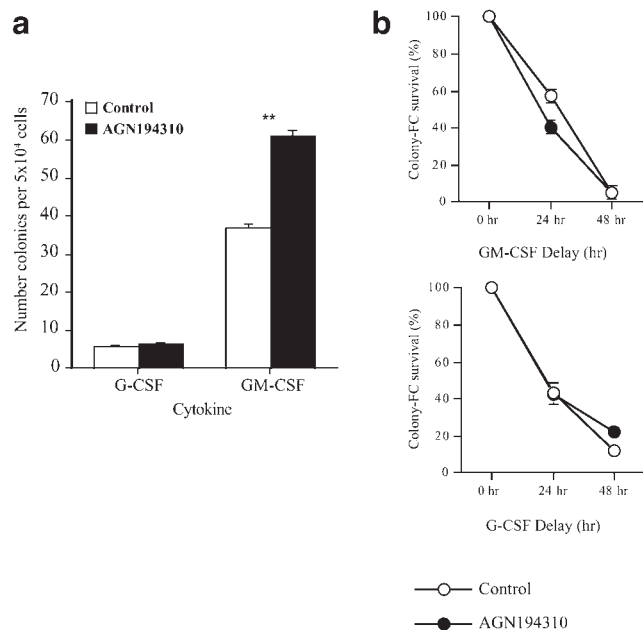


Figure 6 Increased number, but no alteration in apoptosis, of progenitor cells following RAR antagonism. (a) The number of colonies formed from 5×10^4 bone marrow cells derived from AGN194310 ($n=3$) and control ($n=3$) treated mice. Cells were cultured in agar containing either 1000 U/ml rhG-CSF or rmGM-CSF for 7 days and then counted. (b) Bone marrow cells from AGN194310-treated ($n=3$) and control-treated mice ($n=3$) were cultured in semi-solid media as for (a), with the exception that cytokines were added following 24 or 48 h of culture in the absence of cytokine. Colonies were enumerated at day 7 after addition of cytokine. Data expressed as the mean \pm s.e.m. (** $P \leq 0.01$).

relative or absolute amount of cell death was observed between cells derived from mice exposed to AGN194310 and control-treated mice (Table 2). This was the case for T lymphocytes, macrophages and granulocytic cells. Propidium iodide staining for hypodiploid granulocytic cells also failed to show any difference in survival of mature granulocytes between AGN194310-treated and control mice (Table 2). The continued presence of the antagonist in the *ex vivo* cultures did not protect mature granulocytes from death, as cultures from treated mice containing AGN194310 had similar

Table 2 Mature granulocytes from AGN194310-treated and control mice display similar rates of apoptosis *ex vivo*

Cell lineage	% viability (annexin negative/7AAD negative)	
	Control-treated ($n=4$)	AGN194310-treated ($n=4$)
Gr-1	4.6 ± 1.2 3.4 ± 1.1^a	6.2 ± 1.9 3.0 ± 1.2^a
CD11b	2.7 ± 1.1	1.7 ± 0.5
CD4	16.2 ± 6.7	17.7 ± 6.2
CD8	14.3 ± 3.7	10.0 ± 4.3

Results expressed as mean \pm s.e.m. Gr-1, granulocytes; CD11b, macrophages; CD4 and CD8, T lymphocytes. Viability determined by negative Annexin-V staining and 7AAD exclusion.

^a Percentage viable mature granulocytes as determined by propidium iodide staining to exclude cells with hypodiploid DNA content ($n=3$).

amounts of cell death as those that did not contain antagonist (data not shown).

An alternative possibility is that RAR antagonism inhibits the cell death of granulocytic progenitors, a population that is expanded significantly following exposure to AGN194310 (see Figure 5). To assess this, progenitor cell assays were established from bone marrow cells derived from AGN194310 treated and untreated mice and cytokine was withheld for 24 or 48 h and the number of colonies formed assessed after 7 days in culture. There was no increased survival of progenitors stimulated with GM-CSF and a slight, but not significant, increase in survival of progenitors stimulated with G-CSF (48 h delay of cytokine addition, Figure 6b). These data further support the contention that reduced apoptosis is not the underlying cause of the granulocytic expansion observed following treatment with the pan-RAR antagonist AGN194310, and that modulation of cell death does not appear to be a physiological role of RARs during granulopoiesis.

The failure to describe a change in cell death between AGN194310-treated and control mice strongly supports the hypothesis that the effects of AGN194310 are the result of an expanded population of granulocytic precursor cell/s. The expanded precursor population may then lead to the proportional overall expansion of granulocytes in AGN194310-treated mice as cell death rates remained unaffected.

Retinoic acid receptor antagonism delays the maturation of granulocytic precursors *ex vivo*

The data derived from the *in vivo* administration of AGN194310 demonstrated that there was an expansion in the granulocytic precursor pool following RAR antagonism. Such an increase may be the result of a shift in the balance between differentiation and proliferation, with RAR antagonism disrupting the equilibrium by impairing differentiation causing increased proliferation and expansion of the precursor pool. To further analyse the effects of AGN194310 on granulocytic precursors, cells were isolated using the FACS strategy as describe in Figure 2. Following isolation cells were placed in culture and maintained in media containing 10^{-7} M AGN194310 (for cells derived from AGN194310-treated group) or treated with vehicle alone (for cells derived from control-treated group). To determine the kinetics of maturation of these precursor populations, cells were analysed by morphology and for phenotypic maturation as determined by the development of the Gr-1^{bright} surface phenotype that we have demonstrated to be restricted to mature granulocytes (see Figures 2 and 3).

As can be clearly seen in Figure 7, cells cultured in the presence of AGN194310 are delayed in their ability to develop the Gr-1^{bright} phenotype, consistent with the *in vivo* data demonstrating that RAR antagonism changes the differentiation of granulocytic progenitors. Culture with AGN194310 does not alter the expression of Gr-1 on the mature Gr-1^{bright} population, and also does not affect the survival of cells in culture (data not shown). Analysis at later time-points than 3 days (up to 5 days of culture) was difficult as a result of a significant decrease in viability of the cultures, irrespective of the presence of the antagonist. We observed that the AGN194310 treated cultures, however, never attained the same proportion of Gr-1^{bright} cells as the control-treated cultures suggesting that *ex vivo* RAR antagonism does impart a degree of inhibition of maturation on granulocyte precursor populations. This result confirms that RAR activation is important for the differen-

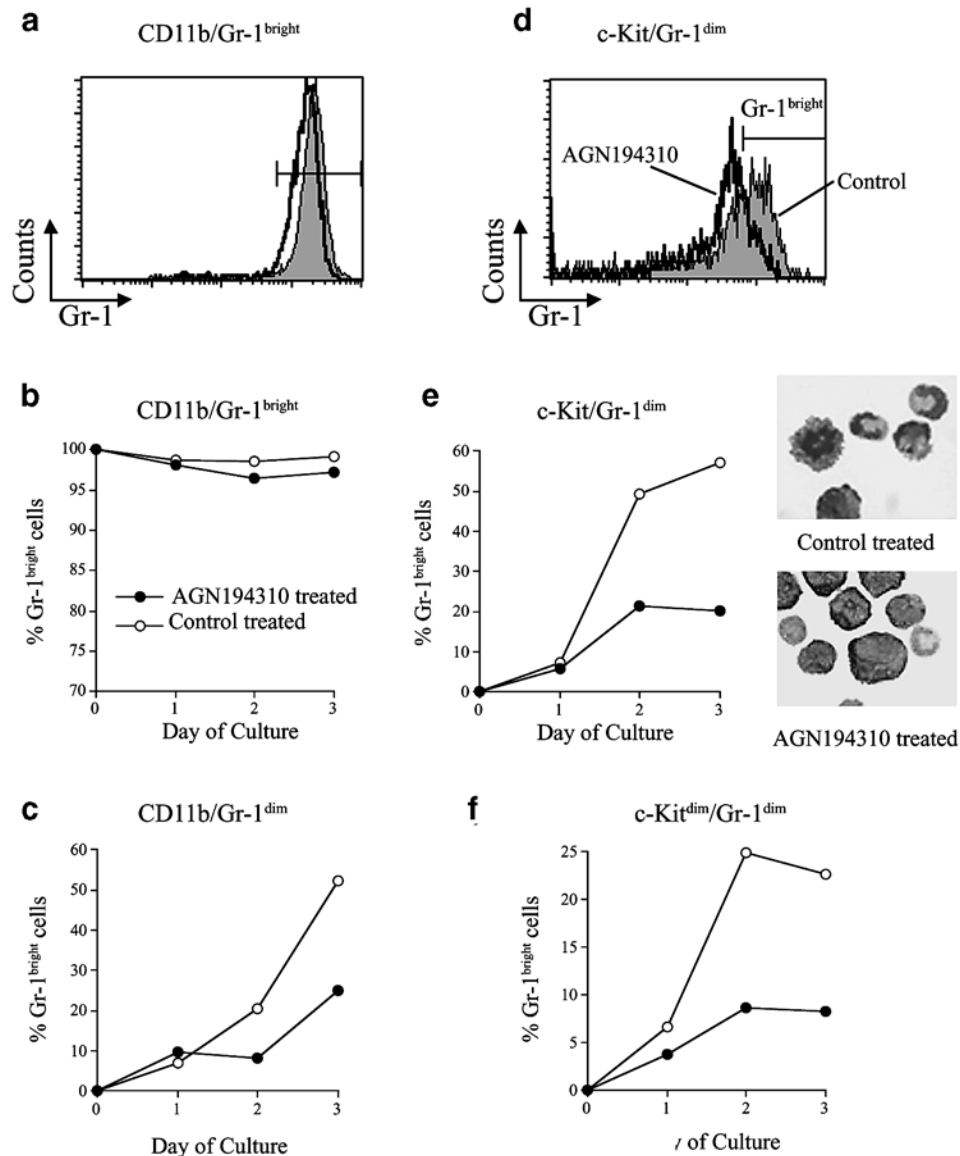


Figure 7 RAR antagonism impairs the differentiation of granulocyte progenitors *ex vivo*. Cells derived from AGN194310-treated and control mice were isolated as described in Figures 2 and 3. Cells were cultured with SCF and rhG-CSF for 3 days in media containing 10^{-7} M AGN194310 (control cells were cultured in media containing vehicle alone). (a) Gr-1 expression at day 3 of culture of the CD11b/Gr-1^{bright} populations from a AGN194310-treated culture and a control culture. (b) Percentage of cells that had the Gr-1^{bright} surface phenotype over 3 days of culture. (The Gr-1^{bright} or mature cell population was defined as indicated by the cursor in A). Culture with AGN194310 did not increase cell death compared with controls in all populations tested. (c) Kinetics of up-regulation of Gr-1 expression on CD11b/Gr-1^{dim} cells. (d, e) Gr-1 expression and morphology of c-Kit/Gr-1^{dim} cells after 3 days of culture. (f) Gr-1 expression of c-Kit^{dim}/Gr-1^{dim} cells.

tiation and maturation of granulocyte precursors, and that specific antagonism of the RARs is able to delay and potentially inhibit the differentiation of granulocytic precursors *ex vivo*.

Discussion

Chronic *in vivo* administration of retinoid agonists results in neutropenia, whilst dietary deprivation of vitamin A results in an expansion of granulocytic cells. Although retinoid ligands are essential for normal granulopoiesis, the specific contribution of their cognate nuclear hormone receptors the RARs and RXRs to granulopoiesis is unclear. It is also unclear if these changes in granulopoiesis are due to changes in differentiation of granulocyte precursors, altered proliferation

and/or changes in cell death. To investigate these mechanisms, we have characterised a strategy utilising flow cytometry to examine the differentiation and proliferative potential of granulocyte precursors. We have used this strategy in conjunction with progenitor cell assays to evaluate the specific role/s of RARs in normal granulopoiesis by utilising a pan-RAR antagonist to block the physiological function of RARs.

We have demonstrated that physiological RAR antagonism results in a rapid expansion of myeloid cells across haemopoietic compartments in all mice treated with the pan-RAR antagonist. This expansion is associated with an expanded pool of granulocyte precursors in the bone marrow, an increase in immature granulocyte fractions (as determined by surface antigen phenotyping) in the bone marrow and no detectable changes in cell death of either the mature granulo-

cytes or granulocytic progenitors. Also observed was a specific increase in the number and percentage of thymic CD8 T lymphocytes. We have described a FACS strategy that enables the isolation and analysis of granulocytic progenitors. Using this protocol we confirm that the expanded cell populations are granulocytic precursors and that pan-RAR antagonism does not alter the proliferative capacity of these precursors *ex vivo*, as determined by BrdU incorporation. We also demonstrate that AGN194310 is able to delay the differentiation of granulocytic progenitors *ex vivo*. These data demonstrate that one of the physiological roles of RAR activation is to ensure the normal differentiation of granulocytic precursors, thus maintaining homeostasis during granulopoiesis.

Through use of the pan-RAR antagonist AGN194310 we observe a significant expansion of granulocytic precursor cells. As can be seen in Figure 5, treatment with AGN194310 results in an increase in both the mature and immature granulocyte populations as determined by use of CD11b/Gr-1 and c-Kit/Gr-1. The expansion of both the immature and mature granulocyte populations observed using surface antigen phenotyping occurs proportionally for both populations. We also observe a significantly expanded number of GM-CFUs in mice treated with the antagonist. The increases in the mature populations of granulocytes appear to be less than would be anticipated given the high proliferative potential of the CD11b/Gr-1^{dim} and c-Kit^{bright}/Gr-1^{dim} populations (see Figure 5) and the significant increase in CFUs, a result that suggests that pan-RAR antagonism *in vivo* alters the balance between differentiation and proliferation during granulopoiesis. This hypothesis is further supported by the delay in maturation and differentiation of the granulocytic precursor populations observed during *ex vivo* culture (Figure 7).

The data presented herein is in contrast to the report of Kuwata *et al*¹⁷ who described a model of murine dietary vitamin A deficiency. One significant difference is the rapidity of the observed granulocytic expansion. Using AGN194310 we observed a significant myeloid expansion after only 10 days of treatment compared with the dietary deprivation model that took approximately 14 weeks before effects on granulopoiesis became evident. Of greater significance, Kuwata and colleagues observed no alteration in the frequency of colony-forming cells between the groups, but describe a significant decrease in the apoptosis of mature granulocytes from vitamin A-deficient mice. The data derived from use of the pan-RAR antagonist AGN194310 do not support changes in cell death as the cause of the observed myeloid expansion. We were unable to detect any differences in cell death of mature granulocytes between the control and AGN194310 treated mice using annexin staining and propidium iodide staining for hypodiploid DNA (see Table 2). The continued presence of the antagonist in cultures was not able to protect the mature granulocytes from death. Altered cell death of granulocytic precursors could result in the significant expansion of mature granulocytes observed after treatment with the pan-RAR antagonist, but no enhanced survival of progenitor cells in response to withdrawal of cytokines was evident following treatment with AGN194310 (see Figure 6b).

The disparity in the reported causes of myeloid expansion may result from the use of an antagonist to RARs compared with dietary deprivation of vitamin A. The pan-RAR antagonist used in this study does not display affinity for RXRs and so will not affect cellular pathways that are independent of RARs, but dependent on RXRs. Vitamin A deficiency results in the depletion of both all-*trans* retinoic acid (ligand for RARs) and 9-*cis* retinoic acid (ligand for both RARs and RXRs), thereby

affecting RXR, as well as RAR signalling. It has previously been demonstrated that RXRs play an important role in regulating apoptosis of T lymphocytes, acting with RARs in the induction of spontaneous apoptosis but cooperating to prevent activation-induced apoptosis (reviewed in Ref. 27). This may also be the case during granulopoiesis, where specific RAR antagonism leads to an expansion of granulocytic precursor cells and a delay of differentiation, whilst loss of both RAR and RXR signalling deregulates apoptosis.

The data obtained using AGN194310 are consistent with the observations of Kastner *et al*,²⁸ who have utilised gene targeting strategies to describe the role of RAR α in granulopoiesis as a key mediator of the response to retinoids during granulopoiesis. RAR α was shown to be a bidirectional modulator of granulopoiesis, acting as a differentiation activator in the presence of ligand or an inhibitor when unliganded (or liganded to an antagonist). Inhibition of RAR function by AGN194310 results in the expansion of an immature granulocytic cell population/s, most likely as a result of a perturbation to the normal differentiation process. An expansion of an immature cell population (pre-CFU-S) was also observed when c-Kit⁺/Sca1⁺/lin⁻ purified precursor populations were cultured with a pan-RAR antagonist.²⁴ These data support the hypothesis that RAR antagonism *in vivo* causes an expansion of an immature granulocytic cell type, due to an altered balance of differentiation and proliferation, that results in the overall expansion of granulopoiesis observed. The altered differentiation and proliferation observed following pan-RAR antagonism is consistent with the effects observed following over-expression of truncated RARs and fusion proteins involving RAR.⁸⁻¹⁰ The more profound block to differentiation observed in these models, distinct to the delay observed using AGN194310, may be due to the degree of transcriptional repression that the mutated receptors are able to impart compared with that of the antagonist.

The use of the pan-RAR antagonist is a specific means to study the effects of RAR signalling *in vivo* and to determine the role/s of this signalling in normal adult development and differentiation of the haemopoietic system. The use of a pan-RAR antagonist has allowed us to delineate a specific role for RAR signalling during granulopoiesis as regulating the differentiation of granulocytic progenitors. As such pan-antagonists and RAR-specific antagonists will prove useful tools to further our understanding of the role/s and relative contributions of the RARs, both collectively and individually, to normal and malignant haemopoiesis.

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